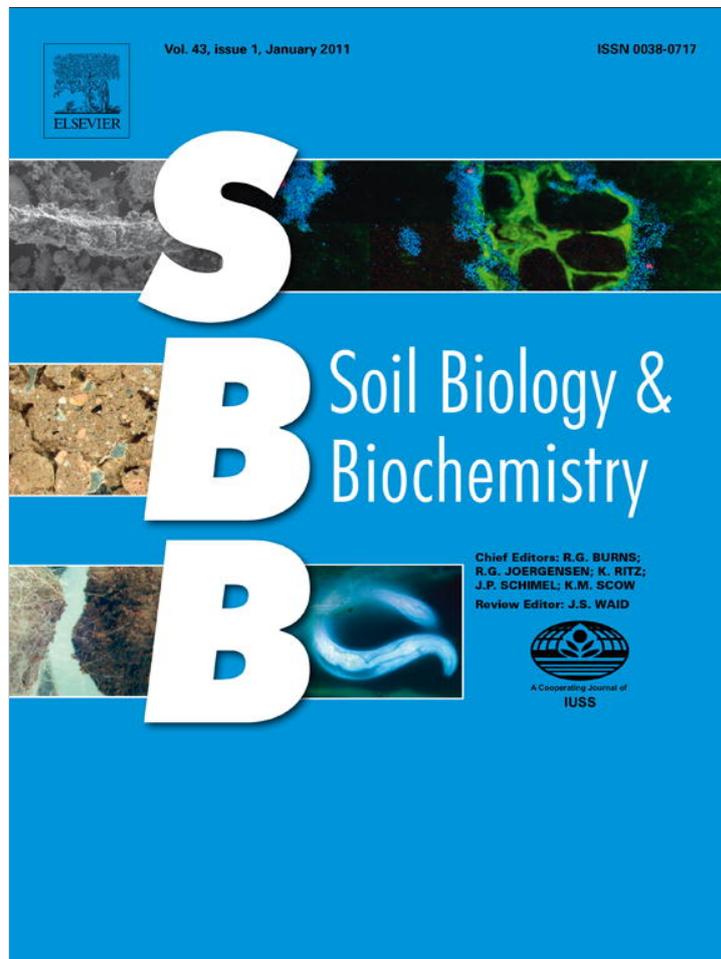


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## A new approach for removing iron interference from soil nitrate analysis

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## ABSTRACT

The accurate measurement of soil nitrate (NO<sub>3</sub><sup>-</sup>) is critical for determining rates of nitrogen (N) cycling and potential N losses from ecosystems. Iron (Fe) can interfere with the colorimetric NO<sub>3</sub><sup>-</sup> analysis of soil extracts to cause the appearance of anomalously low NO<sub>3</sub><sup>-</sup> concentrations. To resolve the interference, imidazole or NH<sub>4</sub>Cl–DTPA has been recommended to replace NH<sub>4</sub>Cl–EDTA as the buffer in the analysis. Here we show that phosphate (PO<sub>4</sub><sup>3-</sup>) filtration can completely remove Fe interference whereas any of these buffers alone may not. Regardless of which buffer was used, 5.5–55 mg Fe L<sup>-1</sup> ferrous iron (Fe<sup>2+</sup>) interfered with NO<sub>3</sub><sup>-</sup> determination in 0.3 mg N–NO<sub>3</sub><sup>-</sup> L<sup>-1</sup> 2 M KCl solutions. Phosphate filtration removed all detectable Fe<sup>2+</sup> in 2 M KCl anaerobic soil slurry extracts with high Fe<sup>2+</sup> concentrations (25.9 ± 1.7 mg Fe<sup>2+</sup> L<sup>-1</sup>). With each of the three buffers tested, the measured NO<sub>3</sub><sup>-</sup> concentrations in the anaerobic soil slurry extracts were significantly higher with PO<sub>4</sub><sup>3-</sup> filtration compared to without filtration. After filtration, the measured NO<sub>3</sub><sup>-</sup> concentrations were similar across all three buffers, suggesting that NO<sub>3</sub><sup>-</sup> concentrations were accurately measured in PO<sub>4</sub><sup>3-</sup> filtered soil extracts regardless of the buffer used. The Fe:N ratio of Fe interference with NO<sub>3</sub><sup>-</sup> determination depended on Fe concentration, NO<sub>3</sub><sup>-</sup> concentration, buffer, and cadmium column age, so that the amount of Fe interference that could occur can be difficult to predict. We suggest comparing measured NO<sub>3</sub><sup>-</sup> concentrations for unfiltered and PO<sub>4</sub><sup>3-</sup> filtered soil extracts to determine the potential for Fe interference in colorimetric NO<sub>3</sub><sup>-</sup> determination as standard additions may not detect all forms of Fe interference.

