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Molybdenum Availability is Key to Nitrate Removal in Contaminated Groundwater Environments

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Abstract

The concentrations of molybdenum (Mo) and of 25 other metals were measured in groundwater samples from 80 wells on the Oak Ridge Reservation (ORR, Oak Ridge, TN), many of which are contaminated with nitrate as well as uranium and various other metals. The concentrations of nitrate and uranium were in the ranges of 0.1 µM to 230 mM and <0.2 nM to 580 µM, respectively. Almost all metals examined had significantly greater median concentrations in a subset of wells that were highly contaminated with uranium (> 0.126 µM). These included cadmium, manganese and cobalt, which were 1,300-2,700 fold higher. A notable exception, however, was Mo, which had a lower median concentration in the uranium contaminated wells. This is significant because Mo is essential in the dissimilatory nitrate reduction branch of the global nitrogen cycle. It is required at the catalytic site of nitrate reductase, the enzyme that reduces nitrate to nitrite. Moreover, more than 85% of the groundwater samples contained less than 10 nM Mo, whereas concentrations of 10 – 100 nM Mo were required for efficient growth by nitrate reduction for two *Pseudomonas* strains isolated from ORR wells and by a model denitrifier *Pseudomonas stutzeri* RCH2. Higher concentrations of Mo tended to inhibit the growth of these strains due to the accumulation of toxic concentrations of nitrite, and this effect was exacerbated at high nitrate concentrations. The relevance of these results to a Mo-based nitrate-removal strategy and the potential community-driving role that Mo plays in contaminated environments are discussed.

Introduction

Environmental contamination from industrial processes, including the release of heavy metals, organic solvents, and nitrogen-containing compounds, can have profound negative impacts on wildlife and human health (1, 2). The Department of Energy oversees the monitoring and restoration of over 90 waste sites at 18 facilities (2). These include facilities at Hanford, WA and Oak Ridge, TN where nuclear production and processing has resulted in the release into the environment of complex mixtures of hazardous waste. For example, between 1951 and 1983, four unlined enclosures known as the S-3 ponds were used for waste disposal at the nuclear processing Y-12 plant on the Oak Ridge Reservation (ORR). The waste material was acidic (pH <2.0) with nitrate, metals such as uranium, aluminum, manganese and chromium, and organic solvents (including acetone, dichloromethane, toluene, and tetrachloroethene) as major contaminants. In 1983 the waste in the ponds was treated by neutralization and biodenitrification, and the ponds were eventually capped and covered by a parking lot (3, 4). Several contaminant plumes have entered the groundwater surrounding the Y-12 S-3 ponds and many wells have been drilled in the contaminated areas as well as in a pristine location 6 km distant from the S-3 ponds (5). The contaminated and pristine wells have been used to conduct geochemical, bioremediation, and ecological studies (4, 6-8). Recently a comprehensive survey of the groundwater was conducted that included 16S rDNA sequencing. The results showed that bacterial communities could be linked to geochemical parameters, showing that bacteria could be used as biosensors to detect contamination (9).

In addition to their presence as contaminants in nitrate-rich environments, some metals play important roles in biological removal of the nitrate by the process of denitrification, a major branch of the global nitrogen cycle, in which nitrate is converted to nitrogen gas. The first of four steps in denitrification, the reduction of nitrate to nitrite, is catalyzed by nitrate reductase, which contains molybdenum (Mo) at its active site (Fig. 1) (10). The second enzyme is nitrite reductase, which catalyzes the reduction of nitrite to nitric oxide, and these are either iron (Fe, NirS) or copper (Cu, NirK)-containing enzymes. Nitric oxide is reduced to nitrous oxide by Fe-containing nitric oxide reductase, while the terminal step, reduction of nitrous oxide to nitrogen gas, is catalyzed by Cu-containing nitrous oxide reductase (10). While denitrifiers are ubiquitous in nature, many microorganisms do not contain the complete pathway. Some nitrate-reducing organisms carry out only the first step, the dissimilatory reduction of nitrate to nitrite while others reduce nitrate to ammonium by a process called nitrate ammonification (11). On the other hand, some microorganisms cannot reduce nitrate but contain later steps of the denitrification pathway (12).

