Nitrification gene ratio and free ammonia explain nitrite and nitrous oxide production in urea-amended soils

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ABSTRACT

The atmospheric concentration of nitrous oxide (N₂O), a potent greenhouse gas and ozone-depleting chemical, continues to increase, due largely to the application of nitrogen (N) fertilizers. While nitrite (NO₂⁻/NO₃⁻) is a central regulator of N₂O production in soil, NO₂⁻/NO₃⁻ and N₂O responses to fertilizer addition rates cannot be readily predicted. Our objective was to determine if quantification of multiple chemical variables and structural genes associated with ammonia (NH₃)- (AOB, encoded by amoA) and NO₂⁻/NO₃⁻-oxidizing bacteria (NOB, encoded by nxrA and nxrB) could explain the contrasting responses of eight agricultural soils to five rates of urea addition in aerobic microcosms. Significant differences in NO₂⁻/NO₃⁻ accumulation and N₂O production by soil type could not be explained by initial soil properties. Biologically-coherent statistical models, however, accounted for 70–89% of the total variance in NO₂⁻/NO₃⁻ and N₂O. Free NH₃ concentration accounted for 50–85% of the variance in NO₂⁻/NO₃⁻ which, in turn, explained 62–82% of the variance in N₂O. By itself, the time-integrated nxrA:amoA gene ratio explained 78 and 79% of the variance in cumulative NO₂⁻/NO₃⁻ and N₂O, respectively. In all soils, nxrA abundances declined above critical urea addition rates, indicating a consistent pattern of suppression of Nitrobacter-associated NOB due to NH₃ toxicity. In contrast, Nitrospira-associated nxrB abundances exhibited a broader range of responses, and showed that long-term management practices (e.g., tillage) can induce a shift in dominant NOB populations which subsequently impacts NO₂⁻/NO₃⁻ accumulation and N₂O production. These results highlight the challenges of predicting NO₂⁻/NO₃⁻ and N₂O responses based solely on static soil properties, and suggest that models that account for dynamic processes following N addition are ultimately needed. The relationships found here provide a basis for incorporating the relevant biological and chemical processes into N cycling and N₂O emissions models.

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1. Introduction

Nitrous oxide (N₂O) has two important ecological impacts: it is the predominate ozone-depleting chemical (Ravishankara et al., 2009) and a potent greenhouse gas (Forster et al., 2007) that has increased in atmospheric concentration by more than 20% since 1750, due largely to the application of N fertilizers and manures (Davidson, 2009; Ciais et al., 2013). It is estimated that 3–5% of anthropogenic nitrogen (N) inputs applied to agricultural ecosystems are eventually emitted to the atmosphere as N₂O (Crutzen et al., 2008). Thus, there is much interest in quantifying the effects of nitrogen (N) fertilizer inputs on soil-to-atmosphere N₂O emissions. In particular, substantial efforts have been made to characterize the functional responses (e.g., linear vs. non-linear) of N₂O emissions to N fertilizer addition rates (Shcherbak et al., 2014). Such responses can be used to parameterize N₂O emission models (Zhou et al., 2015). It is generally understood that an imbalance between N fertilizer inputs and plant N uptake capacity promote N₂O losses, due in large part to elevated soil inorganic N availability, which in turn enhances soil microbial processes including
nitrification and denitrification. Both of these processes can lead to gaseous emissions of N₂O, ammonia (NH₃) and nitric oxide (NO), and also regulate nitrate (NO₃⁻) leaching to ground and surface waters (Firestone and Davidson, 1989; Robertson and Vitousek, 2009). While it is well known that soil processes interact with plant and climatic factors to regulate N₂O emissions (Venterea et al., 2012), few studies have simultaneously quantified multiple chemical variables and genetic markers of specific soil microbial processes following N fertilizer addition.

Production of N₂O in soil can occur via chemo-denitrification (Stevenson et al., 1970), bacterial heterotrophic denitrification (Zumft, 1997) and nitrifier-denitrification (Wrage et al., 2001). In all of these processes, nitrite (NO₂⁻) serves as a proximal substrate for N₂O production. Although soil NO₂⁻ concentrations are commonly low compared to ammonium (NH₄⁺) and NO₃⁻, even low NO₂⁻ concentrations can be important due to rapid N₂O production kinetics (Venterea, 2007). Moreover, due to its role as a central substrate in these multiple N cycling processes, NO₂⁻ concentrations correlate better with N₂O emissions than either NH₄⁺ or NO₃⁻ concentrations under field (Venterea and Rolston, 2000; Maharjan and Venterea, 2013) and laboratory (Ma et al., 2015; Cai et al., 2016) conditions. Accurate determination of soil NO₂⁻ concentrations requires careful consideration with regard to methods of sampling, storage, extraction, and analysis (Stevens and Laughlin, 1995; Maharjan and Venterea, 2013; Homay et al., 2015).