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## 1. Introduction

Nitrate (NO<sub>3</sub><sup>-</sup>) is a mineral form of nitrogen (N) and a major nutrient for plant and microbial metabolism. Nitrate can become a groundwater pollutant if leached from soil, lead to eutrophication of aquatic and coastal ecosystems, and contribute to air pollution and climate change through the emissions of NO<sub>x</sub> and N<sub>2</sub>O from nitrification and denitrification (Townsend et al., 2003). The measurement of soil NO<sub>3</sub><sup>-</sup> concentrations is both commonplace and critical to biogeochemical, ecological, and environmental research. In iron (Fe) rich soils, NO<sub>3</sub><sup>-</sup> can be significantly underestimated due to interference during colorimetric analyses.

Iron interference with colorimetric NO<sub>3</sub><sup>-</sup> analysis has long been recognized (Horita et al., 1997; Vaughan et al., 1993). The interference manifests itself as anomalously low measured NO<sub>3</sub><sup>-</sup> concentrations (Colman et al., 2007; Davidson et al., 2008) as well as

negative peaks, baseline drift, peak shift, calibration check failures, and cadmium (Cd) column poisoning (personal observation). Recently, Colman and Schimel (2010) demonstrated that the likely mechanism for Fe interference is competition by ferrous iron (Fe<sup>2+</sup>) with *N*-(1-naphthyl)-ethylenediamine (NED), the color reagent for the diazo intermediate in the modified Griess–Ilosvay reaction. Ferric iron (Fe<sup>3+</sup>) can be reduced to Fe<sup>2+</sup> in the Cd column, so the interference can occur regardless of the Fe redox state in the soil extract. Experiments using pure solutions show that Fe interference occurs with Fe concentrations above 50 mg Fe L<sup>-1</sup> (Colman et al., 2007; Davidson et al., 2008), but interference at lower Fe concentrations has not been tested.

Various buffers for the modified Griess–Ilosvay reaction have been used to overcome Fe interference. Colman and Schimel (2010) provided a thorough historical review of how ammonium chloride ethylenediaminetetraacetic acid (NH<sub>4</sub>Cl–EDTA) became the standard buffer used. However, the NH<sub>4</sub>Cl–EDTA buffer creates more Fe interference than it resolves. Imidazole is another widely used buffer (e.g., Hales et al., 2004; Patton et al., 2002), but imidazole may cause Fe to precipitate to form Fe hydroxides that would interfere with photometric analysis and/or coat the Cd column to decrease the surface area available to reduce NO<sub>3</sub><sup>-</sup> (Herzprung et al., 2005; Nydahl, 1976). A NH<sub>4</sub>Cl buffer containing 1 g L<sup>-1</sup> of

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diethylenetriaminepentaacetic acid (DTPA) can prevent Fe interference at  $\text{Fe}^{2+}$  concentrations up to  $200 \text{ mg Fe L}^{-1}$ , presumably by the DTPA chelating the Fe so that it cannot react with the diazo intermediate (Colman and Schimel, 2010).

Here we present an alternative solution to the Fe interference problem that has been used successfully in our lab for over a decade. Phosphate ( $\text{PO}_4^{3-}$ ) is added to soil extracts and the resulting Fe precipitate is removed by membrane filtration. We report on a series of four experiments that (1) compare the effectiveness of buffers alone in resolving Fe interference with  $\text{NO}_3^-$  analysis at low Fe concentrations ( $<50 \text{ mg Fe}^{2+} \text{ L}^{-1}$ ), (2) demonstrate that  $\text{PO}_4^{3-}$  does not interfere with colorimetric  $\text{NO}_3^-$  analysis, (3) demonstrate that  $\text{PO}_4^{3-}$  filtration removes Fe and its interference with colorimetric  $\text{NO}_3^-$  analysis, and (4) characterize the Fe:N ratio of Fe interference with  $\text{NO}_3^-$  determination.

## 2. Materials and methods

### 2.1. Experimental design

For the first experiment, we used pure solutions to determine if Fe interference occurs at low Fe concentrations ( $<50 \text{ mg Fe}^{2+} \text{ L}^{-1}$ ). We prepared a  $0.3 \text{ mg N L}^{-1}$   $\text{KNO}_3$  solution in 2 M KCl and added  $100 \text{ mM Fe(II)Cl}_2$  in 0.5 N HCl to achieve 0, 5.5, 27.5, or  $55 \text{ mg Fe}^{2+} \text{ L}^{-1}$  in replicate aliquots ( $n = 5$  per  $\text{Fe}^{2+}$  concentration). The dilution effect of adding the Fe solutions to the aliquots was within expected measurement error ( $0.01 \text{ mg N L}^{-1}$ ) at the concentration of the  $\text{NO}_3^-$  solution,  $0.3 \text{ mg N L}^{-1}$ .

For the second experiment to determine if  $\text{PO}_4^{3-}$  interferes with  $\text{NO}_3^-$  analysis, we prepared 2 M KCl solutions with a  $\text{NO}_3^-$  concentration of  $0.3 \text{ mg N L}^{-1}$  and with  $\text{PO}_4^{3-}$  concentrations of 0, 10, or 20 mM ( $n = 5$  per treatment). We added  $100 \mu\text{L}$  and  $200 \mu\text{L}$  of 1 M  $\text{Na}_2\text{HPO}_4$  to 5 mL aliquots of the  $\text{NO}_3^-$  solution to achieve 10 and 20 mM  $\text{PO}_4^{3-}$ , respectively. The 1 M  $\text{Na}_2\text{HPO}_4$  solution was made by dissolving 26.8 g  $\text{Na}_2\text{HPO}_4$  in 100 mL heated deionized water. The solution was returned to room temperature before it was added to the samples.