Since Mo is a key element in the biological conversion of nitrate to nitrite, the primary goal of this project was to measure the concentrations of Mo in the ORR contaminated groundwater and determine if Mo could influence the degree that biological denitrification plays in removing nitrate from such environments. There was also an interest in the concentration of tungsten (W) in the groundwater. Tungstate and molybdate have very similar physical properties resulting in an inability for many biological systems to fully discriminate between the two (13), and tungstate has been shown to inhibit dissimilatory nitrate reduction by several different microorganisms (14-18), as well as by mixed cultures obtained from the rumen (19). Tungsten could therefore impede environmental nitrate utilization if present in significant concentrations relative to Mo. Herein we show that the contaminated ORR groundwater contains extremely high concentrations of a range of metals. Unexpectedly, however, both Mo and W are present at extremely low concentrations. Moreover, laboratory studies of the Mo requirements of nitrate-reducing microorganisms, including two isolated from the ORR groundwater, demonstrate that Mo availability is likely a major determinant in the efficient removal of environmental nitrate. Consequently, Mo supplementation could stimulate biological nitrate reduction in groundwater. We also show that addition of Mo to the groundwater could lead to a toxic buildup of nitrite, so Mo supplementation experiments should will require careful optimization. This project represents the first step in understanding the potentially community shifting effects that Mo might have on the groundwater community at ORR, and will inform bioreactor experiments that may lead to an improved method of nitrate bioremediation involving Mo supplementation.

Materials and Methods

Strain isolation and sequencing

Strain N2A2 was isolated from groundwater collected from well FW300 on basal medium (using 10mM sodium acetate as the sole carbon source and electron donor and 10mM sodium nitrate as the electron acceptor). Strain N2E2 was isolated from groundwater collected from the same well using the same basal medium, but 10mM sodium propionate as the sole carbon source and electron donor and 10mM sodium nitrate as the electron acceptor. DNA was extracted from the isolates using the MoBio UltraClean Microbial DNA Isolation Kit (MoBio Inc, Carlsbad,CA). We PCR amplified the 16S gene using primers 27F and 1492R and performed Sanger sequencing directly on the PCR products. We prepared Illumina sequencing libraries for N2A2 and N2E2 using two methods: the Nextera XT kit following the manufacturers (Illumina) instructions and 800 bp inserts labeled with Illumina TruSeq adapters. For the Nextera XT libraries, we performed paired end sequencing (2 x 250 bp) on the Illumina MiSeq. For the 800 bp insert libraries, we performed paired end sequencing (2 x 150 bp) on the Illumina MiSeq. We merged the Nextera and 800 bp insert paired end reads using PEAR (PMID 24142950) and assembled the genomes with the SPAdes genome assembler (PMID 22506599). The draft genome sequences for each strain are available in the Integrated Microbial Genomes system: N2A2 (2600255294) and N2E2 (2600255295).

Growth conditions

The basal growth medium had the following composition: 20 mM sodium fumarate, 4.7 mM NH_4Cl, 1.3 mM KCl, 2 mM $MgSO_4$, 0.2 mM NaCl, 1.2 mM $NaHCO_3$, 5 mM NaH_2PO_4 with sterile vitamin and trace elements prepared as described by Widdel and Bak but lacking Mo and W (20). The $NaHCO_3$ (>99.7% pure) used was from Sigma-Aldrich, St. Louis, MO (other sources contained elevated contaminating levels of Mo that interfered with the growth experiments). Either $NaNO_3$ or $NaNO_2$ were used as electron acceptors at the indicated concentrations. Initial cultures were grown aerobically in a medium containing fumarate (20 mM) and nitrate (20 mM) but lacking added Mo and W. These were then diluted 20-fold into the experimental growth medium. For each experiment, the indicated amounts of nitrate, Mo and/or W were added to the basal growth medium. Cultures were grown in a 100 well Bioscreen plate with each well containing 400 µL of diluted preculture. The Bioscreen plate was incubated anaerobically at 30°C with continuous shaking in a Bioscreen C (Thermo Labsystems, Milford, MA) within an anaerobic chamber (Plas Labs, Lansing, MI) in an atmospheric composition of 95% Ar and 5% H_2. Growth was monitored at an optical density of 600 nm. All experiments were performed in biological duplicate or triplicate. Error for the growth experiments was calculated as the standard deviation.

