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## Ammonium sorption and ammonia inhibition of nitrite-oxidizing bacteria explain contrasting soil N<sub>2</sub>O production

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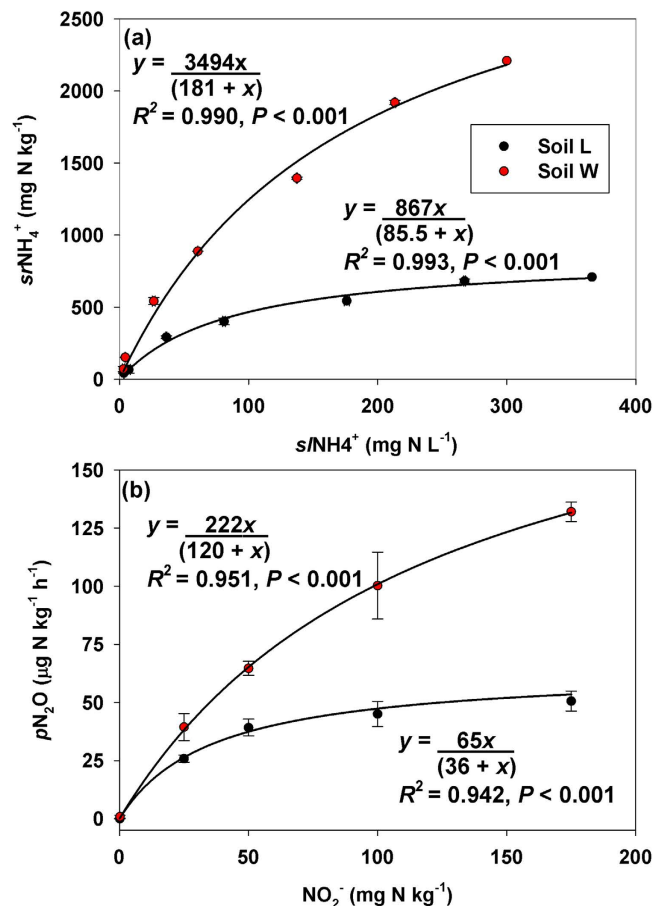
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Rodney T. Venterea<sup>1,2</sup>, Timothy J. Clough<sup>3</sup>, Jeffrey A. Coulter<sup>4</sup> & Florence Breuillin-Sessoms<sup>5</sup>

Better understanding of process controls over nitrous oxide (N<sub>2</sub>O) production in urine-impacted 'hot spots' and fertilizer bands is needed to improve mitigation strategies and emission models. Following amendment with bovine (*Bos taurus*) urine (Bu) or urea (Ur), we measured inorganic N, pH, N<sub>2</sub>O, and genes associated with nitrification in two soils ('L' and 'W') having similar texture, pH, C, and C/N ratio. Solution-phase ammonia (sNH<sub>3</sub>) was also calculated accounting for non-linear ammonium (NH<sub>4</sub><sup>+</sup>) sorption capacities (ASC). Soil W displayed greater nitrification rates and nitrate (NO<sub>3</sub><sup>-</sup>) levels than soil L, but was more resistant to nitrite (NO<sub>2</sub><sup>-</sup>) accumulation and produced two to ten times less N<sub>2</sub>O than soil L. Genes associated with NO<sub>2</sub><sup>-</sup> oxidation (*nxrA*) increased substantially in soil W but remained static in soil L. Soil NO<sub>2</sub><sup>-</sup> was strongly correlated with N<sub>2</sub>O production, and cumulative (c-) sNH<sub>3</sub> explained 87% of the variance in c-NO<sub>2</sub><sup>-</sup>. Differences between soils were explained by greater sNH<sub>3</sub> in soil L which inhibited NO<sub>2</sub><sup>-</sup> oxidation leading to greater NO<sub>2</sub><sup>-</sup> levels and N<sub>2</sub>O production. This is the first study to correlate the dynamics of soil sNH<sub>3</sub>, NO<sub>2</sub><sup>-</sup>, N<sub>2</sub>O and nitrifier genes, and the first to show how ASC can regulate NO<sub>2</sub><sup>-</sup> levels and N<sub>2</sub>O production.

Better understanding of biochemical process controls over soil N<sub>2</sub>O production is needed for improving N<sub>2</sub>O mitigation strategies and emissions models. Incorporation of improved process-related information into models will help improve emissions assessments at field, regional and global scales<sup>1,2</sup>. In cattle grazing systems, urine deposition events typically result in localized N loads equivalent to 1000 kg N ha<sup>-1,3</sup>, and can create 'hot spots' for elevated N<sub>2</sub>O emissions<sup>4</sup>. Similarly, N fertilizers applied in concentrated bands result in localized inorganic N concentrations ranging from several hundred to more than 2000 μg N g<sup>-1,5,6</sup>, and can result in substantially greater N<sub>2</sub>O emissions compared to uniformly applied fertilizer<sup>7-9</sup>. Thus, understanding the processes mediating N<sub>2</sub>O production within these concentrated zones is critical. Urine deposition and Ur application can cause elevated soil NO<sub>2</sub><sup>-</sup> levels which in turn promote elevated N<sub>2</sub>O production<sup>9-11</sup>. Accumulation of NO<sub>2</sub><sup>-</sup> is presumed to occur due to a sequence of chemical and microbial responses. Hydrolysis of Ur results in localized increases in both soil pH and NH<sub>4</sub><sup>+</sup> which together promote the formation of free NH<sub>3</sub><sup>12</sup>. Both groups of autotrophic nitrifying bacteria, i.e., the NH<sub>3</sub>-oxidizing bacteria (AOB) and the NO<sub>2</sub><sup>-</sup> oxidizing bacteria (NOB), are sensitive to NH<sub>3</sub> toxicity, but it is generally believed that NOB are more sensitive than AOB<sup>11-14</sup>; thus, soil NO<sub>2</sub><sup>-</sup> accumulates in the presence of sufficiently high NH<sub>3</sub> levels because NOB are unable to fully process the NO<sub>2</sub><sup>-</sup> produced by AOB.

<sup>1</sup>USDA-ARS, Soil and Water Management Research Unit, St. Paul, MN 55108. <sup>2</sup>Dep. of Soil, Water, and Climate, Univ. of Minnesota, St. Paul, MN 55108. <sup>3</sup>Faculty of Agriculture and Life Science, Lincoln Univ., PO Box 85084, Lincoln 7647, Canterbury, New Zealand. <sup>4</sup>Dep. of Agronomy and Plant Genetics, Univ. of Minnesota, St. Paul, MN 55108. <sup>5</sup>Biotechnology Institute, College of Biological Sciences, Univ. of Minnesota, St. Paul, MN 55108. Correspondence and requests for materials should be addressed to R.V. (email: Venterea@umn.edu)



**Figure 1. Ammonium sorption capacity (ACS) and potential N<sub>2</sub>O production ( $pN_2O$ ).** (a) ACS results showing equilibrium  $NH_4^+$  concentrations in sorbed-phase ( $srNH_4^+$ ) versus solution-phase ( $slNH_4^+$ ) and (b)  $pN_2O$  following addition of nitrite ( $NO_2^-$ ) at 85% field capacity for soils L and W. Symbols are means with vertical standard error bars and lines are regression curves based on replicated data (in form of Eq. (1)). Horizontal error bars are displayed in (a) but are barely visible.