For the third experiment, we compared measured background  $\text{NO}_3^-$  concentrations of unfiltered versus filtered soil extracts as well as evaluated the recovery of a  $0.3 \text{ mg N L}^{-1}$   $\text{NO}_3^-$  standard addition in both types of extracts. We used 2 M KCl extracts of soil (0–10 cm depth) from a humid tropical forest in the Luquillo Experimental Forest, Puerto Rico (5:1 ratio of 2 M KCl volume to g oven dry equivalent soil). We used extracts from fresh soil (referred to as “ambient soil”) as well as soil slurries (4:1 ratio of DI water to dry soil) that had been incubated in an oxygen-free headspace for three days to increase  $\text{Fe}^{2+}$  concentrations and decrease  $\text{NO}_3^-$  concentrations (referred to as “anaerobic soil slurries”) to provide a wider range of concentrations for comparison ( $n = 5$  per extract type). The extracts were frozen for two months before analysis.

For the  $\text{PO}_4^{3-}$  filtration procedure,  $200 \mu\text{L}$  of 1 M  $\text{Na}_2\text{HPO}_4$  solution was added to 10 mL of soil extract contained in a 15 mL polypropylene centrifuge tube to attain a final concentration of  $10 \text{ mM PO}_4^{3-}$  in the soil extract. Previous work showed that  $5 \text{ mM PO}_4^{3-}$  was sufficient to prevent Fe interference in our soil extracts, so  $10 \text{ mM PO}_4^{3-}$  is regularly used to ensure that there is sufficient  $\text{PO}_4^{3-}$  to remove iron from most, if not all, soil extracts analyzed (data not shown). The tube was capped, and the extract and  $\text{PO}_4^{3-}$  were shaken vigorously by hand for 10–20 s to precipitate the Fe. The extract was then syringe-filtered through a  $0.45 \mu\text{m}$  membrane filter. Five mL of the filtered extract and  $50 \mu\text{L}$  of  $30 \text{ mg N L}^{-1}$   $\text{KNO}_3$  were pipetted into a new polypropylene tube. The tube was capped and shaken by hand to mix well. This procedure was repeated with a 10 mL unfiltered aliquot of each soil extract. Nitrate standard addition recovery was calculated as the difference in measured

$\text{NO}_3^-$  concentration with and without a known quantity of  $\text{NO}_3^-$  added.

For the fourth experiment, we characterized the Fe:N ratio of Fe interference using 2 M KCl solutions containing either 0.3 or  $3 \text{ mg N L}^{-1}$   $\text{KNO}_3$ . We separately added  $50 \mu\text{L}$  of 0, 100, 200, 300, 400, or  $500 \text{ mM Fe(II)Cl}_2$  in 0.5 N HCl to  $4.95 \text{ mL}$  of the  $\text{NO}_3^-$  solutions to achieve 0, 55, 110, 165, 220, and  $275 \text{ mg Fe}^{2+} \text{ L}^{-1}$  in replicate 5 mL aliquots ( $n = 5$  per  $\text{Fe}^{2+}$  concentration). We quantified Fe interference as the difference between the actual and measured  $\text{NO}_3^-$  concentrations. Thus, when negative peaks were measured, the amount of Fe interference was greater than the actual  $\text{NO}_3^-$  concentration. We calculated the Fe:N ratio of Fe interference as the Fe molar concentration (mM) divided by the Fe interference expressed in  $\text{NO}_3^-$  molar concentration (mM).

### 2.2. Sample analyses

For all experiments the samples were analyzed on a flow-injection autoanalyzer (Lachat Instruments, Milwaukee, WI) in three repeated runs using the following buffers in this sequence: imidazole,  $\text{NH}_4\text{Cl}$ –DTPA, and  $\text{NH}_4\text{Cl}$ –EDTA. The buffers were made according to the recipes described in Colman and Schimel (2010). Each buffer was allowed to flow through the Cd column for 5–10 min before  $\text{NO}_3^-$  analysis to flush out the previous buffer. For the first three experiments we utilized a well conditioned Cd column (approximately 2000 samples analyzed) because  $\text{PO}_4^{3-}$  interference with  $\text{NO}_3^-$  analysis has been documented under these conditions (Olson, 1980). For the fourth experiment we used a Cd column that had previously been used to analyze approximately 1000 samples. The modified Griess–Ilosvay method used for colorimetric  $\text{NO}_3^-$  analysis measures  $\text{NO}_3^-$  + nitrite ( $\text{NO}_2^-$ ) concentrations, but we report only on  $\text{NO}_3^-$  because our pure solutions did not contain  $\text{NO}_2^-$  and our soil extracts most likely did not contain  $\text{NO}_2^-$ , which is highly reactive and quickly consumed in soils.