To condense large amounts of growth data, maximum growth optical density (O.D.) or % maximum growth O.D. is reported. The maximum growth O.D. was defined as the O.D. at the time point of a curve that corresponds to the same time point at which maximum growth was first obtained by the fastest growing culture. The % maximum growth O.D. is the maximum growth O.D. that was obtained normalized to the fastest growing growth condition (taken as 100%).

Nitrite detection and nitrite reductase assay

Nitrite was measured in the supernatant after cultures had been centrifuged aerobically for 20 min at 5,000 rpm in an Allegra 25R Centrifuge (Beckman Coulter, Fullerton, CA). Samples were mixed with an equal volume of Griess reagent (Sigma-Aldrich, St. Louis, MO) and after incubating aerobically for 15 min at 22°C, the A_{540} of the samples were measured in a

Spectra Max 190 (MTX Lab Systems, Inc., Vienna, Va). For whole-cell nitrite reductase assays, the strains were grown anaerobically for 16 hr in 50 mL of basal medium containing fumarate (20 mM), nitrate (20 mM) and 10 nM Mo. Cells were harvested aerobically and were washed with 10 mL of wash buffer (50 mM KPO_4 buffer, pH 7.0), and were then resuspended to a final volume of 1 mL with wash buffer. An aliquot of cell suspension that contained 1-10 mg protein, as determined by the Bradford method (21), was added to assay buffer (50 mM potassium phosphate containing 40 mM fumarate and 1 mM nitrite, pH 7.0) to give a final volume of 5 ml. The mixture was incubated at 30°C and samples were removed every 5 min for 30 min, diluted 1:10 with ddH_20 and mixed 1:1 with Griess reagent (Sigma-Aldrich, St. Louis, MO). After incubating for 15 min at 22°C, the A_{540} of the samples was measured in a Spectra Max 190 (MTX Lab Systems, Inc., Vienna, Va). One unit of nitrite reductase activity is defined as catalyzing the reduction of 1 nmol of nitrite/min.

ICP-MS analysis

Groundwater samples were stored in sealed serum bottles at 4ºC prior to analysis. A Corning MP-3A distillation apparatus was used to produce pure glass distilled water ($gddH_2O$) and this was used in all dilution and washing steps. All containers used for ICP-MS analysis were acid-washed by submersion in a 2% v/v solution of concentrated nitric acid in $gddH_2O$ for 24 hours and rinsed twice by submersion in $gddH_20$ for 24 hours. Trace metal grade concentrated (70%) nitric acid (Fisher A509-212) was used to acidify samples. To measure soluble elements present, groundwater samples (6 ml) in acid-washed 17 x 20mm Sarstedt polypropylene screw-cap conical tubes (62.554.002-PP) were centrifuged at 7,000 x g for 15 minutes at 4ºC in a Beckman-Coulter Allegra 25R centrifuge. The supernatant was decanted into acid washed polypropylene tubes and acidified with 120 µl of concentrated nitric acid. All samples were briefly vortexed

(30 seconds) and incubated with shaking at 37ºC for 1 hour in a New Brunswick Scientific G24 Environmental Incubator Shaker. All samples were centrifuged at 2000 x g for 10 minutes in a Beckman Allegra 6R centrifuge at 25ºC. Metal analyses were performed in triplicate using an Agilent 7500ce octopole ICP-MS in FullQuant mode using and internal standard with in-line addition and multi-element external standard curve as previously described (22). Samples were loaded via a Cetac ASX-520 autosampler. Control of sample introduction, data acquisition and processing was performed using Agilent MassHunter version B.01.01.