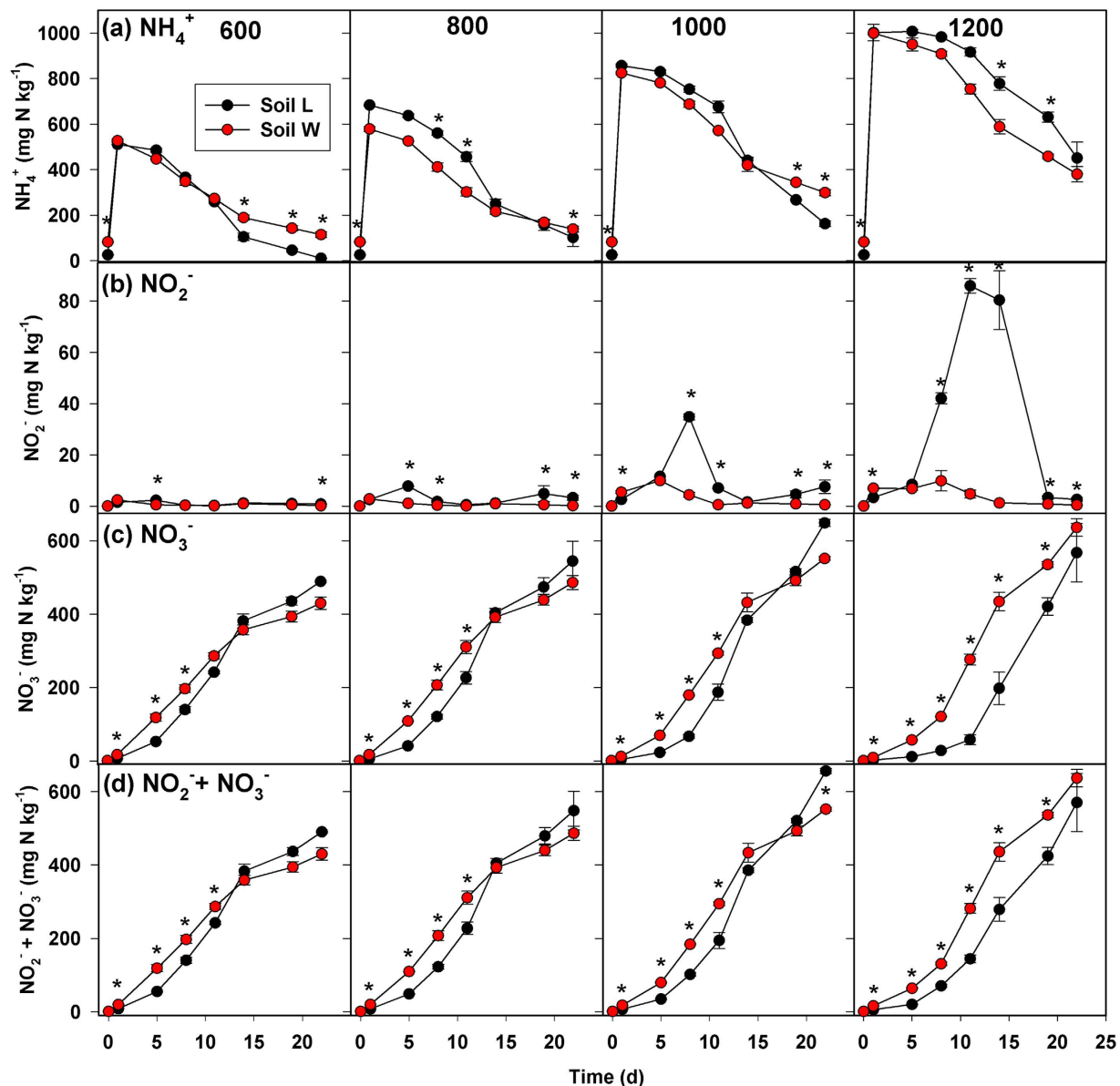
However, beyond this rather general understanding of soil  $NO_2^-$  dynamics, little is known about specific soil properties that regulate  $NO_2^-$  accumulation. While related processes have been well-studied in wastewater systems<sup>14</sup>, the presence of soil surface-solution interactions and other factors complicate our understanding in soils. For example, the partitioning of  $NH_4^+$  between soil surfaces and solution could regulate solution-phase levels and therefore influence nitrifier activity and  $NH_3$  toxicity<sup>15</sup>. Simultaneous quantification of genes associated with activities of NOB, AOB and  $NH_4^+$  oxidizing archaea in soil following amendment with N has been reported in a few studies<sup>16–18</sup>, but more data are needed to understand the role of nitrifier responses in regulating  $NO_2^-$  and  $N_2O$  dynamics. Limited understanding of these and other factors limits our ability to predict  $NO_2^-$  dynamics for a particular soil, management practice, or climate condition, and our ability to predict  $N_2O$  emissions resulting from  $NO_2^-$  transformations<sup>19,20</sup>.

In preliminary experiments, we observed that two soils collected from grazed fields in New Zealand, while having similar texture, pH, C content and C/N ratio, displayed substantially different  $N_2O$  production rates when amended with Ur. Our general hypothesis was that differences in  $NO_2^-$  dynamics were responsible for the contrasting  $N_2O$  production. In this study, we conducted a series of experiments designed to elucidate controls over  $NO_2^-$  and  $N_2O$  production under conditions representative of concentrated BU patches or Ur bands, and to explain the differences in  $N_2O$  production between these soils.

## Results

**Ammonium sorption.** Ammonium sorption, determined in batch equilibrium experiments and modeled using Eq. 1, was significantly greater in soil W, which sorbed more  $NH_4^+$  from solution compared to soil L (Fig. 1a). The modeled sorption parameters  $\mu$  and  $K$  in soil W were four and two times greater, respectively, than in soil L (Fig. 1a).

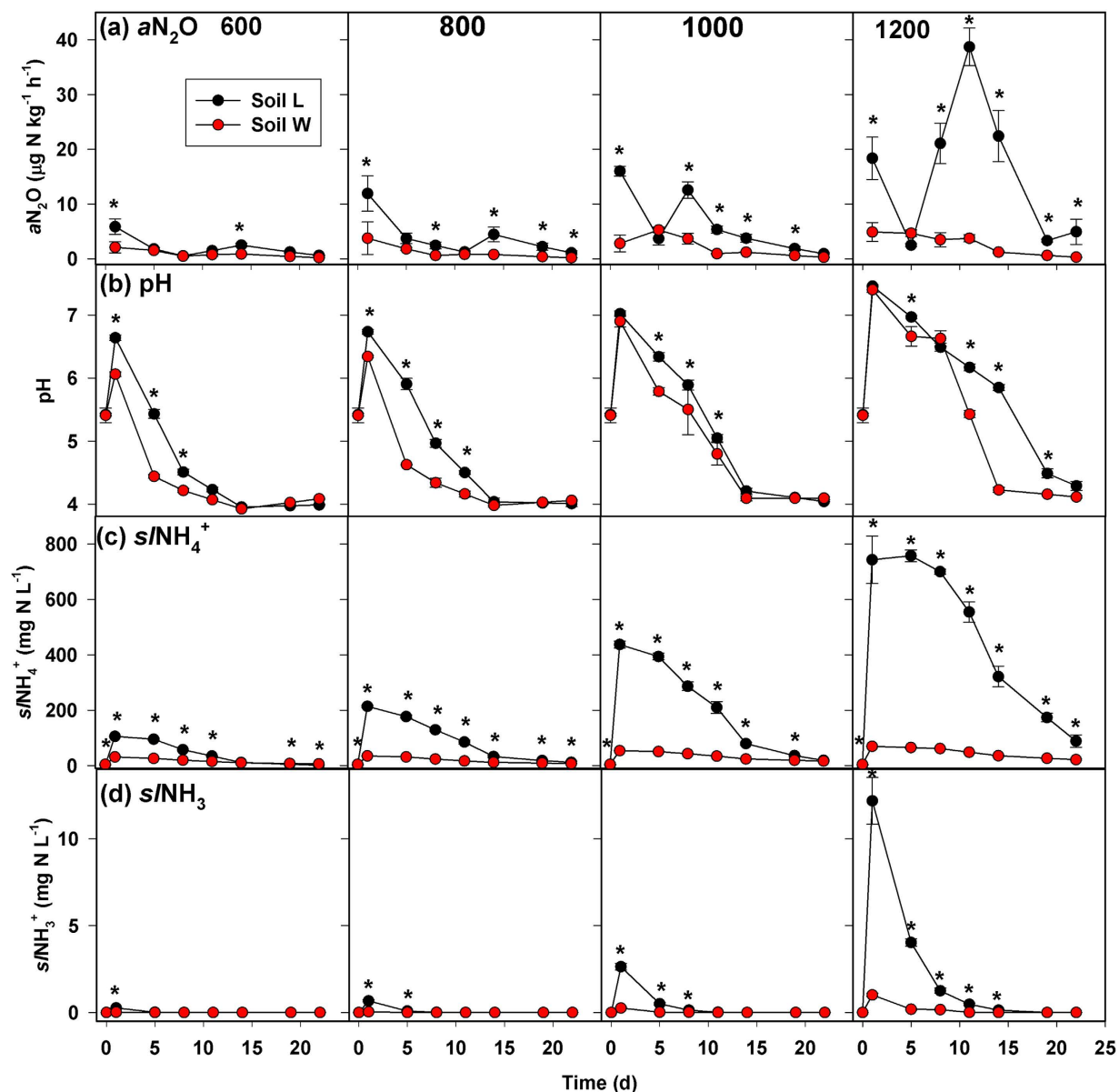
**Nitrite addition experiments.** Soil W produced more  $N_2O$  after amendment with  $NO_2^-$  compared to soil L. The potential  $N_2O$  production rate ( $pN_2O$ ) was well-described by models in the form of Eq. (1)



**Figure 2. Results of Series 1 microcosm experiment.** Concentrations of extractable (a)  $\text{NH}_4^+$ , (b)  $\text{NO}_2^-$ , (c)  $\text{NO}_3^-$  and (d)  $\text{NO}_2^- + \text{NO}_3^-$  following addition of bovine urine at 600, 800, 1000 and 1200  $\text{mg N kg}^{-1}$  at 85% field capacity. Asterisks indicate significant differences between soils at  $P < 0.05$  for a given sampling date.

with sorbed-phase  $\text{NH}_4^+$  ( $s\text{rNH}_4^+$ ) replaced by potential  $\text{N}_2\text{O}$  production rate ( $p\text{N}_2\text{O}$ ), and with solution phase  $\text{NH}_4^+$  ( $s\text{NH}_4^+$ ) replaced by  $\text{NO}_2^-$  concentration (Fig. 1b). The modeled  $\mu$  and  $K$  parameters in soil W were each approximately three times greater than in soil L.

