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Correcting for background nitrate contamination in KCl-extracted samples during isotopic analysis of oxygen and nitrogen by the denitrifier method

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RATIONALE: Previous research has shown that the denitrifying bacteria *Pseudomonas chlororaphis* ssp. *aureofaciens* (*P. aureofaciens*) can be used to measure the $\delta^{15}N$ and $\delta^{18}O$ values of extracted soil nitrate (NO_3^-) by isotope ratio mass spectrometry. We discovered that N_2O production from reference blanks made in 1 M KCl increased relative to blanks made of deionized water (DIW). Further investigation showed that isotopic standards made in KCl yielded $\delta^{15}N$ and $\delta^{18}O$ values different from the standards prepared in DIW.

METHODS: Three grades of crystalline KCl were dissolved in DIW to create solutions of increasing molarity (0.1 M to 2 M), which were added to *P. aureofaciens* broth and measured as blanks. Reference standards USGS-32, USGS-34, and USGS-35 were then dissolved in a range of KCl concentrations to measure isotopic responses to changing KCl molarity. Reference blanks and standards created in DIW were analyzed as controls to measure the impact of KCl on the $\delta^{15}N$ and $\delta^{18}O$ values.

RESULTS: The amount of N_2O in the KCl blanks increased linearly with increasing molarity, but at different rates for each KCl grade. The isotopic values of the reference standards measured in KCl were systematically different from those measured in DIW, suggesting contamination by background NO_3^- in the KCl reagents. However, we also noted reduced conversion of NO_3^- into N_2O as the KCl molarity increased, suggesting there is a physiological response of *P. aureofaciens* to KCl.

CONCLUSIONS: There is a small amount of NO_3^- present in crystalline KCl, which can bias isotopic measurement of NO_3^- at low sample concentrations. This can be minimized by making standards and blanks in the same KCl as is used in samples, diluting all samples and standards to the appropriate NO_3^- concentration using matched KCl solutions, and adding samples and standards to the broth at a constant volume to standardize the KCl molarity in the reaction vial.

Mass spectrometry of the stable isotopes of oxygen (O) and nitrogen (N) of nitrate (NO_3^-) through bacterial denitrification has advanced our understanding of reactive nitrogen in terrestrial, freshwater and marine ecosystems.[1–4] Anthropogenic and naturally produced NO_3^- have distinct isotopic signatures of both $\delta^{15}N$ and $\delta^{18}O$.[5] Mixing models based on these values and background NO_3^- concentrations can help identify the sources and transformation of nitrogen within a local system.[4,6,7] Recent studies have analyzed potassium chloride (KCl) solutions containing NO_3^- extracted from soil samples using the denitrifying bacteria *Pseudomonas chlororaphis* ssp. *aureofaciens* (*P. aureofaciens*) without identifying any isotopic artifacts,[7–9] although earlier work by Stark and Hart[10] measured NO_3^- contamination by 2 M KCl while performing diffusion digests. While conducting similar analyses on KCl-extracted soils in southern California, we noted an increase in N_2O production in blanks prepared in KCl and worrying shifts in the expected isotopic values of reference standards prepared in KCl relative to blanks and standards prepared in deionized water (DIW).

KCl is commonly used as an extraction reagent to measure bio-available NO_3^- and NH_4^+ in soil samples[11] and to elute NO_3^- from ion-exchange resins.[12,13] *Pseudomonas aureofaciens* was initially used in seawater to study nitrogen cycling in marine ecosystems,[14,15] suggesting that high salt concentrations should not affect the method. Standard reagent grade KCl has a label purity of 99–100.5% KCl. According to the American Chemical Society (ACS), reagent grade KCl has an allowable limit of <0.003% NO_3^- by weight.[16] At the 0.003% level, a 1 M solution of KCl would have a NO_3^- concentration of 2.24 μg mL^{-1} which is enough to significantly alter the isotopic composition of samples prepared in a KCl matrix which have low NO_3^- concentrations.