Ferrous iron concentrations for unfiltered and filtered soil extracts were measured using a modified ferrozine assay (Liptzin and Silver, 2009). The samples were analyzed manually on a spectrophotometer (Spectronic 20, Milton Roy, Ivyland, PA for the first three experiments and Genesys 20, Thermo Fisher Scientific, Waltham, MA for the fourth experiment). Standards were prepared using  $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$  in 0.5 N HCl.

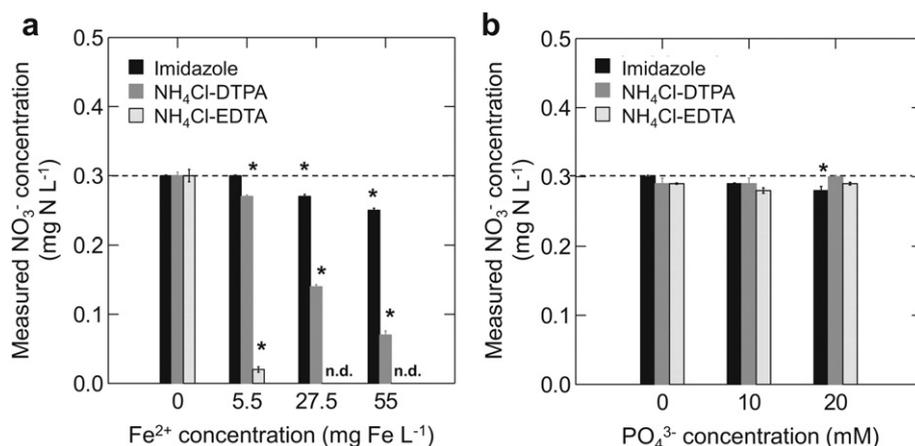
### 2.3. Statistical analysis

We used SYSTAT Version 10 (SPSS Inc., Evanston, IL) to perform statistical analyses. Analysis of variance and Tukey tests were used to identify statistically significant differences in measured  $\text{NO}_3^-$  concentrations,  $\text{NO}_3^-$  standard addition recovery, and Fe:N ratio of Fe interference among buffers. Measured  $\text{NO}_3^-$  concentrations and  $\text{NO}_3^-$  standard addition recovery for unfiltered and filtered soil extracts were compared using *t*-tests. Mean values are reported in the text followed by standard errors ( $\pm\text{SE}$ ). Statistical significance was determined at  $p < 0.05$  unless otherwise noted.

## 3. Results

### 3.1. Fe interference in pure solutions

We detected Fe interference in colorimetric  $\text{NO}_3^-$  determination at  $\text{Fe}^{2+}$  concentrations between 5.5 and  $55 \text{ mg Fe}^{2+} \text{ L}^{-1}$  for all buffers tested ( $p < 0.05$ ; Fig. 1a). The imidazole buffer was least sensitive to Fe interference, with a small but statistically significant under-determination of  $\text{NO}_3^-$  concentration at  $27.5 \text{ mg Fe}^{2+} \text{ L}^{-1}$ . In contrast, the measured  $\text{NO}_3^-$  concentration using the  $\text{NH}_4\text{Cl}$ –DTPA buffer was only half of the actual concentration of  $0.3 \text{ mg N L}^{-1}$



**Fig. 1.** Measured  $\text{NO}_3^-$  concentrations in 2 M KCl solutions containing (a)  $\text{Fe}^{2+}$  or (b)  $\text{PO}_4^{3-}$  at different concentrations ( $n = 5$  per treatment). The dashed line indicates the actual  $\text{NO}_3^-$  concentration of the solutions, and asterisks indicate measured  $\text{NO}_3^-$  concentrations that differed significantly from the actual concentration. n.d. indicates  $\text{NO}_3^-$  concentrations that were not determined due to baseline shifts from Fe interference that prevented peak integration. Errors bars represent standard errors.

with an Fe concentration of 27.5  $\text{mg Fe}^{2+} \text{ L}^{-1}$ . The  $\text{NH}_4\text{Cl-EDTA}$  buffer was highly sensitive to Fe interference, with the measured  $\text{NO}_3^-$  concentration 93% less than the actual concentration in solutions containing only 5.5  $\text{mg Fe}^{2+} \text{ L}^{-1}$ .

### 3.2. $\text{PO}_4^{3-}$ effects on $\text{NO}_3^-$ analysis

Phosphate did not interfere with  $\text{NO}_3^-$  analysis at 10 mM concentration, which is the concentration we use for  $\text{PO}_4^{3-}$  filtration (Fig. 1b). There were no significant differences in measured  $\text{NO}_3^-$  concentrations among all  $\text{PO}_4^{3-}$  treatments using the  $\text{NH}_4\text{Cl-DTPA}$  and  $\text{NH}_4\text{Cl-EDTA}$  buffers. The measured  $\text{NO}_3^-$  concentration at 20 mM  $\text{PO}_4^{3-}$  was significantly lower than at 0 mM  $\text{PO}_4^{3-}$  when using the imidazole buffer ( $p = 0.008$ ). However, the measured  $\text{NO}_3^-$  concentration was only 0.02  $\text{mg N L}^{-1}$  lower than expected.