Results

Concentrations of metals and nitrate in ORR groundwater

In a previous study 93 groundwater samples taken from 80 wells at various distances from the S-3 ponds on the ORR were analyzed for a number of geochemical parameters including pH, nitrate and dissolved O_2 and several metals (9). Herein, the same groundwater samples were further processed by centrifugation and the soluble fractions were then analyzed by ICP-MS to determine the concentrations of 26 metals, including Mo, W, Fe and Cu, using a previously described method (23). Metal concentrations are reported in Table S1 with standard deviations reported in Table S2 and detection limits in Table S3.

Our analysis shows high concentrations of a range of metals in the ORR groundwater, in addition to the expected uranium (U). This may have been anticipated due to the complexity and diversity of the waste deposited in the S-3 ponds. For example, Figure 2A shows the concentrations of metals in a groundwater sample from one of the most highly contaminated wells (FW126), together with the data from a pristine well (FW300) located 6 km from the contaminated S-3 ponds. Both groundwater samples were acquired on the same date. As might be expected, the concentrations of most metals (21 out of 26) were higher in the FW126 well, with Mn, U, and Ni above 0.1 mM. Interestingly, the concentration of Mo was extremely low, below 10 nM, in both wells.

In order to look at the effects of contamination on the concentrations of the 26 metals across all 93 groundwater samples, the samples were divided into two categories, highly contaminated, that had levels of U above the U.S. Environmental Protection Agency (EPA) maximum contaminant level (MCL) for drinking water (126 nM; 27 samples) and samples with lower amounts of U (66 samples). The minimum, maximum and median concentrations of each metal in the two categories of groundwater samples were calculated and are reported in Figure 2B. The median concentration of U in the highly contaminated samples was 1,600-fold higher than in the other groundwater samples. For all other metals there is a significant overlap in their range of concentrations between the two categories. However, the median concentration of many of the metals varies greatly between the two subsets of groundwater samples. For example, manganese (Mn) was present at the highest concentration (maximum 3.2 mM) and the median concentration of Mn was 1,700 fold higher in the highly contaminated wells. Other metals that had significantly greater median concentrations in the highly contaminated groundwater samples were cadmium (Cd; 2,700-fold), cobalt (Co; 1,300-fold), nickel (Ni; 230-fold), Tl (60 fold), Re (38 fold), Cu (25 fold), and Fe (20 fold).

Although most of the metals were present in higher median concentrations in samples from wells highly contaminated with U (> 125 nM), there were three metals that had lower median concentrations, and these included Mo, which was 7-fold lower. The other metals were vanadium (V; 2-fold lower) and gallium (Ga; 1.4-fold lower). Indeed, the concentrations of Mo were very low in all wells. They ranged from <10 pM to 330 nM, with 85% of the groundwater samples containing Mo at concentrations below10 nM. The concentrations of W, an antagonist of Mo-utilization in biological systems, were even lower in the groundwater samples, ranging from 10 pM to 20 nM with 98% of the samples below 10 nM.

The Mo and nitrate concentrations within each groundwater sample are compared in Figure 3. As reported previously (9), the concentration of nitrate in the wells varied over an extremely large range, from 100 nM to 230 mM. Of note is a cluster of groundwater samples in the lower right hand quadrant of Figure 3 that have extremely low concentrations of Mo (< 1 nM) and very high concentrations of nitrate (> 10 mM). The extent to which sub-nanomolar levels of Mo might limit nitrate removal in the contaminated groundwater is discussed below along with the effect nitrate has on Mo solubility. The uranium concentrations are also depicted in Figure 3 and show that in general the wells containing U above the drinking water standard (126 nM) also contain high nitrate (> 10 mM), although not all wells contaminated with nitrate are contaminated with U.