Our study examines the response of *P. aureofaciens* to KCl and determines if the interaction measured is the result of the mixing of trace amounts of NO_3^- in the KCl reagent or if the ionic matrix is producing a biological response in the bacteria. We measured the rate of N_2O production in DIW blanks and standards with

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increasing molarity of three chemical grades of KCl to quantify the amount of background NO_3^- impurity. Next, we determined the $\delta^{15}N$ and $\delta^{18}O$ values of N_2O produced from international reference standards across the same KCl concentration gradient to detect any isotopic bias introduced by KCl and determine if NO_3^- conversion efficiency was affected by KCl additions. We hypothesized that increasing the molarity of KCl would result in an increased amount of N_2O produced due to the presence of background NO_3^- in the reagent. We also hypothesized that the isotopic response of the reference standards would change in accordance with a two source mixing model (Source 1 = standard NO_3^- and Source 2 = background NO_3^- in reagent KCl), unless *P. aureofaciens* was physiologically affected by KCl additions.

EXPERIMENTAL

Sample analysis

To determine the $\delta^{15}N$ and $\delta^{18}O$ values of NO_3^-, the microbial denitrifier method was used to convert NO_3^- into N_2O.[14,15] The bacteria *P. aureofaciens* (ATCC# 13985) lack nitrous oxide reductase activity and therefore produce N_2O gas as a final product of denitrification which allows the isotopic ratios of N and O to be simultaneously determined. Briefly, the bacteria were grown in a modified soy broth solution for 7–10 days, concentrated, and sealed in a 20 mL headspace vial and sparged with helium gas for 2 h. Samples were then added to the reaction vials, which were inverted overnight to allow for complete conversion of NO_3^- while minimizing N_2O loss. The following morning 0.1 mL of 10 N NaOH was added to each vial to raise the pH of the solution, lysing the bacteria to stop denitrification and immobilizing CO_2 gas as dissolved inorganic carbon (elapsed time was <5 min from first to last sample). Using a GC-PAL autosampler (CTC Analytics, Lake Elmo, MN, USA), the vials were sequentially sparged with helium gas forcing N_2O gas in the headspace to be cryogenically focused in a liquid nitrogen trap. The samples were then directed into a gas chromatography column through a Thermo GasBench II (Thermo Fisher Scientific, Waltham, MA, USA) to separate the N_2O gas from residual CO_2 within the system. The *m/z* 44, 45, and 46 ions of the N_2O were then measured using a Delta V isotope ratio mass spectrometer (Thermo Fisher Scientific). Initial isotopic calibration was accomplished by replicated pulses of N_2O reference gas and used to derive raw $\delta^{18}O$ and $\delta^{15}N$ values for each sample.

Three international reference standards (USGS-32, USGS-34, and USGS-35) were used in our study for secondary calibration of raw instrument values. USGS-32 and USGS-34 were used as endmembers to create a secondary calibration curve for all $\delta^{15}N$ measurements and USGS-34 and USGS-35 were used to create a secondary calibration curve for all $\delta^{18}O$ measurements. Isotope ratio values are reported using Eqn. (1) where X = nitrogen or oxygen.

$$\delta X = \left[\left(\frac{R_{Sample}}{R_{Standard}}\right) - 1\right] * 1000 \quad (1)$$

where R_{Sample} is the ratio of heavy isotope to light isotope in a sample and $R_{Standard}$ is the ratio of heavy to light isotope in the international standard reference material (atmospheric N_2 or Vienna Standard Mean Ocean Water (VSMOW)).

Experiment 1: Effect of KCl molarity on DIW blanks

To evaluate if N_2O production was affected by KCl addition, we prepared KCl solutions in DIW (18 megaohm) ranging in strength from 0.1 to 2 molar (M) KCl. Three grades of crystalline KCl (Fisher Chemical, Crystalline/USP/FCC (denoted Fisher KCl); Fisher Chemical, Crystalline/EP/BP/USP/FCC (denoted Fisher-Reagent KCl); and Acros Organics, Extra Pure (denoted Acros KCl)) were individually used to prepare each molarity of KCl (all ordered from Thermo Fisher Scientific). Aliquots of 3 mL from each batch of KCl were injected into helium-sparged vials of *P. aureofaciens* broth and the quantity and $\delta^{18}O$ and $\delta^{15}N$ values of the evolved N_2O were measured using the previously described methods. Comparing the amplitude of the *m/z* 44 peak of the KCl blanks with the amplitude of known amounts of NO_3^- allowed us to compute the mass of NO_3^- injected into the vials; from the mass injected we were able to calculate the background level of NO_3^- in each of the crystalline reagents.