### 3.3. Fe interference in soil extracts

For the anaerobic soil slurry extracts, the measured background soil  $\text{NO}_3^-$  concentrations were significantly lower without  $\text{PO}_4^{3-}$  filtration compared to with filtration for all three buffers ( $p < 0.02$ ). In contrast, there were no significant differences for the ambient soil extracts using any of the buffers (Fig. 2a, b). The

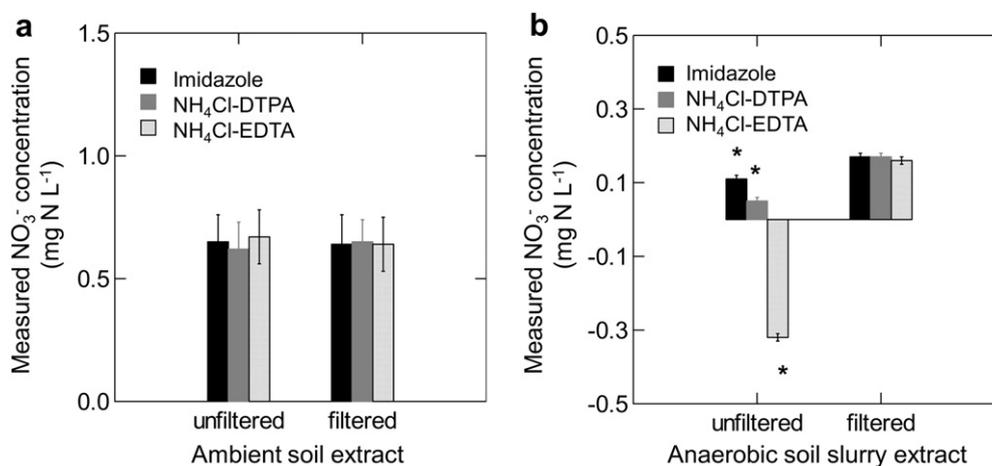
$\text{NH}_4\text{Cl-EDTA}$  buffer resulted in large negative peaks measured for the unfiltered anaerobic soil slurry extracts.

Phosphate filtration using 10 mM  $\text{PO}_4^{3-}$  removed all detectable  $\text{Fe}^{2+}$  from the soil extracts (data not shown) and resulted in similar measured  $\text{NO}_3^-$  concentrations using all three buffers (Fig. 2a, b). The anaerobic soil slurry extracts contained  $25.9 \pm 1.7 \text{ mg Fe}^{2+} \text{ L}^{-1}$  before filtration and no detectable  $\text{Fe}^{2+}$  after filtration. The ambient soil extracts contained no detectable  $\text{Fe}^{2+}$  before or after filtration.

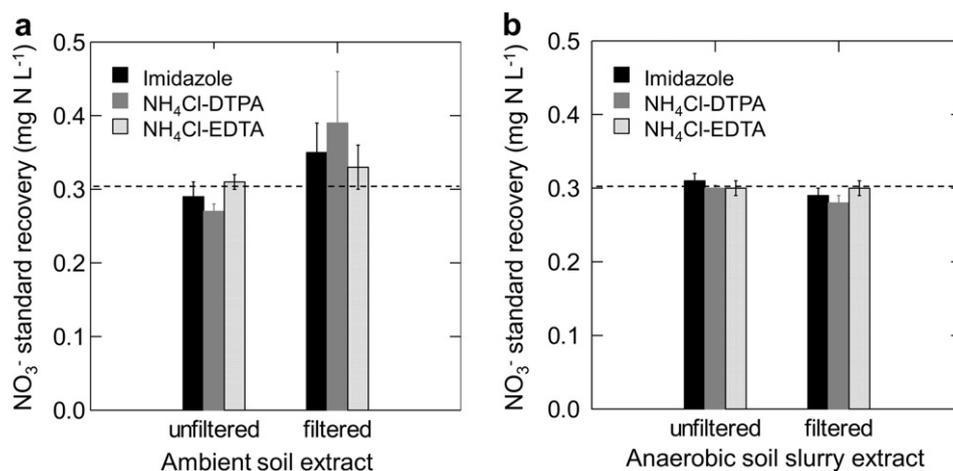
Unexpectedly, we completely recovered the  $\text{NO}_3^-$  standard addition in all soil extracts, regardless of filtration, Fe concentration, or buffer (Fig. 3). This result was particularly strange because, for the unfiltered anaerobic soil slurry extracts analyzed using the  $\text{NH}_4\text{Cl-EDTA}$  buffer, the peaks remained negative with the standard addition but were less negative by an amount corresponding to the intended increase in  $\text{NO}_3^-$  concentration.