**Microcosm experiments.** *Series 1 - Effect of BU addition rate at 85% of field capacity (FC).* There were significant soil-by-BU addition rate-by-time interaction effects on all point-in-time (Figs 2, 3) and cumulative variables (Table 1). Most notably for point-in-time concentrations, soil L had greater  $\text{NO}_2^-$  and actual  $\text{N}_2\text{O}$  production rate ( $a\text{N}_2\text{O}$ ) compared to W on at least two sampling dates at all N rates, and the frequency and magnitude of significant differences in  $\text{NO}_2^-$  and  $a\text{N}_2\text{O}$  by soil increased with increasing BU addition (Figs 2b, 3a). Following BU addition, both soils showed a similar temporal pattern of increasing total extractable  $\text{NH}_4^+$  ( $t\text{NH}_4^+$ ) followed by decreasing  $t\text{NH}_4^+$  (Fig. 2a). Differences in  $t\text{NH}_4^+$  by soil were not observed until at least Day 8, and the timing and direction of significant differences varied by BU addition rate. Soil W had more nitrate ( $\text{NO}_3^-$ ) and ( $\text{NO}_2^- + \text{NO}_3^-$ ) present compared to soil L during at least the first 8 d, and the magnitude and duration of significant differences in  $\text{NO}_3^-$  by soil increased with increasing BU addition (Figs 2c–d). Soil L consistently produced more  $\text{N}_2\text{O}$  on Day 1 compared to W. Soil pH,  $s\text{NH}_4^+$  and  $s\text{NH}_3$  were frequently greater in soil L compared



**Figure 3. Results of Series 1 microcosm experiment (continued).** (a) Actual  $N_2O$  production rate ( $aN_2O$ ), (b) pH, and theoretical concentrations of solution-phase (c) ammonium ( $s/NH_4^+$ ) and (d) ammonia ( $s/NH_3$ ) following addition of bovine urine at 600, 800, 1000 and 1200  $mg\ N\ kg^{-1}$  at 85% field capacity. Asterisks indicate significant differences between soils at  $P < 0.05$  for a given sampling date.

to *W* (Figs 3b–d). Most notably for cumulative indices (Table 1), soil *L* had consistently greater  $c-NO_2^-$ ,  $c-s/NH_4^+$ ,  $c-NH_3$ , and  $c-aN_2O$  compared to *W* at all BU addition rates. During the first 5 d, soil *W* had greater  $NO_2^- + NO_3^-$  accumulation rate (NAR) compared to soil *L* at all BU addition rates, and both soils showed reduced NAR at  $N \geq 1000\ mg\ N\ kg^{-1}$  compared to  $N < 1000\ mg\ N\ kg^{-1}$  (Table S1). During Days 5 through 11, significant differences in NAR by soil were only present at 1000 and 1200  $mg\ N\ kg^{-1}$ . During Days 11 through 19, soil *L* had greater NAR compared to *W* at all BU addition rates except 1200  $mg\ N\ kg^{-1}$ .

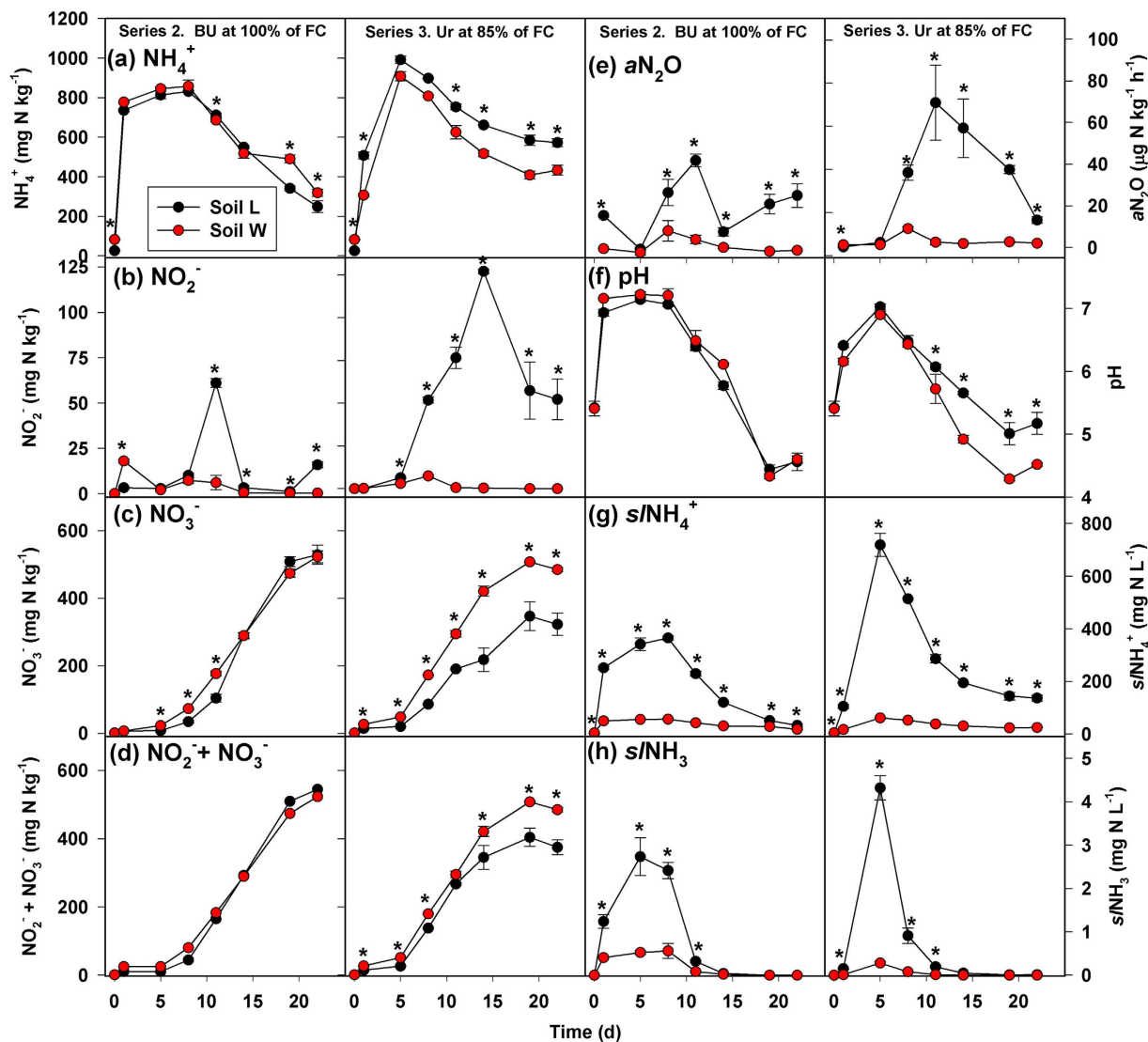
**Series 2 - Effect of soil water content with 1000  $mg\ N\ kg^{-1}$  of BU added.** There were significant soil-by-water content-by-time interaction effects on point-in-time concentrations of  $tNH_4^+$ ,  $NO_2^-$ ,  $NO_3^-$ ,  $aN_2O$ ,  $s/NH_4^+$ , and  $s/NH_3$ . Differences by soil in the 100% of FC treatment were similar to differences by soil in the 85% of FC treatment with 1000  $mg\ N\ kg^{-1}$ , except for pH and  $NO_2^- + NO_3^-$  where no differences were observed (Fig. 4). For cumulative indices, there were significant soil-by-water content-by-time interaction effects on  $c-tNH_4^+$ ,  $c-(NO_2^- + NO_3^-)$ ,  $c-s/NH_4^+$  and  $c-aN_2O$  (Table S2). Differences in  $c-aN_2O$  by soil were similar at 85% and 100% of FC, but both soils had greater  $c-aN_2O$  at 100% compared to 85% of FC. Across both soils,  $c-NO_2^-$  and  $c-s/NH_3$  were greater at 100% compared