Experiment 2: Effect of KCl molarity on USGS-32, -34 and -35 standards

To measure the isotopic bias caused by the KCl matrix, the reference standards (USGS-32, USGS-34, and USGS-35) were dissolved in varying molarities of Fisher KCl (0.25 to 1 M). All standards were diluted to a concentration of 1 μg NO_3^- mL^{-1} and were injected into helium-sparged vials of *P. aureofaciens* in 3 mL aliquots, thus providing approximately 48.4 nanomoles of NO_3^- to the bacteria for conversion into N_2O. This amount of NO_3^- created an *m/z* 44 peak of between 12,000 and 14,000 mV. Using the same injected volume for each sample allowed any response due to the KCl to be consistent across each set of samples. Isotopic values were calculated using calibrations developed with the same standards prepared in DIW.

Experiment 3: Effect of KCl molarity on the conversion efficiency of *P. aureofaciens*

To determine if there was a physiological response of *P. aureofaciens* to KCl we measured the *m/z* 44 peak amplitude of the three standards across a gradient of KCl molarity (0.25 to 2 M). A series of blanks was prepared at identical KCl molarities and used to correct the peak amplitudes of the standards for background NO_3^- in the KCl. This correction allowed us to observe the efficiency of NO_3^- conversion into N_2O independent of the background NO_3^- contained in the KCl reagent.

Experiment 4: Effect of KCl molarity on isotope fractionation

To measure the linearity of the isotopic bias across a range of isotopic values, five working standards were created by mixing precisely measured amounts of USGS-32 and USGS-34 reference solutions. These reference standards were chosen because they represent the two endmembers of our isotope calibration curve and they do not contain any excess ^{17}O isotope, which can introduce error into $\delta^{15}N$ measurements of NO_3^-.[14] The reference samples were prepared in DIW as well as in a 1 M KCl solution made with Fisher-Reagent KCl, and then precisely mixed to create a gradient of isotope working standards in both DIW and KCl matrices. All standards were

diluted to a concentration of 1 μg NO_3^- mL^{-1} (molarity held constant) and were injected into helium-sparged *P. aureofaciens* vials in 3 mL aliquots. The $\delta^{15}N$ and $\delta^{18}O$ values were measured as previously described.

Since we knew the true isotopic values of all the working standards, and the results from Experiment 1 provided the isotopic values of the background NO_3^- in KCl, we attempted to determine if a simple two-component mixing equation could be used to correct for the bias caused by background NO_3^- in the KCl reagent:

$$\delta X_{CORR} = \frac{((A_{KCl\ Sample} - A_{KCl\ Blank})^{*}\delta X_{Cert}) + (A_{KCl\ Blank})^{*}\delta X_{KCl\ Blank}}{A_{KCl\ Sample}} \quad (2)$$

where $A_{KCl\ Sample}$ is the amplitude of the *m/z* 44 peak of the working standard in 1 M KCl, $A_{KCl\ Blank}$ is the amplitude of the *m/z* 44 peak of a 1 M KCl blank sample, δX_{Cert} is the known isotopic ratio value of the working standard (N or O), and $\delta X_{KCl\ Blank}$ is the measured delta value of O or N associated with the KCl blank sample. This model assumes that all the NO_3^- added with the KCl is denitrified by the bacteria.

RESULTS AND DISCUSSION

Background NO_3^- in crystalline KCl

The amount of N_2O produced in the KCl blanks increased linearly with increasing molarity for each grade of KCl (Fig. 1). The *m/z* 44 peak amplitudes for the blanks prepared in Fisher-Reagent and Acros KCl were generally more consistent than those for the blanks prepared in Fisher KCl. We speculate that this phenomenon might be due to increased reagent purity reducing other background molecules that interfere with the bacterial conversion of NO_3^- into N_2O. Each of the three KCl types (Fisher, Fisher-Reagent, and Acros) had unique rates of N_2O production with increasing molarity (ANCOVA Effects: df = 2, F = 15.78, $p < 0.0001$). The linear increase of N_2O production suggests that there was a characteristic level of background NO_3^- in each type of crystalline KCl. Calculating the amount of NO_3^- present in the KCl based on the amplitude of the *m/z* 44 peak indicates that each mL of 1 M KCl contained between 0.0390 and 0.0953 μg of NO_3^- equaling 0.000052 to 0.000128% NO_3^- by weight in the crystalline reagent (Table 1). These levels are similar to the value of 0.025 μg mL^{-1} measured by Stark and Hart.[10] This level of impurity is much lower than the labeled ACS certification, but still represents between 3.9 and 9.5% of the total NO_3^- in a 1 μg mL^{-1} sample and therefore may affect the isotopic analysis of low NO_3^- extracts.