Nitrite can be produced and consumed both aerobically, via nitrification, and anaerobically via denitrification (Burns et al., 1996). In the days to weeks following application of urea, the accumulation of NO₃⁻ is mainly regulated by nitrification, even in the presence of NO₂⁻ and over a range of soil water contents (Van Cleemput and Samater, 1995; Smith et al., 1997; Shen et al., 2003). Nitrification is generally considered to be a two-step process, wherein NH₃ is first oxidized to NO₂⁻ by ammonia-oxidizing bacteria (AOB) and/or archaea (AOA), followed by the oxidation of NO₂⁻ to NO₃⁻ by nitrite-oxidizing bacteria (NOB) (Heil et al., 2016). Recently, some NOB within the genus Nitrospira have been found to be capable of oxidizing both NH₃ and NO₂⁻ (Daims et al., 2015; van Kessel et al., 2015), although the prevalence of bacteria with this metabolic capability, referred to as “complete nitrification” or “comammox,” in agricultural soils is unknown. While the two steps of nitrification are often tightly coupled, both temporally and spatially, the presence of free NH₃ can promote their decoupling, wherein NH₃ inhibits NOB such that the NO₂⁻ generated by NH₃ oxidation cannot be immediately processed and therefore accumulates (Stoianov and Alexander, 1958; Smith et al., 1997; Park and Bae, 2009).

Because elevated pH favors NH₃ in its equilibrium with NH₄⁺, initial soil pH is often considered an indicator of the soil NO₂⁻ accumulation potential (Shen et al., 2003). However, Venterea et al. (2015) observed that soil pH, and other basic soil properties including texture and carbon content, did not explain highly contrasting NO₂⁻ and N₂O production in two soils amended with urine or urea. In that study, greater accumulation of NO₂⁻ was associated with greater abundances of the amoA gene that encodes for ammonia monooxygenase in AOB, and lower abundances of the nxrA gene that encodes for nitrite oxidoreductase in Nitrobacter-associated NOB, while abundances of amoA that encodes for ammonia monooxygenase in AOA did not explain any of the variation. Venterea et al. (2015) also found that reductions in nxrA gene abundances were associated with increased free NH₃ concentrations which accounted for differences in soil NH₄⁺ sorption capacity (ASC). Few, if any, studies have examined relationships among AOB- and NOB- gene copies, NH₃, NO₂⁻ and N₂O in N-amended soils. Improved understanding of NOB response to land management has recently been identified as an important research need (Koch et al., 2015; Bertagnoli et al., 2016; Daims et al., 2016). Quantification of the relative responses of Nitrobacter and Nitrospira, the two major NOB genera considered important in soil, has been facilitated by the development of polymerase chain reaction (PCR) primers that target the nxrA genes of Nitrobacter (Wertz et al., 2008) and, more recently, the nxrB genes of Nitrospira (Pester et al., 2014).

Consistent with Venterea et al. (2015), several recent studies have found that AOB are the dominant regulators of nitrification and N₂O production in non-acidic soils receiving N inputs equivalent to fertilizer or urine deposition rates (Di et al., 2009; Wertz et al., 2012; Chen et al., 2013; Banning et al., 2015; Giguere et al., 2015; Sterngren et al., 2015; Wang et al., 2016). In contrast, AOA have been found to be more important relative to AOB in acid soils (Prosser and Nicol, 2012; Shen et al., 2012; Zhang et al., 2012). Some studies have shown AOA to be important in regulating nitrification in non-acidic soils amended with manure (Schauss et al., 2009), wastewater biosolids (Kelly et al., 2011), or relatively low concentrations (<50 µg N g⁻¹ soil) of inorganic N (Giguere et al., 2017). Based on the large number of studies, cited above, indicating the likely importance of AOB in non-acidic soils receiving larger N inputs, the current investigation focused on quantifying AOB-associated amoA, together with NOB-associated nxrA and nxrB, in several non-acidic, urea-amended agricultural soils.