Soil	BU added (mg N kg <sup>-1</sup> )			
	600	800	1000	1200
<i>c-t</i> NH <sub>4</sub> <sup>+</sup> (g N d kg <sup>-1</sup> )				
<i>L</i>	5.48 D‡	8.78 C	12.42 B	18.05 A
<i>W</i>	6.28 D	7.21 C	12.12 B	15.61 A
<i>P</i> >   <i>t</i>	<0.001§	<0.001	0.216	<0.001
<i>c</i> -NO <sub>2</sub> <sup>-</sup> (mg N d kg <sup>-1</sup> )				
<i>L</i>	23.95 D	59.55 C	209.37 B	761.04 A
<i>W</i>	15.43 B	18.72 B	72.64 A	94.38 A
<i>P</i> >   <i>t</i>	0.005	<0.001	<0.001	<0.001
<i>c</i> -NO <sub>3</sub> <sup>-</sup> (g N d kg <sup>-1</sup> )				
<i>L</i>	5.35 A	5.53 A	5.43 A	3.64 B
<i>W</i>	5.56 B	6.03 AB	6.22 AB	6.25 A
<i>P</i> >   <i>t</i>	0.502	0.128	0.023	<0.001
<i>c</i> -(NO <sub>2</sub> <sup>-</sup> + NO <sub>3</sub> <sup>-</sup> ) (g N d kg <sup>-1</sup> )				
<i>L</i>	5.38 A	5.59 A	5.64 A	4.40 B
<i>W</i>	5.57 B	6.05AB	6.29 A	6.35 A
<i>P</i> >   <i>t</i>	0.448	0.102	0.029	<0.001
<i>c</i> -H <sup>+</sup> (mol H <sup>+</sup> d kg <sup>-1</sup> )				
<i>L</i>	0.315 A	0.295 A	0.256 B	0.129 C
<i>W</i>	0.267 A	0.274 A	0.243AB	0.221 B
<i>P</i> >   <i>t</i>	0.010	0.217	0.433	<0.001
<i>c</i> -aN <sub>2</sub> O (mg N <sub>2</sub> O-N kg <sup>-1</sup> )				
<i>L</i>	0.90 D	1.72 C	2.90 B	8.08 A
<i>W</i>	0.44 B	0.52 B	1.10 A	1.29 A
<i>P</i> >   <i>t</i>	0.005	<0.001	<0.001	<0.001
<i>c</i> - <i>s</i> NH <sub>4</sub> <sup>+</sup> (g N d kg <sup>-1</sup> )				
<i>L</i>	0.94 D	2.02 C	4.45 B	10.29 A
<i>W</i>	0.35 C	0.41 C	0.74 B	1.01 A
<i>P</i> >   <i>t</i>	0.020	<0.001	<0.001	<0.001
<i>c</i> - <i>s</i> NH <sub>3</sub> (mg N d kg <sup>-1</sup> )				
<i>L</i>	0.72 D	1.99 C	8.79 B	50.26 A
<i>W</i>	0.055 C	0.11 C	0.76 B	3.70 A
<i>P</i> >   <i>t</i>	<0.001	<0.001	<0.001	<0.001

**Table 1. Cumulative indices for total extractable ammonium (*c-t*NH<sub>4</sub><sup>+</sup>), nitrite (*c*-NO<sub>2</sub><sup>-</sup>), nitrate (*c*-NO<sub>3</sub><sup>-</sup>), the sum of nitrite and nitrate (*c*-[NO<sub>2</sub><sup>-</sup> + NO<sub>3</sub><sup>-</sup>]), actual N<sub>2</sub>O production rate (*c*-aN<sub>2</sub>O), acidity (*c*-H<sup>+</sup>) and solution-phase ammonium (*c*-*s*NH<sub>4</sub><sup>+</sup>) and ammonia (*c*-*s*NH<sub>3</sub>) as affected by soil type and rate of bovine urine (BU) in Series 1 experiments. Statistical analyses are based on logarithm base 10 transformed data, and back-transformed means are reported. ‡Within a row, means followed by the same letter are not significantly different at  $P \leq 0.05$ . §Significance of *t* test comparing the means from the two soils for a given rate of BU addition.**

to 85% of FC, while *c*-NO<sub>3</sub><sup>-</sup> and *c*-H<sup>+</sup> were greater at 85% compared to 100% of FC (Table S3). Across both water content treatments, *c*-NO<sub>2</sub><sup>-</sup> was greater in soil *L* compared to *W*.

*Series 3- Effect of BU versus Ur added at 1000 ug N g<sup>-1</sup> at 85% of FC.* There were significant soil-by-N source-by-time interaction effects on all point-in-time variables, and differences by soil following Ur addition were generally consistent with differences following BU addition at 1000 mg N kg<sup>-1</sup> (Fig. 4). However, differences by soil tended to be more consistent and/or to persist longer with Ur compared to BU. Also, *t*NH<sub>4</sub><sup>+</sup> and pH each took longer to reach their maximum values and did not decrease as rapidly with Ur compared to BU. Cumulative *t*NH<sub>4</sub><sup>+</sup>, *c*-N<sub>2</sub>O, and *c*-*s*NH<sub>3</sub> were greater with Ur, while *c*-H<sup>+</sup> was lower with Ur, compared with BU, in both soils (Table S4). Cumulative NO<sub>2</sub><sup>-</sup> was more than five times greater with Ur compared to BU for soil *L*.

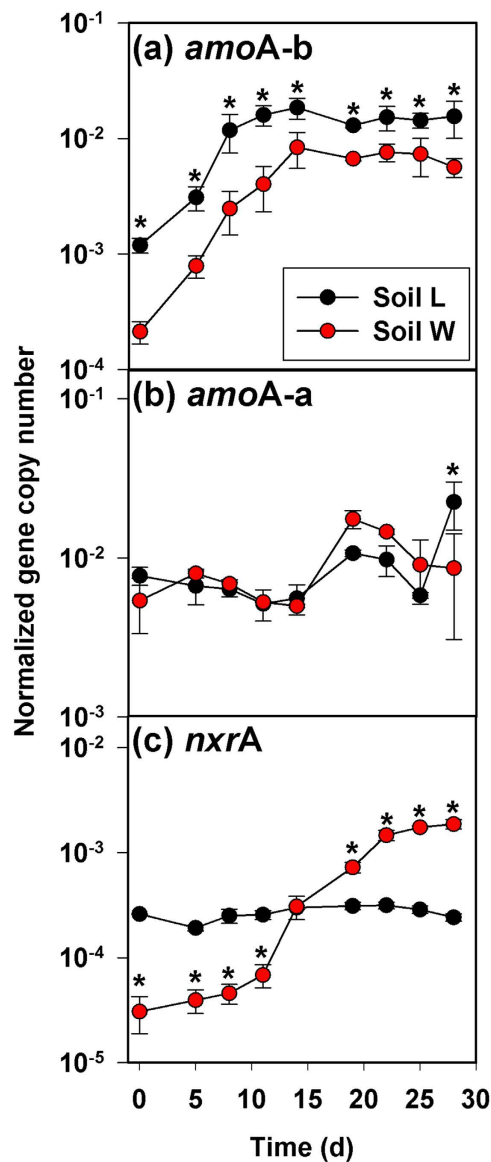


**Figure 4. Results of Series 2 and 3 microcosm experiments.** Concentrations of extractable (a)  $\text{NH}_4^+$ , (b)  $\text{NO}_2^-$ , (c)  $\text{NO}_3^-$  and (d)  $\text{NO}_2^- + \text{NO}_3^-$ , (e) actual  $\text{N}_2\text{O}$  production rate ( $a\text{N}_2\text{O}$ ), (f) pH and theoretical concentrations of solution-phase (g) ammonium ( $s\text{NH}_4^+$ ) and (h) ammonia ( $s\text{NH}_3$ ) in Series 2 (left-hand plates for each variable) and Series 3 (right-hand plates). Series 2 used bovine urine (BU) at  $1000\text{ mg N kg}^{-1}$  with soils at 100% of field capacity (FC), and Series 3 used urea (Ur) at  $1000\text{ mg N kg}^{-1}$  with soils at 85% of FC. Asterisks indicate significant differences between soils at  $P < 0.05$  for a given sampling date.