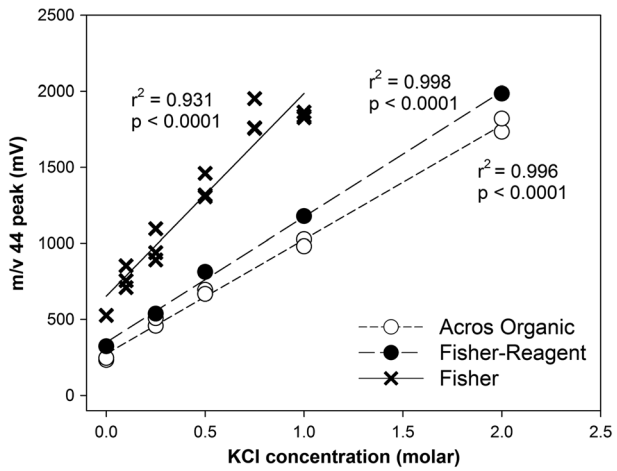

Figure 1. Relationships between peak amplitude (*m/z* 44) of DIW blanks and KCl molarity for three grades of KCl.

The 1 M and 2 M KCl blanks from Experiment 1 produced *m/z* 44 peaks of sufficient amplitude (>800 mV), that we could accurately estimate the isotopic composition of the background NO_3^-. The $\delta^{15}N$ and $\delta^{18}O$ values for the blanks ranged from –13.0‰ (±0.08) to 3.0‰ (±0.44) and from –8.3‰ (±0.03) to 10.3‰ (±0.22) (Table 1). We noted a progression towards increased values of $\delta^{15}N$ and $\delta^{18}O$ as the background level of NO_3^- in the KCl declined. The fact that each type of KCl had a distinct isotope composition suggests that the isotopic bias caused by background NO_3^- in KCl cannot be fully removed unless the same bottle (or lot) of KCl is used in extractions, dilutions, and the creation of standards.

Responses of standards to KCl

When USGS standards were prepared in varying molarities of Fisher-Reagent KCl, the precision of the raw $\delta^{15}N$ and $\delta^{18}O$ values was typically not as good as the precision for duplicate standards prepared in DIW (Fig. 2). The measured $\delta^{15}N$ and $\delta^{18}O$ values of the reference standards responded linearly to the increasing molarity of KCl in solution which suggested two-source mixing behavior (Source 1 = NO_3^- in standard and Source 2 = background NO_3^- in KCl) (Fig. 2). The $\delta^{15}N$ and $\delta^{18}O$ values of the background NO_3^- in Fisher-Reagent KCl were measured at –6.9‰ and +4.5‰, respectively. The $\delta^{15}N$ value is consistent with the relationships shown in Fig. 2, which shows the $\delta^{15}N$ values of samples decreasing with increasing additions of the NO_3^- from the KCl. Similarly, the $\delta^{18}O$ values of the standards were altered toward +4.5‰ as KCl concentrations increased. These results indicated that the background NO_3^- concentration in KCl is high enough to significantly alter the isotopic value of a low concentration samples, emphasizing the need to use the same KCl in extraction, dilutions, and the creation of standards.