Isolation of bacteria

The finding of low to sub-nanomolar concentrations of Mo in the vast majority of the ORR groundwater samples begged the question of whether such concentrations would limit microbial denitrification. To address this issue, two different nitrate-reducing microbial strains were isolated from groundwater samples taken from a pristine well (FW300) at ORR. The well contained 60 µM nitrate and 9 nM Mo (and 1 nM W). The strains were designated N2A2 and N2E2 and by 16S rDNA analysis their closest relatives were *Pseudomonas migulae* str. T19 (EU111700.2; 99.72% similarity) and *Pseudomonas corrugata* str. P94 (EF153018.1; 99.13% similarity), respectively. Also included in the following analysis of Mo requirements was *P. stutzeri* strain RCH2, a model denitrifying microorganism that was isolated from chromate-contaminated groundwater at the nuclear site at Hanford, WA (24). The denitrification pathway in *P. stutzeri* has been well studied (10). All three strains exhibit nitrate-dependent growth using fumarate as the carbon and electron source (Fig. S1).

Effect of Mo and W concentrations on nitrate-dependent growth

The three *Pseudomonas* strains were grown on fumarate in the presence of nitrate (60 mM) in a medium containing added Mo (100 pM to 10 µM). In addition, W was also added (0 – 1 µM W). The results are shown in Supplementary Figure 2.   In the absence of added Mo and W the medium contained 3 nM Mo and 2 nM W as contaminants from other medium components.

For each strain, maximal growth required at least 10 nM Mo (in the absence of added W). This is much higher than the concentrations of Mo measured in most of the groundwater samples (Fig. 3). When 10 nM W was added to the growth medium, an amount greater than that seen in 98% of the groundwater samples, growth of all strains was largely unaffected compared to when W was not added. Concentrations of W of at least 100 nM were required to inhibit growth, but the inhibition was relieved by adding comparable concentrations of Mo (Fig. S2). Hence, for efficient nitrate reduction, the three strains require much higher concentrations of Mo than are found in the vast majority of the ORR wells, but nitrate removal would not be affected by W at the concentrations found in those wells.

Effect of high concentrations of Mo on denitrification

Given that the concentration of Mo in the ORR groundwater potentially limits nitrate reduction, the process could be potentially stimulated by Mo supplementation. To determine the potential effects of high Mo concentrations, the three *Pseudomonas* strains were grown on fumarate media containing up to 2 mM Mo with 60 mM or 120 mM nitrate. Note that, as shown in Figure 3, many wells contain at least 60 mM nitrate. The results of the growth study are summarized in Figure 4. In order to condense the large amount of data, composite growth graphs are shown based on the percentage of the maximum growth observed. Surprisingly, the growth of the two ORR isolates was inhibited at Mo concentrations above 100 nM (strain N2A2) and 1 µM (strain N2E2), with 2 mM Mo resulting in less than 10% of maximum growth (in the presence of 120 mM nitrate). Strain RCH2 was less sensitive to inhibition by high Mo concentrations and inhibition was observed only in the presence of the higher nitrate concentration (120 mM). While strains RCH2 and N2E2 displayed increased Mo inhibition at the higher nitrate concentration, strain N2A2 was severely inhibited by Mo at both of the nitrate concentrations that were used.

To confirm that the inhibitory effects of Mo were related to nitrate reduction, the effect of adding W, the Mo antagonist, was investigated. When cells were grown in the presence of added W (1 µM) under conditions where Mo caused inhibition of growth (1 mM Mo, 120 mM nitrate), the inhibition was relieved (Fig. S3). Since W is a Mo antagonist and results in inactive nitrate reductase (14), it is proposed that the inhibition by high concentrations of Mo is due to a buildup of toxic nitrite (Fig. 1). High concentrations of Mo in the growth medium result in increased amounts of active nitrate reductase, this in turn can lead to increased nitrite production. If these nitrite concentrations were beyond the capacity of rest of the denitrification pathway to remove, then nitrite would accumulate and inhibit growth. Hence we propose that W has a positive effect on growth by inhibiting nitrate reductase thereby lowering the amount of nitrite produced.