### 3.4. Fe:N ratio of Fe interference

The Fe:N ratio of Fe interference was significantly greater for the imidazole buffer than for the  $\text{NH}_4\text{Cl-DTPA}$  and  $\text{NH}_4\text{Cl-EDTA}$  buffers ( $p < 0.001$ ). It ranged from 395 to 7529 for imidazole, from 33 to 132 for  $\text{NH}_4\text{Cl-DTPA}$ , and from 8 to 51 for  $\text{NH}_4\text{Cl-EDTA}$ . This wide range in Fe:N ratios observed in this study for each buffer was



**Fig. 2.** Measured  $\text{NO}_3^-$  concentrations in unfiltered versus  $\text{PO}_4^{3-}$  filtered 2 M KCl extracts of (a) ambient soil and (b) anaerobic soil slurries ( $n = 5$  per treatment). Asterisks indicated significantly different measured  $\text{NO}_3^-$  concentrations between unfiltered and  $\text{PO}_4^{3-}$ -filtered extracts. Error bars represent standard errors.



**Fig. 3.** Recovery of 0.3 mg N L<sup>-1</sup> NO<sub>3</sub><sup>-</sup> standard addition in 2 M KCl extracts of (a) ambient soil and (b) anaerobic soil slurries (*n* = 5 per treatment). The dashed line indicates the NO<sub>3</sub><sup>-</sup> standard addition concentration (i.e., 100% recovery). Error bars represent standard errors.

due in part to a non-linear increase in Fe interference as Fe<sup>2+</sup> concentration increased (Fig. 4). The Fe:N ratio was also greater for the 0.3 mg N L<sup>-1</sup> solution than the 3 mg N L<sup>-1</sup> solution for all three buffers (*p* < 0.05). Negative peaks measured in the presence of high Fe concentrations (>50 mg Fe<sup>2+</sup> L<sup>-1</sup>) caused Fe interference, defined as the difference between actual and measured NO<sub>3</sub><sup>-</sup> concentrations, to be greater than the actual NO<sub>3</sub><sup>-</sup> concentration as shown in Fig. 4.

#### 4. Discussion

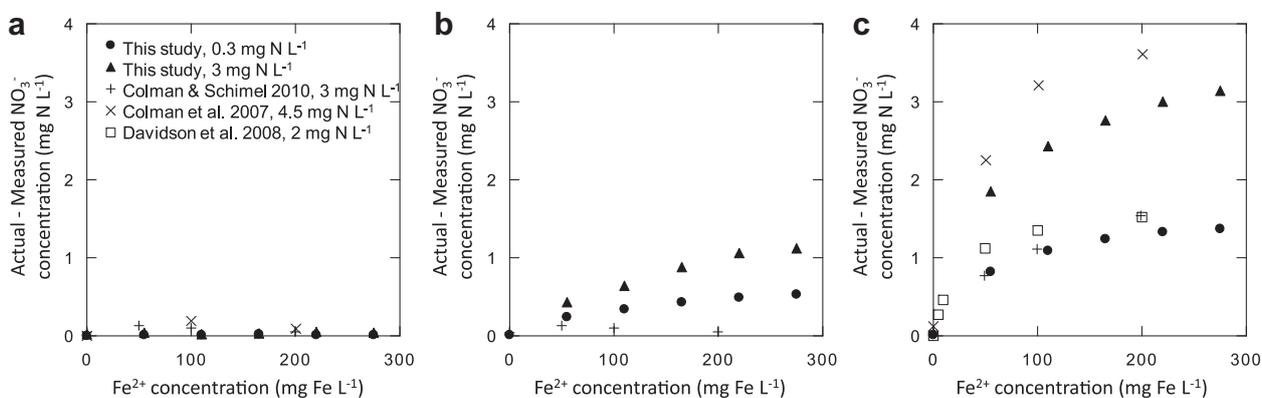
##### 4.1. Fe interference in pure solutions

We documented Fe interference in colorimetric NO<sub>3</sub><sup>-</sup> determination for solutions with Fe<sup>2+</sup> concentrations below 55 mg L<sup>-1</sup> for all three buffers tested, suggesting that buffers alone cannot resolve Fe interference. This finding differs from other studies that have shown that the imidazole and NH<sub>4</sub>Cl-DTPA buffers can remove all of the Fe interference for 3–4.5 mg N L<sup>-1</sup> standards in water with up to 200 mg Fe L<sup>-1</sup> (Colman et al., 2007; Colman and Schimel, 2010). The NO<sub>3</sub><sup>-</sup> solution concentrations used in these studies translate into high soil NO<sub>3</sub><sup>-</sup> concentrations, roughly equivalent to 15–22.5 mg N kg<sup>-1</sup> if we assume a soil extraction ratio of 5:1 (mL of 2 M KCl to g dry soil, Silver et al., 2001). Soil NO<sub>3</sub><sup>-</sup> concentrations can range from 0 to 100 mg N kg<sup>-1</sup>, but they are generally less than