Effects of KCl on NO_3^- conversion efficiency

Increasing the molarity of KCl appeared to decrease the NO_3^- conversion efficiency of *P. aureofaciens* (Fig. 3). While all the relationships shown in Fig. 3 are best described by linear functions, there did appear to be a break in the slope at a concentration of 1 M suggesting that there may be a physiological threshold above which *P. aureofaciens* has difficulty metabolizing NO_3^-. This is consistent with previous research that showed only 30–50% of NO_3^- was denitrified over 50 h in a 2 M KCl matrix, while there was no change in a 0.1 M matrix.[17] The difference between the expected N_2O production and measured N_2O production apparent in Fig. 3 may be the result of incomplete conversion of sample NO_3^- into N_2O due to the KCl matrix or, alternatively, a secondary reaction related to high KCl concentrations may have removed N_2O from the headspace of the vial (e.g., chemodenitrification). Morkved *et al.*[17] hypothesized that, when creating a soil slurry, the

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Table 1. The amount and isotopic composition of background NO_3^- present in 1 M KCl solutions and in crystalline reagent of three grades of KCl. Nitrate values were calculated by comparing the size of the *m/z* 44 peak with those of known NO_3^- standards. Delta values are the average (± standard error) for blanks prepared in 1 M and 2 M KCl which yielded *m/z* 44 peak amplitudes >800 mV

KCl type	Nitrate concentration in 1 M KCl solution (μg mL^{-1})	Nitrate % by weight in KCl reagent	Approximate $\delta^{15}N$ value of background NO_3^- (‰ vs N_2)	Approximate $\delta^{18}O$ value of background NO_3^- (‰ vs VSMOW)
Fisher	0.0953	0.000128	−13.0 ± 0.08	−8.3 ± 0.03
Fisher-Reagent	0.0557	0.000075	−6.9 ± 0.24	4.5 ± 0.15
Acros	0.0390	0.000052	3.0 ± 0.44	10.3 ± 0.22

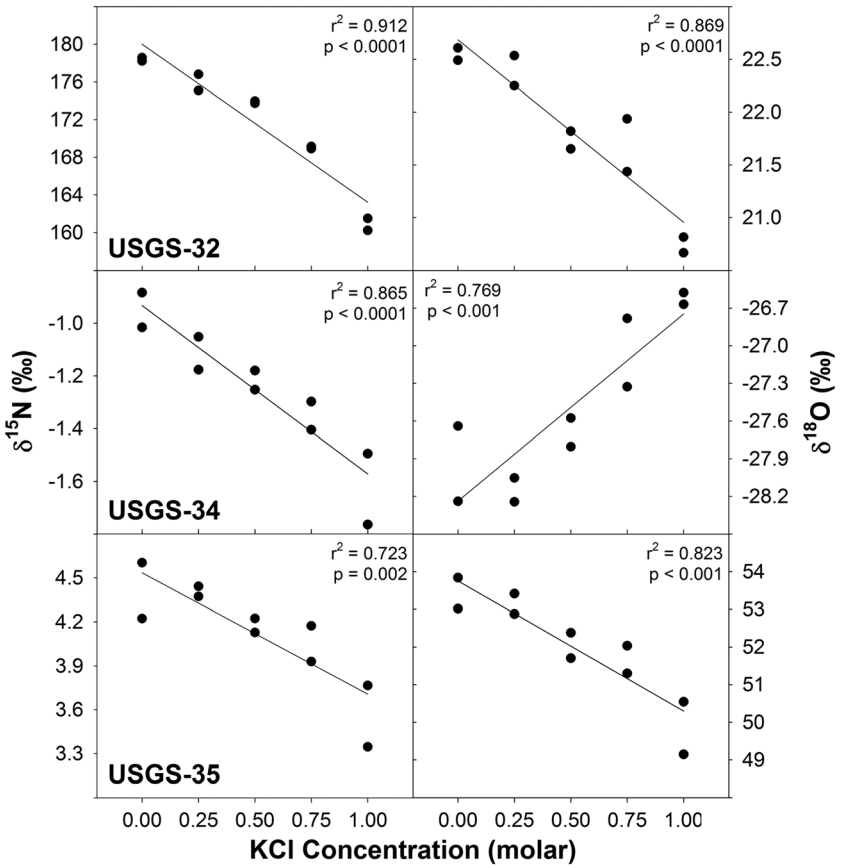

Figure 2. Relationship between $\delta^{15}N$ and $\delta^{18}O$ values of USGS standards 32, 34, and 35 in DIW and in increasing KCl molarity (prepared using Fisher-Reagent KCl).

presence of soil denitrifiers can reduce headspace N_2O to N_2 gas over time, and this needs to be taken into account for soil extracts. We are conducting additional experiments to determine the underlying causes of lower than expected N_2O production in high molarity KCl extracts.