To investigate the proposed model of W stimulation of growth and its relevance to environmental nitrate removal, the concentration of nitrite was measured in the medium after all three *Pseudomonas* strains had been grown with increasing concentrations of added nitrate. As shown in Supplementary Figure 4, all three strains accumulated nitrite, and the two ORR strains, which were more readily inhibited by high Mo concentrations, accumulated significantly higher concentrations of nitrite than did RCH2. Accordingly, as shown in Supplementary Figure 5, the two ORR strains were significantly more sensitive to inhibition by added nitrite than was strain RCH2. Whole cell nitrite reductase assays were performed for the three strains grown anaerobically on fumarate nitrate (20 mM) medium in the presence of 10 nM Mo. Strains RCH2 and N2A2 had higher nitrite reductase activity (15.0 ± 1.0 and 29 ± 2 U/mg, respectively) than did strain N2E2, which had little to no activity (1.0 ± 0.2 U/mg). These results are consistent with genome sequencing data showing that strains RCH2 (NC_019936.1) and N2A2 have the complete denitrification pathway while strain N2E2 lacks dissimialatory nitrite reductase (although it contains both nitric oxide reductase and nitrous oxide reductase, see Figure 1).

The observed accumulation of nitrite by all three nitrate-reducing strains, along with the nitrite sensitivity data, therefore supports the proposed nitrite toxicity model for W-stimulated growth at the higher Mo concentrations. Ironically, while W in its role as a Mo-antagonist inhibits growth when the concentration of Mo is low (Fig. S2), it has a beneficial effect on growth of all three strains at high Mo concentrations by inhibiting nitrate reductase thereby reducing the amounts of toxic nitrite that is produced.

Discussion

The results presented herein show that it is likely that the availability of Mo, an essential metal for nitrate reductase, limits nitrate reduction in the environment and particularly in contaminated ecosystems. In general, the concentrations of Mo present in the ORR groundwater samples are much lower than those required for optimum nitrate-dependent growth of *Pseudomonas* strains in the laboratory, including two strains isolated from the ORR groundwater. Of the ORR groundwater samples investigated, 85% contained less than 10 nM Mo, and Mo was below the limit of detection (< 10 pM) in many wells. Moreover, Mo was one of the few metals that had a lower median concentration in the subset of highly contaminated wells. Such wells also tended to contain higher concentrations of nitrate and have a lower pH. The sorption of molybdate to soil in aqueous suspensions is dependent on pH, with increased sorption in the lower pH range, which could further limit Mo solubility and availability (25). However Mo seems to be limited in the ORR environment in general since the median concentration of Mo measured in the sediment portion of the highly contaminated ORR groundwater samples was still extremely low (equivalent to 0.4 nM).

Both Fe and Cu are also involved in environmental nitrate removal as, like Mo, they are essential metal cofactors of enzymes of the denitrification pathway. The median concentrations of Fe and Cu in the non-contaminated wells were 30 nM and 4 nM respectively, and their median concentrations were increased 20 and 25 fold in the highly contaminated wells. Both Fe and Cu are used in numerous other processes in *Pseudomonas* strains, and it is possible that in some wells containing very low concentrations of these metals (<< 10 nM), growth may be limited by these metals. Like Mo, W is also present at very low concentrations (typically << 10 nM) in the ORR environment and likely does not impact nitrate reduction to any extent. In the laboratory, 10 nM W, which is greater than that measured in 98% of the ORR groundwater samples, did not have an inhibitory effect on nitrate-dependent growth of the *Pseudomonas* strains (Fig. S2). On the other hand, it is also shown here that W can stimulate growth of some nitrate-reducing strains under conditions of excess Mo (≥ 1 µM) by relieving the accumulation of toxic nitrite (Fig. S3). It is noteworthy that the EPA MCL for nitrite in drinking water is 22 µM, compared to 161 µM for nitrate.