Gene copies of bacterial ammonia monooxygenase (*amoA-b*) associated with AOB were greater in soil *L* compared to *W*, but increased over time in a similar manner in both soils until Day 14, after which the abundances did not change (Fig. 5). There was a significant soil-by-time interaction effect on gene copies of archaeal ammonia monooxygenase (*amoA-a*), but a difference by soil was observed only on the final sampling date (Fig. 5b). In both soils, *amoA-a* numbers did not change until after Day 14, corresponding to the cessation of any increases in *amoA-b*. Most notable was a significant soil-by-time interaction effect on gene copies of bacterial nitrite oxidoreductase (*nxrA*) associated with NOB ( $P < 0.001$ ), which were initially lower in soil *W* than *L*, and subsequently increased in soil *W* by a factor of 60 while remaining static in soil *L* (Fig. 5c).

**Correlation and regression analyses.** Soil  $\text{NO}_2^-$  was positively correlated with  $t\text{NH}_4^+$ ,  $s\text{NH}_4^+$  and  $s\text{NH}_3$ , and  $c\text{-NO}_2^-$  was positively correlated with  $c\text{-}t\text{NH}_4^+$ ,  $c\text{-}s\text{NH}_4^+$  and  $c\text{-}s\text{NH}_3$  (Table S5). Across all experiments (Series 1-3),  $c\text{-NO}_2^-$  was most strongly correlated with  $c\text{-}s\text{NH}_3$ , which explained 87% of the total variance in  $c\text{-NO}_2^-$  (Fig. 6a). Soil  $\text{NO}_2^-$  was also positively correlated with  $a\text{N}_2\text{O}$ , and  $c\text{-NO}_2^-$  was positively correlated with  $c\text{-}a\text{N}_2\text{O}$ . Across all experiments,  $c\text{-NO}_2^-$  explained 82% of the total variance in  $c\text{-}a\text{N}_2\text{O}$  (Fig. 6b). Soil  $\text{NO}_3^-$  and  $(\text{NO}_2^- + \text{NO}_3^-)$  tended to be negatively correlated with  $\text{N}_2\text{O}$  (Table S5).





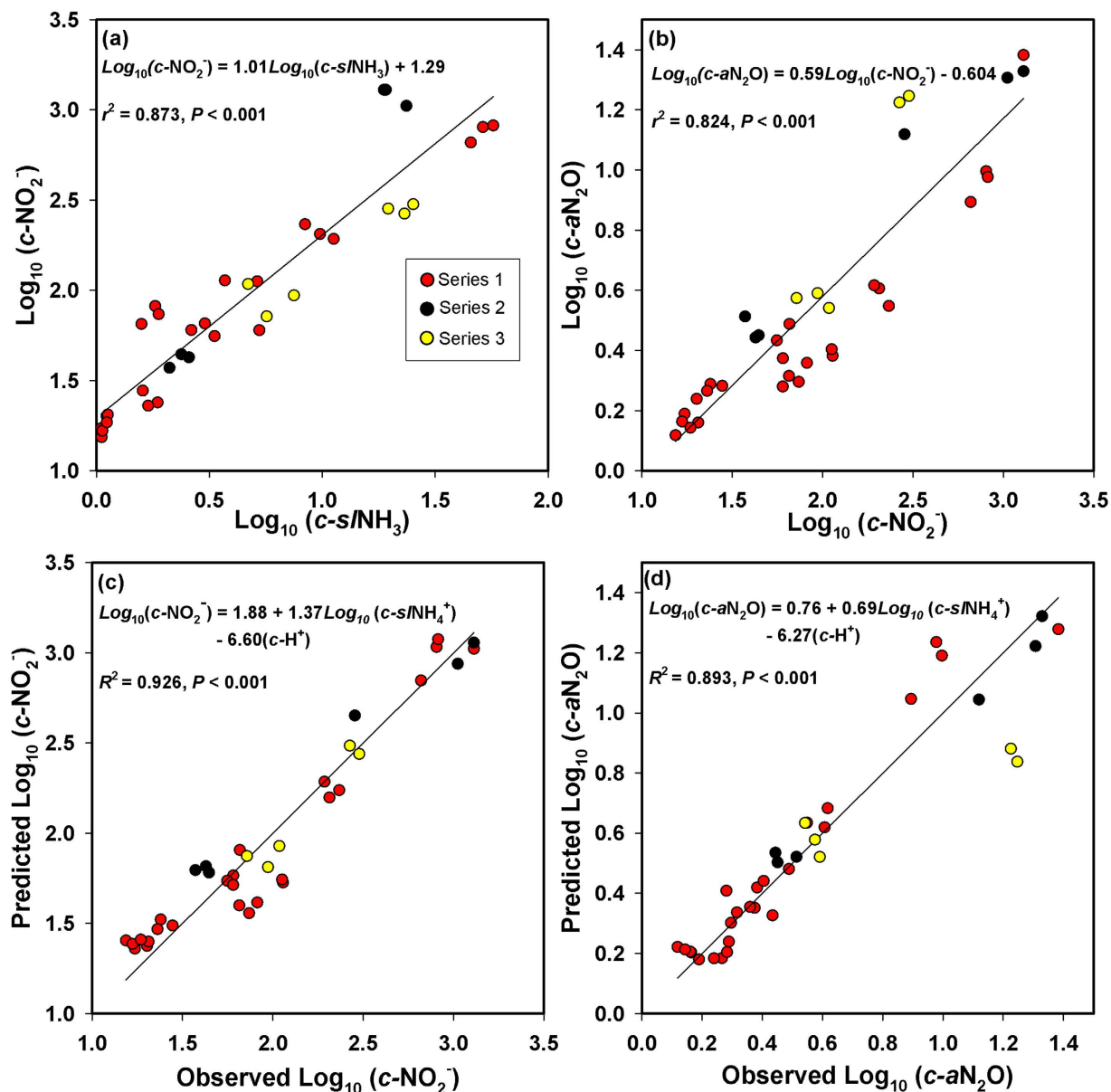
**Figure 5.** Gene copy abundances in Series 3 microcosm experiment. (a) *amoA-b*, (a) *amoA-a*, and (c) *nxrA* following addition of Ur at  $1000 \text{ mg N kg}^{-1}$  soil with soils at 85% of FC. Asterisks indicate significant differences between soils at  $P < 0.05$ . Normalized gene abundances are expressed relative to the number of copies of prokaryotic (bacteria + archaea) 16S rRNA genes in each sample<sup>47</sup>.

Multiple regression models with  $c\text{-}s\text{NH}_4^+$  and  $c\text{-H}^+$  as independent variables explained 93 and 89% of the variance in  $c\text{-NO}_2^-$  and  $c\text{-}a\text{N}_2\text{O}$ , respectively (Figs 5c,d).

## Discussion

This is the first study to correlate the dynamics of  $s\text{NH}_3$ ,  $\text{NO}_2^-$ ,  $\text{N}_2\text{O}$  and nitrifier genes in incubating soil. The strong relationship ( $r^2 = 0.87$ ) between  $c\text{-}s\text{NH}_3$  and  $c\text{-NO}_2^-$  suggests that  $\text{NH}_3$  toxicity acting more strongly on NOB than AOB, and more strongly in soil L than in soil W, was responsible for the contrasting  $\text{NO}_2^-$  and  $\text{N}_2\text{O}$  dynamics in the two soils<sup>11–14</sup>. The greater  $\text{N}_2\text{O}$  production in soil L appeared to be driven by a greater  $\text{NO}_2^-$  accumulation which in turn resulted from greater  $s\text{NH}_3$  accumulation due to its lower ASC (Fig. 7). This explanation is further supported by the static *nxrA* gene copies in soil L in contrast to substantial increases in *nxrA* observed in soil W.