15 mg N kg<sup>-1</sup> (Booth et al., 2005; Taylor and Townsend, 2010; Vitousek and Matson, 1988). Moreover, soils that experience reducing conditions typically have lower NO<sub>3</sub><sup>-</sup> concentrations (<1 mg N kg<sup>-1</sup>) due to low nitrification rates and high rates of denitrification and dissimilatory NO<sub>3</sub><sup>-</sup> reduction to NH<sub>4</sub><sup>+</sup> (e.g., Silver et al., 2001). Using solutions with NO<sub>3</sub><sup>-</sup> concentrations at 0.3 mg N–NO<sub>3</sub><sup>-</sup> L<sup>-1</sup> (roughly equivalent to 1.5 mg N kg<sup>-1</sup>), we were able to detect Fe interference that may be exceeded by measurement error or natural variability at higher NO<sub>3</sub><sup>-</sup> concentrations. For example, at 55 mg Fe<sup>2+</sup> L<sup>-1</sup> and using the imidazole buffer, the measured NO<sub>3</sub><sup>-</sup> concentration was 0.05 ± 0.003 mg N L<sup>-1</sup> or 0.25 mg N kg<sup>-1</sup> less than the actual NO<sub>3</sub><sup>-</sup> concentration. Small under-determinations of NO<sub>3</sub><sup>-</sup> concentrations such as this can lead to large errors in estimates of gross N cycling rates determined via <sup>15</sup>N pool dilution, especially when NO<sub>3</sub><sup>-</sup> pools are small.

##### 4.2. PO<sub>4</sub><sup>3-</sup> effects on NO<sub>3</sub><sup>-</sup> analysis

We did not observe an effect of PO<sub>4</sub><sup>3-</sup> on colorimetric NO<sub>3</sub><sup>-</sup> analysis for PO<sub>4</sub><sup>3-</sup> concentrations up to 20 mM. This contrasts with a previous study that demonstrated that up to 50 mM PO<sub>4</sub><sup>3-</sup> interfered with NO<sub>3</sub><sup>-</sup> reduction on the Cd column for 1–20 mg N L<sup>-1</sup> NO<sub>3</sub><sup>-</sup> standards in water (Skicko and Tawfik, 1988). Olson (1980) also observed PO<sub>4</sub><sup>3-</sup> interference in seawater that was greater on older Cd columns compared to newer ones. These studies provided no



**Fig. 4.** Iron interference in NO<sub>3</sub><sup>-</sup> determination (i.e., actual – measured NO<sub>3</sub><sup>-</sup> concentration) versus Fe concentration using different buffers: (a) imidazole, (b) NH<sub>4</sub>Cl-DTPA, and (c) NH<sub>4</sub>Cl-EDTA. Symbols represent data from this study and other studies that used pure solutions at different NO<sub>3</sub><sup>-</sup> concentrations.

mechanism for the interference, but suggested that it was a surface effect on the Cd column based on the observations that the interference was reversible (Olson, 1980; Skicko and Tawfik, 1988). We used an older Cd column to accentuate any potential interference in our experiment, but we did not find any  $\text{PO}_4^{3-}$  interference with colorimetric  $\text{NO}_3^-$  analysis in 2 M KCl solutions. Our data suggest that the use of  $\text{PO}_4^{3-}$  to precipitate Fe in 2 M KCl soil extracts will not cause additional interferences in colorimetric  $\text{NO}_3^-$  analysis.

#### 4.3. Fe interference in soil extracts

We demonstrated that  $\text{PO}_4^{3-}$  filtration quantitatively removed  $\text{Fe}^{2+}$  from soil extracts, completely resolving the Fe interference problem. Measured  $\text{NO}_3^-$  concentrations were comparable for all three buffers after  $\text{PO}_4^{3-}$  filtration, suggesting that  $\text{NO}_3^-$  concentrations were accurately measured following the removal of Fe. Phosphate filtration did not increase measurement errors or significantly change measured  $\text{NO}_3^-$  concentrations in the ambient soil extracts with no detectable  $\text{Fe}^{2+}$ .

Standard addition is a common approach for identifying interference in chemical analyses, but we completely recovered 0.3 mg  $\text{N L}^{-1}$   $\text{KNO}_3$  standard additions in both the ambient soil and anaerobic soil slurry extracts using all three buffers. We know that the anaerobic soil slurry extracts exhibited Fe interference because we measured different  $\text{NO}_3^-$  concentrations in the extracts using the three buffers without  $\text{PO}_4^{3-}$  filtration (i.e., Fe removal) and comparable  $\text{NO}_3^-$  concentrations using the three buffers with  $\text{PO}_4^{3-}$  filtration. The complete standard recovery in the presence of Fe was unexpected because the previously described Fe interference pathways would cause a decrease in the slope between expected and measured  $\text{NO}_3^-$  concentrations across a range of standard additions (i.e., due to direct chemical interference with the colorimetric reaction, Colman et al., 2008; Colman and Schimel, 2010) or lower than expected measured concentrations (i.e., from over-reduction of  $\text{NO}_3^-$  due to clogging in fine-grained Cd columns, Gales and Booth, 1975; or inefficient reduction of  $\text{NO}_3^-$  to  $\text{NO}_2^-$  due to column poisoning). There are several possible reasons for the unexpected standard addition recovery: (1) the ratio of  $\text{Fe}^{2+}$  to  $\text{NO}_3^-$  to cause interference in colorimetric analysis is very high, (2) the added  $\text{NO}_3^-$  does not interact with the Fe in the same way that the native  $\text{NO}_3^-$  does, and (3) the mode of Fe interference with the modified Griess–Ilosvay method is more complex than previously described. Regardless of the reason, this means that  $\text{NO}_3^-$  standard additions may not be the optimal method for detecting Fe interference in colorimetric  $\text{NO}_3^-$  analysis. Instead, we suggest comparing measured  $\text{NO}_3^-$  concentrations for unfiltered and  $\text{PO}_4^{3-}$  filtered soil extracts.