During microbial nitrate removal in natural environments, the product of the nitrate reductase enzyme is nitrite, which in an organism containing the complete denitrifying pathway is further reduced to nitric oxide, nitrous oxide, and then finally to nitrogen gas (Fig. 1). Of these metabolites, both nitrite and nitric oxide are known to be toxic to biological systems with nitric oxide being highly toxic (10). However, herein we show that under Mo-sufficient conditions (10 nM Mo), *Pseudomonas* strains are inhibited by nitrite rather than by nitric oxide. In particular, strain N2E2, which is readily inhibited by increasing Mo concentrations, lacks dissimilatory nitrite reductase activity and cannot produce nitric oxide. The presence of nitrite in the growth media of the *Pseudomonas* strains (Fig. S4) support its role as the growth inhibitor. A factor that could be contributing to the accumulation of nitrite is that nitrate may be competing with nitrite for the active site of nitrite reductase. During denitrification by the bacterium *A. brasilense* SP7, nitrite reduction only occurred when nitrate concentrations were below 50 µM. In fact, the addition of nitrate caused nitrite to accumulate and it was proposed that nitrate inhibited nitrite reductase (14, 26, 27). Preferentially using nitrate over nitrite may be competitively advantageous under certain circumstances since the action of nitrate reductase conserves energy but that of nitrite reductase does not (11). Under conditions where nitrate is limiting, it may be beneficial for microorganisms to first use solely nitrate as an acceptor in the environment, as the

ORR isolates appear to do, whereas RCH2, which uses nitrite concurrently with nitrate, may have an advantage in contaminated environments by having the complete denitrification pathway thereby avoiding the accumulation of nitrite.

The high nitrate and high Mo concentrations used in this study represent a very different environment than the pristine groundwater from which the ORR *Pseudomonas* strains were isolated and presumably adapted to. Indirect control of gene expression can commonly result in fitness defects for organisms exposed to non-native conditions such as those used in the laboratory (28). Another difference between the experimental conditions and the ORR groundwater environment is carbon availability. The strains were grown in high concentrations (20 mM) of a readily usable carbon source (fumarate) whereas the groundwater environment is limiting in carbon (9) which, in turn limits nitrate reduction. The experiments described herein are probably more relevant to nitrate-contaminated environments where carbon stimulation experiments could potentially have different results depending on whether Mo is supplemented. In terms of Mo supplementation of ground water environments, it is noteworthy that chronic toxicity tests conducted on multiple diverse freshwater organisms found that molybdate had a low degree of toxicity compared to other metals with the most sensitive organism tested being the freshwater fish *Pimephales promelas* with a chronic effect level of 162 μM (29).

The virtual absence of Mo in nitrate-contaminated ground water effectively limits the use of nitrate as an electron acceptor for microbial action. This could hinder nitrate removal, and result in the preferential survival of fermentative organisms and organisms that can grow using alternate electron acceptors. Previously, chromate (CrO_4^{2-}) contaminated groundwater was used as an inoculum for a continuous-flow reactor supplemented with fumarate (30 mM) and only a low concentration of sulfate (0.05 mM) as the electron acceptor (30). Diversity in the reactor decreased from 63 to 11 bacterial genera and the reactor was dominated by *Pelosinus* species (30), which are known to ferment various compounds including fumarate (31). They are also able to use nitrate, Fe(III), CrO_4^{2-} and UO_2^{2+} as electron acceptors (30, 31). The diversity of mechanisms that they use to dispose of reductant may be one of the reasons they are found in great abundance in many supplemented groundwater studies including lactate amended enrichments, some of which were conducted with ORR groundwater (30, 32, 33). The results presented herein suggest that Mo-supplementation of ORR nitrate-contaminated ground water enrichments may favor a different set of organisms that efficiently use nitrate as a terminal electron acceptor.

Nitrate contamination of groundwater is an issue in many other locations besides ORR and is a result of geologic origin, poor waste disposal, and most predominantly, the use of nitrate containing fertilizers (34). The concentrations of Mo and W may be an important factor to consider when looking at other such nitrate contaminated sites. Removal of toxic chemicals such as nitrate, UO_2^{2+} and CrO_4^{2-} from environments by stimulation of the microbial community is a complex process that depends on the types and concentrations of electron donors and acceptors that are used. A factor not yet considered in these types of *in situ* studies is the pivotal role that Mo might play due to its essential role in the dissimilatory nitrate reductase. In a previous study at ORR the contaminated groundwater was stimulated with ethanol over a two-year period (6). The process was dependent on a preemptive *ex situ* pH adjustment (from pH 3.6 to 6.0) and nitrate removal with a denitrification bioreactor (from >100 mM to 0.5 mM). This resulted in the reductive immobilization of U(VI) to below EPA drinking water standards (126 nM). Community analysis after the experiment revealed an increase in the diversity of microorganisms present, including denitrifying, iron-reducing and sulfate-reducing species of *Desulfovibrio, Geobacter*, *Anaeromyxobacter*, *Desulfosporosinus*, and *Acidovorax*(6).