Differences in ASC were related to differences in cation exchange capacity (CEC); i.e., the ratio (W:L) of  $K_d$  values in the two soils was 1.91 which was nearly identical to the ratio of their CEC values (1.93). Thus, while soil L and W had similar organic C and clay contents, differences in ASC were likely due to differences in chemical composition of soil organic matter and/or mineralogical composition of clay which control CEC<sup>21</sup>. It is not likely that a difference in the capacity of the two soils to fix N in clay minerals was an important factor; because  $\text{NH}_4^+$  fixed by clay is not readily extracted by 2 M KCl nor is

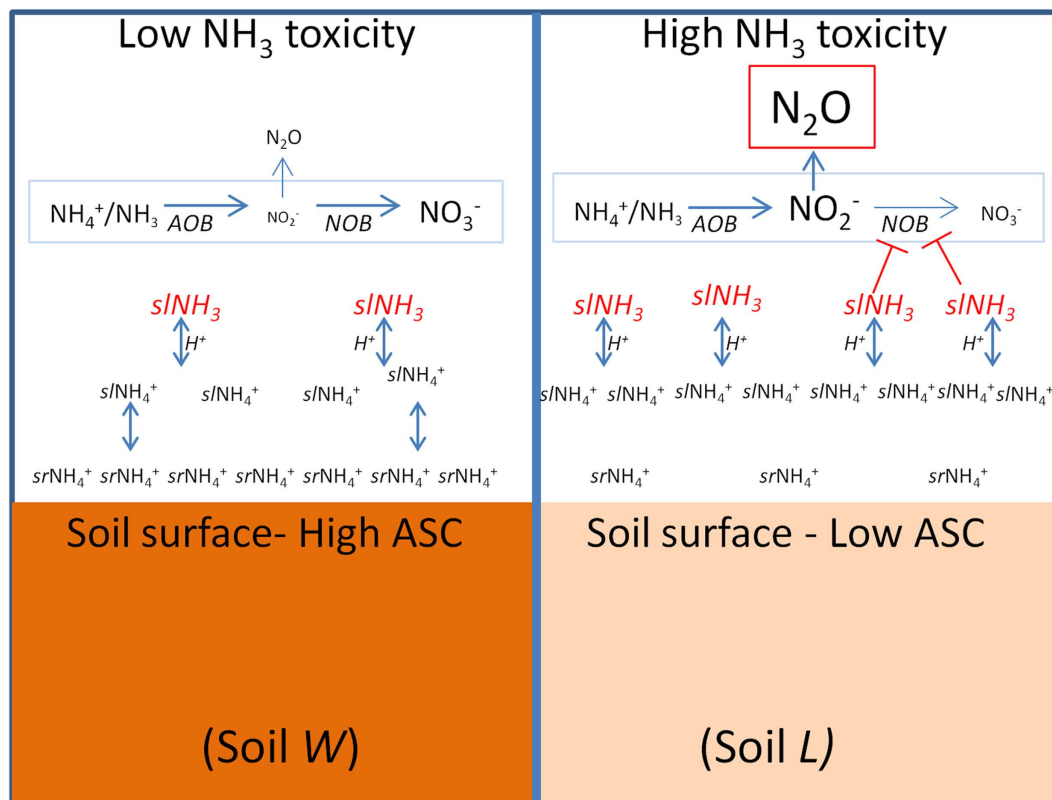


**Figure 6. Regression results.** Single-factor regression models of (a) cumulative nitrite ( $c\text{-NO}_2^-$ ) versus cumulative solution-phase ammonia ( $c\text{-s/NH}_3$ ) and (b) cumulative actual  $\text{N}_2\text{O}$  production ( $c\text{-aN}_2\text{O}$ ) versus  $c\text{-NO}_2^-$  with regression lines, and multiple regression models describing (c)  $c\text{-NO}_2^-$  and (d)  $c\text{-aN}_2\text{O}$  as functions of cumulative solution-phase ammonium ( $c\text{-s/NH}_4^+$ ) and cumulative acidity ( $c\text{-H}^+$ ) with 1:1 lines, for all microcosm data (Series 1–3).

it readily available to microbes, such an effect would have been evident in differences by soil in  $t\text{NH}_4^+$  that were not associated with differences in NAR and/or urea hydrolysis rates<sup>22</sup>.

Our calculations of  $s\text{NH}_3$  concentrations are theoretical approximations. Because soil pH is by its nature operationally defined, any subsequent calculations are also operationally defined; e.g., a different range of absolute  $\text{NH}_3$  values would have resulted if a different pH solvent were used<sup>23</sup>, although  $s\text{NH}_3$  levels based on a single solvent provide a basis for relative comparison. Our methods assumed that the  $\text{NH}_4^+$ -sorption equilibria in the isotherm experiments also described the solid-liquid  $\text{NH}_4^+$  partitioning in the microcosm experiments. Nonetheless, it is interesting that the maximum  $s\text{NH}_3$  concentrations observed in soil  $L$  (approximately 0.5 to 10 mg N  $\text{L}^{-1}$ ) were within the range observed to inhibit NOB and cause  $\text{NO}_2^-$  accumulation in nitrifying wastewater systems<sup>14</sup>. Apart from any theoretical  $s\text{NH}_3$  calculations, the multiple regression model (Fig. 6c) is further suggestive of  $\text{NH}_3$  toxicity; that is, increased  $s\text{NH}_4^+$  combined with reduced acidity (which together are the ‘raw ingredients’ for  $\text{NH}_3$  formation) explained 93% of the variance in  $c\text{-NO}_2^-$ .





**Figure 7. Conceptual schematic.** Decreased ammonium (NH<sub>4</sub><sup>+</sup>) sorption capacity (ASC) results in increased ratio between solution-phase (*sl*) and sorbed-phase (*sr*) NH<sub>4</sub><sup>+</sup>, which increases the potential formation of free ammonia (*sl*NH<sub>3</sub>). When *sl*NH<sub>3</sub> differentially inhibits nitrite (NO<sub>2</sub><sup>-</sup>) oxidizing bacteria (NOB) to a greater extent than NH<sub>3</sub> ammonia-oxidizing bacteria (AOB), NO<sub>2</sub><sup>-</sup> accumulates, leading to increased NO<sub>2</sub><sup>-</sup>-driven N<sub>2</sub>O production in the low ASC soil.

The *sl*NH<sub>3</sub> levels reached maximum values the day after N addition, but NO<sub>2</sub><sup>-</sup> did not reach maxima until at least Day 5 and generally remained elevated for longer than *sl*NH<sub>3</sub>. These results are reflected in stronger correlations between *c*-NH<sub>3</sub> and *c*-NO<sub>2</sub><sup>-</sup> compared with correlations between point-in-time concentrations. This result could have been due to residual inhibitory effects of *sl*NH<sub>3</sub> on NOB that persisted even after *sl*NH<sub>3</sub> had subsided. It also is possible that once NO<sub>2</sub><sup>-</sup> started to accumulate, NO<sub>2</sub><sup>-</sup> itself (or its protonated form [HNO<sub>2</sub>]) became a source of toxicity to NOB. Kinetic models that account for NH<sub>3</sub> and NO<sub>2</sub><sup>-</sup>/HNO<sub>2</sub> as separate sources of toxicity affecting AOB and NOB have been developed for wastewater systems<sup>14</sup>.

Previous studies have found a significant correlation between NO<sub>2</sub><sup>-</sup> and N<sub>2</sub>O dynamics<sup>9,11,24</sup>. The NO<sub>2</sub><sup>-</sup> molecule is an immediate precursor substrate for N<sub>2</sub>O produced via nitrifier-denitrification<sup>19</sup> and chemo-denitrification<sup>20,25</sup>, and there is evidence that N<sub>2</sub>O can be produced via reaction of NO<sub>2</sub><sup>-</sup> with hydroxylamine (NH<sub>2</sub>OH)<sup>26</sup>. Previous studies have also found significant differences among soils in *p*N<sub>2</sub>O following amendment with NO<sub>2</sub><sup>-</sup><sup>20,27</sup>. The current results show that *p*N<sub>2</sub>O is not necessarily a reliable indicator of actual N<sub>2</sub>O production (*a*N<sub>2</sub>O) following amendment of soil with BU or Ur; i.e., although soil W had greater *p*N<sub>2</sub>O after being artificially amended with NO<sub>2</sub><sup>-</sup>, soil L had greater *a*N<sub>2</sub>O because it accumulated more NO<sub>2</sub><sup>-</sup> biologically than soil W following amendment with BU or Ur.