Methods for correcting for the KCl matrix

The gradient of working standards prepared from mixtures of USGS-32 and USGS-34 allowed us to determine if the isotopic bias induced by the KCl matrix was consistent across the range of values. When the working standards were made in a DIW solution, the raw $\delta^{15}N$ values (prior to secondary calibration) were linearly consistent with the expected values (Fig. 4(A)). When the working standards were made in a 1 M KCl solution, the measured $\delta^{15}N$ values were increasingly biased from the expected values as the $\delta^{15}N$ values increased, resulting in the curvilinear response evident in Fig. 4(A). Due to the shift in $\delta^{15}N$ values for the samples in KCl, using the calibration equation created with reference standards dissolved in DIW to correct the measured $\delta^{15}N$ values of working standards prepared in KCl produced inaccurate results, with lower $\delta^{15}N$ values than expected (Table 2(A)). When a calibration equation was created using reference standards dissolved in 1 M KCl, the corrected values of the KCl working standards were much closer to their true values in the natural abundance range, but were significantly higher at larger $\delta^{15}N$ values (Table 2(A)). This indicates that reference standards must be dissolved in the same KCl solution as the KCl-extracted unknowns to obtain accurate isotope ratio

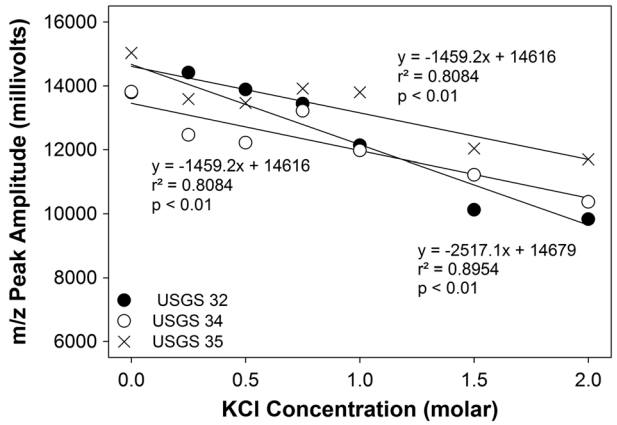

Figure 3. Relationship between corrected peak amplitude (*m/z* 44) of standards and KCl molarity (prepared using Fisher-Reagent KCl). Peak amplitudes were corrected by subtracting the amplitude of DIW blanks from the peak amplitude of the standards at a given KCl concentration.

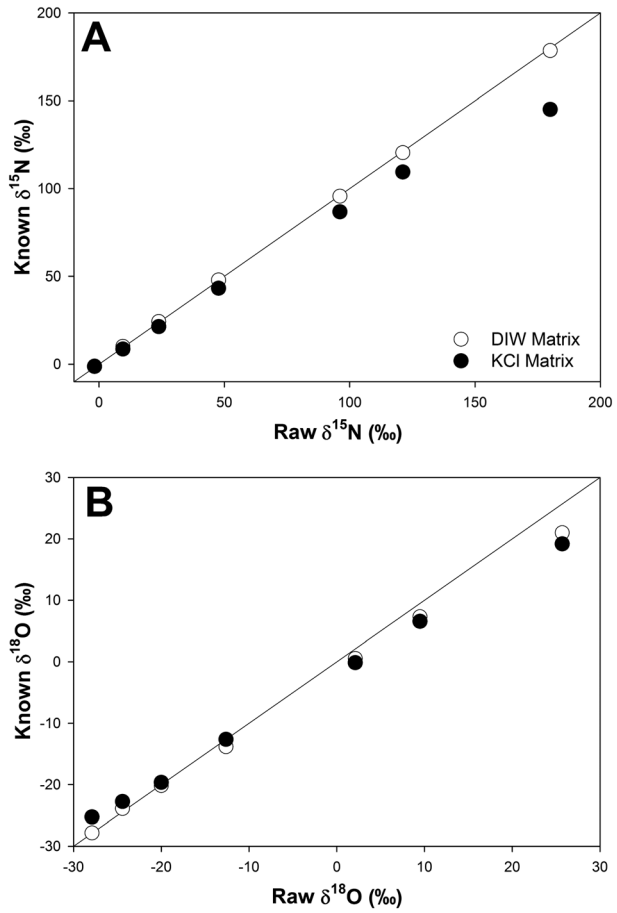

Figure 4. Relationship between corrected peak amplitude (*m/z* 44) of standards and KCl molarity (prepared using Fisher-Reagent KCl). Peak amplitudes were corrected by subtracting the amplitude of DIW blanks from the peak amplitude of the standards at a given KCl concentration.