One wonders how the results of this and other *in situ* studies (6, 35, 36) might have been affected by the presence of sufficient concentrations of Mo. The Mo concentration of wells in the same area of ORR (FW-100 area) in which the ethanol stimulation experiment was performed were in most cases much lower (pM range) than the Mo concentration required for laboratory denitrifying growth of the *Pseudomonas* isolates. The laboratory experiments reported herein, in which the carbon source was more than sufficient (20 mM fumarate) but Mo was limiting, are closer to conditions in the ethanol-supplemented groundwater than unstimulated groundwater (6). Clearly, a future direction is to use groundwater-fed bioreactors supplemented with carbon and various concentrations of Mo to investigate Mo-induced community changes and the effect these have on the removal of nitrate. From our results using three different *Pseudomonas* species, Mo in the concentration range of 10-100 nM Mo was needed for efficient nitrate-dependent growth, although as we demonstrate, a further increase in the Mo concentration could have inhibitory effects due to nitrite accumulation. We predict that in such bioreactor experiments, high concentrations of Mo would result in community changes favoring nitrite-utilizing or nitrite-resistant microorganisms, like strain RCH2. The properties of these organisms including nitrate, nitrite and metal tolerance may make them important players in a more comprehensive study of *in situ* nitrate removal.

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Figure Legends

Figure 1. Model of the effect of NO_3^-, Mo, and W on the growth of *Pseudomonas* isolates under denitrifying conditions. The first enzyme in the denitrification pathway, nitrate reductase, requires a molybdopterin guanine dinucleotide cofactor for activity. Activity of nitrate reductase can be increased during growth of a *Pseudomonas* isolate by supplementing the growth medium with Mo or NO_3^-. This effect is beneficial to growth up to a certain point, however over supplementation with Mo and NO_3^- can result in an inhibitory buildup of nitrite decreasing growth. Supplementation of the growth medium with W results in antagonistic interactions with Mo that decrease nitrate reductase activity.

Figure 2. Metals in pristine and contaminated wells at ORR. A) Concentrations of metals and nitrate (inset) measured in the soluble fractions of groundwater samples from pristine well FW-300 (black) and contaminated well FW-126 (grey). Measurements at or below detection limits are indicated with an asterisk. **B)** The groundwater samples were divided into two subsets based on the soluble U concentration: those below (black) and those above (grey) the maximum amount (126 nM) allowed in drinking water by the EPA. For each subset of groundwater samples, the low, median and high concentrations are represented. Metals whose median concentrations are higher in U contaminated than non-contaminated wells are represented with circles, and those whose median concentrations are lower are represented with triangles.

Figure 3. Nitrate and Mo concentrations in ground water wells at ORR. Each point represents a groundwater sample taken from a well at ORR. A total of 93 groundwater samples were taken from 80 wells. Duplicate samples from the same well were taken either on different dates or at different well depths. The concentrations of nitrate and Mo are plotted on log scales. Wells containing U above (blue) or below (green) a concentration of 126 nM are indicated. The red dashed line crosses the Mo concentration axis at 10 nM and the purple dashed line crosses at the limit of detection for Mo. Nitrate data were collected as part of a previous study (9).

Figure 4. Effect of high concentrations of Mo on the growth of *Pseudomonas* strains in the presence high concentrations of nitrate. The three strains (RCH2, N2A2 and N2E2) were grown anaerobically on basal medium with 60 mM nitrate (black circles) or 120 mM nitrate (grey squares) at various Mo concentrations (expressed on a log scale). Growth is represented as a percentage of the maximum growth measured by the strain.