These experiments were not designed to precisely distinguish among all potential pathways of N<sub>2</sub>O production, e.g. nitrification, nitrifier-denitrification, heterotrophic denitrification, or chemodenitrification<sup>19,20,28,29</sup>. The experiments were designed to maintain aerobic conditions, and therefore the influence of denitrification of NO<sub>3</sub><sup>-</sup> as an N<sub>2</sub>O source was expected to be minimal. This was supported by the lack of positive correlation between *a*N<sub>2</sub>O and soil NO<sub>3</sub><sup>-</sup> levels; in fact, NO<sub>3</sub><sup>-</sup> was negatively correlated with *a*N<sub>2</sub>O in many cases. A previous study<sup>20</sup> found that headspace O<sub>2</sub> levels <5% were required for N<sub>2</sub>O production to proceed in NO<sub>3</sub><sup>-</sup>-amended soil; in contrast, NO<sub>2</sub><sup>-</sup>-amended soil readily produced N<sub>2</sub>O at ambient O<sub>2</sub> and displayed increasing N<sub>2</sub>O per unit NO<sub>2</sub><sup>-</sup> as O<sub>2</sub> decreased below 20%. Similar findings have been shown in culture studies examining nitrifier-denitrification<sup>30</sup>. Thus, in the current study, greater N<sub>2</sub>O production at greater water content was likely due to N<sub>2</sub>O derived from nitrifier-denitrification. At 100% of FC, nitrification proceeded more slowly than at 85% of FC, possibly due decreased O<sub>2</sub> availability to support NH<sub>4</sub><sup>+</sup> oxidation. Because *t*NH<sub>4</sub><sup>+</sup> and pH did not decrease as

quickly (owing to slower nitrification-induced  $H^+$  production), this resulted in greater  $sNH_3$ , which in turn could have caused the greater  $NO_2^-$  at 100% of FC compared to 85% of FC. Thus, greater availability of  $NO_2^-$ , as well as increased potential for nitrifier-denitrification to produce  $N_2O$  with reduced  $O_2$ , likely enhanced  $N_2O$  at 100% of FC.

Both soils responded differently to Ur compared to BU. With BU, it took 1 d for  $tNH_4^+$  and pH to reach their maximum values compared to 5 d with Ur; this was likely due to compounds such as hippuric acid present in BU which accelerate Ur hydrolysis<sup>31</sup>. Soil pH also remained elevated for a longer period with Ur (again, indicating slower nitrification-induced  $H^+$  production), which resulted in a doubling of  $c-sNH_3$  compared to BU. In soil *L*, this resulted in a 4- to 5-fold increase in  $NO_2^-$  and a 7-fold increase in  $c-N_2O$ . These results highlight the interactions involving several processes and substrates following BU and Ur addition that can regulate  $N_2O$  production.

On the first day following addition of BU to soil *L* (in Series 1 and 2),  $N_2O$  production was elevated without a corresponding increase in soil  $NO_2^-$  or  $NO_3^-$ , and then declined on Day 3. This result was not observed with Ur, nor was it observed with soil *W*. Further research would be needed to explain this result, but it is possible that any  $NO_2^-$  produced during the initial onset of nitrification was consumed in the  $N_2O$ -producing reactions and therefore was not measurable. Urine addition could have stimulated 'co-denitrification' reactions<sup>32</sup>, or alternatively,  $N_2O$  may have been produced from reactions involving  $NH_2OH$  produced during AOB activity<sup>28</sup>.

While  $NH_3$  is the main substrate utilized by AOB<sup>33</sup>, at higher levels,  $NH_3$  can itself inhibit AOB activity. Decreasing NAR with increasing  $sNH_3$  was observed in a grassland soil amended with BU and attributed to  $NH_3$  toxicity effects on AOB<sup>34</sup>. Similar results were found for NAR in Series 1 during Days 0–5. The qPCR data from Series 3 indicate that any  $NH_3$  toxicity effects on AOB were not large enough to inhibit *amoA*-b genes from increasing during Days 0–14. The *amoA* data also are consistent with results in New Zealand grasslands soils<sup>35</sup>, where *amoA*-b genes increased following N addition but *amoA*-a genes did not. Here, we found an increase in *amoA*-a numbers occurred only after *amoA*-b gene copies stopped increasing. This could indicate that *amoA*-b growth was inhibited after Day 14 by increasing acidity<sup>36</sup> or  $NO_2^-/HNO_2$ <sup>14</sup> which might have favored *amoA*-a activity.

These results have implications for increased fundamental understanding as well as pointing in new directions for modeling and mitigating  $N_2O$  emissions. The current experiments were aimed at studying processes occurring in localized zones receiving concentrated N inputs representative of potential  $N_2O$  production hot spots. Under field conditions, these processes may be further modified by water infiltration or evaporation, temperature dynamics and other factors. Nonetheless, this study shows that soils having largely similar physical and chemical properties but differing in ASC can display dramatically different potentials for  $NH_3$  toxicity,  $NO_2^-$  accumulation and  $N_2O$  production. A previous study showed effects of altering CEC on nitrification dynamics<sup>15</sup>, but effects on  $NO_2^-$  or  $N_2O$  dynamics have not been considered. Further research comparing soils differing in ASC, and examining the effects of altering soil CEC, should be performed under both lab and field conditions to further investigate these variables as modeling parameters and potential  $N_2O$  mitigation factors.

## Methods

**Soils.** Soil 'L' was collected from a research field at Lincoln University, New Zealand (43.648 S; 172.454 E) that had been under pasture (*Lolium perenne* L.) for at least 5 yr. Soil 'W' was collected from a farmer's field east of Waikari, New Zealand (42.964 S; 172.629 E) planted to alfalfa (*Medicago sativa* L.) and rotationally grazed by sheep (*Ovis aries*). Soils were collected from the upper 0.10 m and allowed to dry at 25 °C, and then ground and sieved (2 mm). Soils *L* and *W* were both classified as silt loams and had similar clay content (116 and 130 g kg<sup>-1</sup>, respectively), organic C (26, 33 g kg<sup>-1</sup>), C/N ratio (11.2, 10.6), and pH in H<sub>2</sub>O (6.3, 6.1), but differing CEC (14 and 27 cmol<sub>c</sub> kg<sup>-1</sup>) (Table S6).

**Ammonium sorption capacity and solution-phase concentrations.** Ammonium sorption isotherms were obtained using a batch equilibrium method<sup>37</sup>. Solutions (15 mL) containing  $NH_4^+$  as  $NH_4Cl$  (5, 10, 50, 100, 200, 300 and 400 mg  $NH_4^+-N$  L<sup>-1</sup>) were added to 50-mL polyethylene tubes containing 0.75 g soil. Three replicate tubes of each  $NH_4^+$  concentration were equilibrated on a reciprocating shaker for 18 h at 100 rpm. Mixtures were filtered (Whatman 42) and the filtrate analyzed for  $NH_4^+$ <sup>38</sup> with a flow-injection analyzer (FIA) (Lachat QuikChem 8500 or Alpkem FS3000). The amount of  $srNH_4^+$  was calculated from the difference in  $sNH_4^+$  at the beginning and end of equilibration and accounting for the initial 2 M KCl-extractable  $NH_4^+$  content of the soil. The resulting data were not consistent ( $R^2 < 0.6$ ) with commonly used models<sup>39</sup> but were well-described ( $R^2 \geq 0.99$ ) by models of the form

$$srNH_4^+ = \frac{\mu (sNH_4^+)}{K + (sNH_4^+)} \quad (1)$$

where  $\mu$  (mg N kg<sup>-1</sup>) is the maximum sorption capacity and  $K$  (mg N L<sup>-1</sup>) is the  $sNH_4^+$  concentration at which  $srNH_4^+$  equals one-half of  $\mu$ . Eq. (1) is similar in form to the Langmuir model and has a 'linear portion' for the case where  $sNH_4^+ \ll K$ , such that  $srNH_4^+ = (sNH_4^+)$  where  $K_d = \mu/K$  is the slope with

units ( $\text{L kg}^{-1}$ ). Eq. (1) was used to calculate theoretical concentrations of  $s\text{NH}_4^+$  and  $s\text{NH}_3$  by expressing the total extractable ammonium ( $t\text{NH}_4^+$ ,  $\text{mg N kg}^{-1}$ ) under equilibrium conditions as follows:

$$t\text{NH}_4^+ = sr\text{NH}_4^+ + \theta (s\text{NH}_4^+) + \theta (s\text{NH}_3) \quad (2)$$

where  $\theta$  is the soil water content ( $\text{L kg}^{-1}$ ). Eq. (1) was used to replace  $sr\text{NH}_4^+$  in Eq. (2), and  $s\text{NH}_3$  in Eq. (2) was replaced by:

$$s\text{NH}_3 = (s\text{NH}_4^+) K_A 10^{\text{pH}} \quad (3)$$

where  $K_A$  is the acid dissociation constant (9.245) for the  $\text{NH}_4^+$ - $\text{NH}_3$  acid-base pair at  $25^\circ\text{C}^{40}$ . This resulted in the quadratic equation:

$$\sigma (s\text{NH}_4^+)^2 + \nu (s\text{NH}_4^+) + \delta = 0 \quad (4)$$

where  $\sigma$ ,  $\nu$  and  $\delta$  are constants containing the parameters  $\mu$ ,  $K$ ,  $\theta$ ,  $K_A$ ,  $t\text{NH}_4^+$  and  $10^{\text{pH}}$ . For each sampling event, measured values of  $t\text{NH}_4^+$  and  $\text{pH}$  were substituted into the constant terms, Eq. (4) was solved for  $s\text{NH}_4^+$  using the quadratic formula, and  $s\text{NH}_3$  was calculated using Eq. (3). Example calculations are provided as Supplementary Information (SI). The above procedure was compared to another method where  $\theta(s\text{NH}_3)$  was omitted from Eq. (2), and the results agreed within  $<1\%$ . Thus, assumptions regarding whether  $s\text{NH}_3$  was captured in the  $t\text{NH}_4^+$  analysis affected the results to a negligible extent.