values. However, even by matching the matrices between samples and standards, the bias at the higher $\delta^{15}N$ values suggests that there was a secondary interaction between the KCl and the bacteria that must be addressed. Identifying a physiological interaction between *P. aureofaciens* and KCl was beyond the scope of this experiment, but when we manipulated the initial growth medium to have a 1 M KCl concentration, no bacterial growth was evident. This suggests that the KCl creates an environment that limits *P. aureofaciens* growth.

The measured $\delta^{18}O$ values of samples in DIW and in 1 M KCl both maintained a linear relationship with their expected values ($r^2 = 0.999$ for both regressions; Fig. 4(B)). The slopes of the lines were different due to the background NO_3^- in the KCl solution. The discrepancy between the large bias in $\delta^{15}N$ values and the negligible bias in $\delta^{18}O$ values may be evidence for incomplete conversion of sample NO_3^- at high KCl molarities. Since N is balanced during denitrification ($2NO_3^- \rightarrow 2NO_2^- \rightarrow 2NO \rightarrow N_2O$), the isotopes of the N molecules within NO_3^- are more likely to suffer from fractionation due to incomplete conversion into N_2O. $^{14}NO_3^-$ requires less kinetic energy during the denitrification reaction and would be expected to produce a final product with an isotopic signature less enriched in ^{15}N if the entire pool was not denitrified. Only one in six O atoms is conserved during denitrification by *P. aureofaciens*, suggesting any fractionation of isotopologues of O in NO_3^- may be buffered by O from the sample solution.

Since isotopic bias is minimal at lower $\delta^{15}N$ values, an alternate method would be to use a reference standard with a $\delta^{15}N$ value between 5‰ and 10‰, e.g. IAEA-5 (4.7‰) for KCl-extracted samples. We also used a calibration curve with endmembers USGS-34 and working standard 9.35‰ to compute the KCl reference samples. By extrapolating the calibration curve through the typical natural abundance range of $\delta^{15}N$ values of soil NO_3^- (–5 to 25‰[2]), these standards provided accuracy within 0.4‰ and improved the precision of measurements at higher $\delta^{15}N$ values (Table 2). If enriched isotopic tracers are going to be used in conjunction with KCl extracts, as in ^{15}N tracer studies, it will be imperative to determine the effects of KCl on higher $\delta^{15}N$ values.

Effects of KCl on bacterial denitrification

Since we knew the true isotopic values of the working standards, we were able to further evaluate the relationship between the KCl matrix and bacterial denitrification process using the two-source mixing model. If background NO_3^- in the KCl was the only factor causing isotopic bias, Eqn. (2) should correct the isotopic values of the working standards to their true values. Table 2 shows the results from attempting to use Eqn. (2) to correct the $\delta^{15}N$ and $\delta^{18}O$ values of the working standards in Experiment 4. Equation (2) shifted the raw isotopic values toward their true values, but there remained a bias between the measured and expected values that increased as the isotopic signature of the working standard increased (Table 2).

We evaluated the degree of bias outside the direct mixing of sample NO_3^- and contaminated NO_3^- by calculating the fractionation factor of each of the working standards. First, we calculated the change in the $\delta^{15}N$ and $\delta^{18}O$ values at each working standard by subtracting the δ value corrected by Eqn. (2) from the expected δ value (Eqn. (3)). We then used Eqn. (4) to calculate the fractionation factor, α, for each working standard (Table 2).