#### 4.4. Fe:N ratio of Fe interference

If the Fe:N ratio of Fe interference is predictable, then it could be possible to identify and even correct for Fe interference by measuring Fe concentrations in soil extracts. Theoretically, 1 mol of  $\text{Fe}^{2+}$  interferes with color development from 1 mol of  $\text{NO}_3^-$ . However, we found that the Fe:N ratio of Fe interference was highly variable and dependent on multiple factors. First, at a given  $\text{Fe}^{2+}$  concentration, the  $\text{NH}_4\text{Cl}$ –EDTA buffer yielded the greatest underestimates of  $\text{NO}_3^-$  concentrations and the imidazole buffer the least. Second, the amount of Fe interference varied non-linearly with  $\text{Fe}^{2+}$  concentration so that the Fe:N ratio of Fe interference changed with  $\text{Fe}^{2+}$  concentration. Third, at a given Fe concentration, the amount of Fe interference was greater for solutions with higher  $\text{NO}_3^-$  concentrations. Thus, it would be necessary to know the actual  $\text{NO}_3^-$  concentration to predict the Fe:N ratio of Fe interference. These factors together suggest that it would be difficult to predict the occurrence and degree of Fe interference.

We compared the Fe:N ratios of Fe interference measured in our study to those measured in other studies to determine if the patterns in the ratios were consistent. The amount of Fe interference observed across Fe concentrations was similar between this study and Colman and Schimel (2010) for the imidazole buffer (Fig. 4a). However, Colman and Schimel (2010) reported little Fe interference across Fe concentrations for a 3 mg  $\text{N L}^{-1}$  water solution analyzed with  $\text{NH}_4\text{Cl}$ –DTPA whereas we found significant Fe interference under the same analytical conditions for a 2 M KCl solution (Fig. 4b). A difference among studies was even more pronounced when comparing Fe interference observed using the  $\text{NH}_4\text{Cl}$ –EDTA buffer (Fig. 4c). A 2 mg  $\text{N L}^{-1}$  0.5 M  $\text{K}_2\text{SO}_4$  solution tested by Davidson et al. (2008), a 3 mg  $\text{N L}^{-1}$  water solution tested by Colman and Schimel (2010), and a 0.3 mg  $\text{N L}^{-1}$  2 M KCl solution tested in this study exhibited similar Fe:N ratios of Fe interference despite different  $\text{NO}_3^-$  concentrations. All studies were performed using Cd columns on Lachat auto-analyzers with reagents made from the same recipes, but differences in the ionic strength of the sample matrix, instrument operation, or Cd column properties may have contributed to the different Fe:N ratios of Fe interference measured in these studies.

The expression of Fe interference also appears to depend on the age of the Cd column used, with less interference exhibited in newer columns. In the Fe:N ratio experiment, we used a Cd column that had 1000 samples previously run on it as opposed to the Cd column in the other experiments that had 2000 samples previously run on it. With the newer column, we did not observe Fe interference with the use of the imidazole buffer at any of the  $\text{Fe}^{2+}$  concentrations tested ( $<275$  mg  $\text{Fe}^{2+} \text{ L}^{-1}$ ). However, we observed similar Fe interference between old and newer Cd columns with the  $\text{NH}_4\text{Cl}$ –DTPA and  $\text{NH}_4\text{Cl}$ –EDTA buffers. Given that imidazole alone could resolve Fe interference when using newer Cd columns, we suggest the use of imidazole as the buffer for colorimetric  $\text{NO}_3^-$  analysis.

## 5. Conclusion

Our study demonstrates that  $\text{PO}_4^{3-}$  filtration can be used to completely remove Fe and its interference from colorimetric  $\text{NO}_3^-$  analysis in 2 M KCl soil extracts whereas buffers alone may not be sufficient. The removal of Fe interference is particularly important in low  $\text{NO}_3^-$  soils because a small amount of Fe interference can cause a large relative error in measured  $\text{NO}_3^-$  concentrations. The Fe:N ratio of Fe interference is sensitive to the buffer used, the age of the Cd column, and the actual  $\text{NO}_3^-$  concentration. Thus, while Fe interference increases predictably with Fe concentration under given analytical conditions, it may be not be possible to estimate the amount of Fe interference that could occur. In addition, the complete  $\text{NO}_3^-$  standard addition recovery in the presence of Fe suggests that the chemistry involved in the modified Griess–Ilosvay reaction with soil extracts may be more complex than previously described. However,  $\text{NO}_3^-$  concentrations can be accurately measured in  $\text{PO}_4^{3-}$  filtered soil extracts regardless of the buffer used. We strongly encourage investigators to test for Fe interference in their samples and verify the absence of  $\text{PO}_4^{3-}$  interference when using  $\text{PO}_4^{3-}$  filtration to remove Fe interference.

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