**Nitrite-addition experiments.** The potential for each soil to produce  $\text{N}_2\text{O}$  when amended with  $\text{NO}_2^-$  was determined<sup>20</sup>. Soil was amended with solutions containing  $\text{KNO}_2$  to achieve concentrations of 0, 25, 50, 100 and  $175 \text{ mg NO}_2^- \text{ N kg}^{-1}$  at a water content equivalent to 85% of FC. Solutions were added to 10.0 g of air-dried soil in ‘wide-mouth’ 250 mL glass jars (69 mm diameter by 65 mm) and homogenized with a spatula. Jars were sealed with septum-equipped caps and incubated for 1 h at  $25^\circ\text{C}$  with sampling of the headspace at 0, 30 and 60 min. Gas samples were immediately transferred to evacuated glass vials which were analyzed for  $\text{N}_2\text{O}$  with a gas chromatograph (8610, SRI Instruments, CA) equipped with an electron capture detector and interfaced to an autosampler (Gilson 222XL, Middleton, WI)<sup>41</sup>. The rate of increase in headspace  $\text{N}_2\text{O}$  concentration, headspace volume and soil mass were used to calculate  $p\text{N}_2\text{O}^{20}$ .

**Microcosm experiments.** Three series of microcosm experiments were conducted. Series 1 used each soil amended with four rates of BU equivalent to 600, 800, 1000 and  $1200 \text{ mg N kg}^{-1}$  at a water content equivalent to 85% of FC. Series 2 used each soil amended with BU at  $1000 \text{ mg N kg}^{-1}$  at 100% of FC. Series 3 used each soil amended with Ur at  $1000 \text{ mg N kg}^{-1}$  at 85% of FC. At the start of the experiment, 21 replicate 250-mL glass jars of each treatment were established by adding solutions by pipette to 10.0 g of dry soil and homogenizing with a spatula such that the wetted soil occupied a thin layer ( $\sim 3 \text{ mm}$ ) in the bottom of the jar. Three replicate jars of each treatment were sacrificed for destructive analysis after 1, 5, 8, 11, 14, 19 and 22 d. An additional three jars containing soil amended with deionized  $\text{H}_2\text{O}$  were used to represent ‘Day 0’. Jars were incubated in the dark at  $25^\circ\text{C}$ . On each sampling day, three randomly selected jars of each treatment were opened for 5 min to equilibrate the jar headspace with lab air and then sealed with caps, equipped with rubber septa, for 1 h. The jar headspace was sampled at 0, 30 and 60 min by syringe and  $a\text{N}_2\text{O}$  was determined using methods described above. Nitrous oxide measured in the microcosm experiments is referred to as ‘actual’  $\text{N}_2\text{O}$  production rate ( $a\text{N}_2\text{O}$ ) to distinguish from  $p\text{N}_2\text{O}$ . Immediately following gas sampling, approximately one-half of the soil mass in each jar was gravimetrically transferred to a polyethylene tube and extracted in 40 mL of 2 M KCl for 1 h. The extracts were filtered and stored at  $4^\circ\text{C}$  until determination of  $t\text{NH}_4^+$ <sup>38</sup>. Subsamples of the extract also were used to determine soil pH. Soil remaining in each jar was amended with 40 mL of a separate 2 M KCl solution and extracted for 10 min and then filtered for determination of  $\text{NO}_2^-$  and the sum of  $\text{NO}_2^- + \text{NO}_3^-$ . The pH of the 2 M KCl used for  $\text{NO}_2^-$  and  $\text{NO}_2^- + \text{NO}_3^-$  extraction was adjusted so that, during extraction, the pH of the soil-solution mixture was  $\geq 8.5$ <sup>42</sup>. The  $\text{NO}_2^-$  analysis was performed within 3 h with a spectrophotometer (Shimadzu UV mini-1240)<sup>38</sup>. The  $\text{NO}_2^- + \text{NO}_3^-$  analysis was conducted within 24 h using a FIA preceded by Cd-reduction of  $\text{NO}_3^-$  to  $\text{NO}_2^-$ , and  $\text{NO}_3^-$  was determined by difference<sup>38</sup>.

The microcosms were designed to maintain aerobic conditions with minimal need for aeration due to the high ratio of headspace volume to soil mass and the high ratio of jar diameter to volume. The jars were opened for 10 min on Days 1, 8 and 15. This procedure minimized evaporative moisture losses while maintaining headspace  $\text{O}_2$  above 18% as determined by gas chromatographic analysis with a thermal conductivity detector. Opening of the jars on Day 1 also allowed release of  $\text{CO}_2$  produced during hydrolysis of Ur. Field capacities ( $0.35$  and  $0.45 \text{ kg H}_2\text{O kg}^{-1}$  for soil *L* and *W*, respectively) were determined by incremental water addition until free water was observed. Bovine urine was collected from the Lincoln University dairy farm where cows were grazing perennial ryegrass (*Lolium perenne* L.)/white clover (*Trifolium repens* L.). Urine was kept frozen until the day prior to setting up the experiment, at which time the urine was thawed and analyzed for total N content. Appropriate volumes of BU and  $\text{H}_2\text{O}$  were added to soil in each jar to achieve target N concentrations and water contents. Because these

grazed soils are commonly dry at the surface for days at a time prior to receiving urine deposition, we did not add water or pre-incubate the soils prior to amendment.

**Quantitative polymerase chain reaction (qPCR).** For Series 3, additional soil sub-samples were collected for DNA isolation and quantification of nitrifier gene abundances. On Days 0, 5, 8, 11, 14, 19, 22, 25 and 28, sub-samples (0.25 g) were extracted using a PowerLyzer PowerSoil DNA isolation Kit (MoBio, Carlsbad, CA) in accordance with manufacturer recommendations except for the final washing step which was performed twice rather than once. Abundances of 16S ribosomal RNA (16S rRNA), *amoA*-b, *amoA*-a and *nxrA* were determined using appropriate primers<sup>43–46</sup>. Abundances of *amoA*-b, *amoA*-a and *nxrA* were normalized to recovered 16S rRNA abundances<sup>47</sup>. Additional details are provided as SI.

**Data analysis.** Concentrations of all N species and production of N<sub>2</sub>O are expressed on a dry weight soil basis. Chemical concentrations and pH determined at individual times were used to calculate cumulative 'exposure' indices using trapezoidal integration of concentration versus time data<sup>7,9,48</sup>. We use a 'c-' prefix to distinguish cumulative variables (e.g. c-NO<sub>2</sub><sup>-</sup>) from point-in-time concentrations (e.g. NO<sub>2</sub><sup>-</sup>). For cumulative acidity (c-H<sup>+</sup>), pH was first converted to theoretical hydrogen ion concentration using  $H^+ = 10^{-pH}$  prior to integration. Integration of aN<sub>2</sub>O versus time also was performed, but in this case the resulting variable (c-aN<sub>2</sub>O) represents cumulative N<sub>2</sub>O production. The NAR was calculated over different time intervals from the difference in NO<sub>2</sub><sup>-</sup>+NO<sub>3</sub><sup>-</sup> concentration divided by elapsed time. Three sets of data from the microcosm experiments were analyzed independently. The first set included all data from Series 1, the second set included data from Series 2 plus the 1000 mg N kg<sup>-1</sup> treatment from Series 1 (to examine water content effects) and the third set included data from Series 3 plus the 1000 mg N kg<sup>-1</sup> treatment from Series 1 (to examine N source effects). Each set was analyzed as a completely randomized design at  $P \leq 0.05$  using the MIXED procedure of SAS [Version 9.2, SAS Institute, Cary, NC] with time as a repeated measurement. Additional details are provided as SI.

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## Author Contributions

R.V. and T.C. designed and conducted the experiments. J.C. performed the statistical analyses. F.B-S. conducted the qPCR analyses. R.V. wrote the main manuscript. All authors reviewed and contributed to writing specific sections of the manuscript.

## Additional Information

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