$$\Delta_{KCl} = \delta_{STD} - \delta_{CORR} \quad (3)$$

$$\alpha = (\Delta_{KCl}/1000) - 1 \quad (4)$$

Table 2. Methods for calculating $\delta^{15}N$ and $\delta^{18}O$ values for working standards made in KCl. The first column shows the true isotopic values of the KCl working standards. The second and third columns show the values of the working standards in 1 M KCl being calibrated against reference standards made in DIW and reference standards made in KCl, respectively. The fourth column shows the values of the KCl working standards using Eqn. (2). The fifth column shows the values of α as calculated using Eqns. (3) and (4). The last column in (A) shows the values of the KCl working standards after secondary calibration using USGS 32 and working standard 9.53‰

A	Reference standards in DIW (True Values)	Calculated using USGS-32 and USGS-34 in DIW	Calculated using USGS-32 and USGS-34 in KCl	Calculated using Eqn. (2)	α	Calculated using endmembers USGS-32 and Working Standard 9.53
USGS-34	–1.80	–2.03	–1.80	–2.21	0.999753	–1.8
W.S. 9.53	9.53	7.96	10.46	7.80	1.001199	9.53
	23.85	20.94	26.37	20.41	1.003065	24.24
	47.69	43.09	53.55	41.70	1.005878	49.35
	96.10	87.07	107.50	84.51	1.012002	99.21
	121.21	110.03	135.67	105.36	1.016528	125.24
USGS-32	180.01	146.17	179.99	158.64	1.022683	166.21

B	Reference standards in DIW (True Values)	Calculated using USGS-32 and USGS-34 in DIW	Calculated using USGS-32 and USGS-34 in KCl	Calculated using Eqn. (2)	α
USGS-34	–27.90	–25.08	–27.90	–24.36	0.996462
	–24.42	–22.34	–24.88	–21.29	0.996872
	–20.00	–18.92	–21.12	–17.88	0.997877
	–12.63	–11.21	–12.66	–11.67	0.999045
	2.11	2.51	2.42	0.52	1.001595
	9.48	9.86	10.49	6.16	1.003322
USGS-32	25.70	23.71	25.70	20.22	1.005485

Instead of observing a consistent level of fractionation across the working standards, we noted a linear increase in α as the $\delta^{15}N$ and $\delta^{18}O$ values of the working standards increased (N: $r^2 = 0.999$, $p < 0.0001$; O: $r^2 = 0.999$, $p < 0.0001$; Table 2). We hypothesize that a secondary interaction occurring between the KCl and the bacteria caused a non-constant α. Unfortunately, this equation cannot be used to remove bias from actual KCl-extracted samples, since it requires an unknown to be analyzed in both H_2O and KCl matrix. Still, these results highlight the fact that exposure of *P. aureofaciens* to high molarity solutions of KCl introduces substantial error in isotopic analysis beyond those caused by background levels of NO_3^- in reagent KCl and that extra caution must be shown when using the microbial denitrifier method for ^{15}N-tracer studies.

CONCLUSIONS

Measurement of the $\delta^{15}N$ and $\delta^{18}O$ values of NO_3^- in KCl extracts is complicated by background levels of NO_3^- in KCl reagents and the physiological effects of KCl on *P. aureofaciens*. These artifacts can be corrected within the natural abundance range of $\delta^{15}N$ values using certified isotopic standards prepared in KCl solution identical to the samples. When analyzing samples in a KCl matrix, it is imperative that the samples are all diluted to the same concentration so that the same amount of KCl is added to each vial of broth. In this way, each sample will be equally spiked by background NO_3^- and the amount of fractionation that occurs due to physiological effects of KCl on *P. aureofaciens* will be consistent. Furthermore, the same batch of KCl that was used to extract the samples needs to be used to make the dilutions as the background NO_3^- in each variety of KCl has a unique isotopic signature. Before running a full set of samples it is recommended that KCl blanks be analyzed to determine the level of background contamination and its isotopic signature. The accuracy of the measurements allows the $\delta^{15}N$ and $\delta^{18}O$ values to be measured to an accuracy of 0.2‰ within the typical natural abundance range. This technique will allow for the accurate measurement of $\delta^{15}N$ and $\delta^{18}O$ values in low concentration NO_3^- (<1 μg m^{-3}) samples within a KCl matrix. This can help determine nitrogen cycling in nutrient-deficient environments including the impacts of anthropogenic inputs to soil systems.

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