While the major processes regulating soil NO₃⁻ production are largely understood, NO₂⁻ dynamics, and associated N₂O production, for any given soil and management regime cannot be predicted. It is expected that NO₂⁻ and N₂O production will increase with increasing N input, but neither the magnitude nor functional nature of the responses to N addition rate have been well-characterized across a variety of soils. Our objective was to determine if simultaneous measurement of multiple chemical variables (NH₄⁺, NH₃, NO₂⁻, NO₃⁻, N₂O and pH) and gene copy numbers of amoA, nxrA and nxrB could be used to elucidate controls over NO₂⁻ and N₂O production in eight agricultural soils following urea addition in aerobically incubated microcosms. We hypothesized that responses would vary widely across individual soil types and that the variation in these responses would be explained by a combination of these chemical and genetic variables, including the nxrA:amoA and nxrB:amoA gene ratios, which to our knowledge have not been evaluated previously.

2. Material and methods

2.1. Soil collection and characterization

Eight agricultural soils were collected from the University of Minnesota Research and Outreach Centers distributed geographically across the state. These sites included Becker (B), Crookston (C), Lamberton (L), Rosemount (R), St. Paul (S) and Waseca (W), representing a range of soil types used for crop production in the state (Table 1). Soil samples were collected following crop harvest in fall 2014 from the upper 0.15 m of plots that received no N fertilizer during the previous growing season. At Rosemount, two soils were collected from plots that had been under contrasting long-term tillage management since 1990, either conventional (soil R-CT) or no tillage (soil R-NT) (Venterea et al., 2006). All other soils were managed with conventional tillage practices for the region. At St. Paul, two soils were collected from plots that had been under contrasting crop management, either continuous corn (soil S-C) since 1973, or continuous soybean (soil S-S) since at least 1996. Samples were dried at room temperature for 7–10 d, ground, sieved (2 mm), homogenized, and stored at 4 °C prior to use in experiments.
2.2. Microcosm design and chemical analysis

Microcosm experiments were conducted using all eight soils. Ten to thirteen-g aliquots of air-dried soil were placed into 165 mm diameter by 130 mm height wide-mouth 250-mL glass 'wide-mouth' jars. Soil in each jar was brought to a moisture content representing 85% of water-holding capacity by adding solutions containing reagent grade urea dissolved with room air for 10 min on days 2, 10 and 24. This proportion was extended beyond 31 d to allow for additional data collection. The incubation period for soil B was extended beyond 31 d to allow for additional data collection. On each sampling day, jars were opened for 5 min to allow equilibration of the headspace with lab air and then sealed. Jar headspace was manually sampled (10 mL) after 0, 30 and 60 min using a polypropylene syringe inserted through a rubber septum. Gas samples were transferred to glass vials that were analyzed within 96 h for N₂O using a gas chromatograph (model 5890, Agilent/Hewlett-Packard) equipped with a Porapak Q column, an electron capture detector and interfaced to an autosampler (model 7000, Teledyne Tekmar) (Maharjan and Venterea, 2013). The N₂O production rate was calculated from the rate of increase in N₂O concentration, headspace volume and dry soil mass. The jar contents were subsequently split into four subsamples. One (~5 g dry mass) subsample was transferred to a plastic vial and stored at −80 °C for subsequent DNA extraction and analysis which was performed on samples from nine of the 11 sampling dates (excluding days 24 and 27).
2.3. Quantitative polymerase chain reaction

Soil DNA was extracted from 0.25 g of previously frozen soil using a PowerSoil DNA isolation Kit (MoBio, Carlsbad, CA) in accordance with manufacturer protocol, except for the final washing step which was performed twice rather than once. Extraction yields were in the range of 10–30 ng DNA μL⁻¹ quantified using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA). Prior to the qPCR analyses, dilutions and reaction conditions were optimized for each gene. Following 10-fold dilution of extracts, 5 μL- aliquots were used for qPCR analyses using the 7500 Fast Real Time PCR system (Applied Biosystem, USA) and iTaq Universal SYBR Green Supermix (Bio-Rad, USA). All analyses were run in triplicate sets in 20-μL reaction mixtures containing 10 μL of SYBR Green supermix, 0.4 μM of primer and 5 μL of diluted template DNA in DNase free H₂O. The amoA, nxrA and nxrB genes were quantified using the primer pairs amoA-1F/amoA-2R (Rothhauwe et al., 1997), F1nxrA/R2nxrA (Wertz et al., 2008), and nxrB169f/nxrB638r (Pester et al., 2014), respectively. For nxrB, the master mix was supplemented with 1 μL of bovine serum albumin (20 μg μL⁻¹), and the annealing temperature was increased to 60 °C to improve primer specificity. The PCR conditions were as follows: 95 °C for 5 min and 35 cycles of 15 s at 95 °C for all three genes, followed by (i) for amoA, 30 s at 57 °C and 45 s at 72 °C, (ii) for nxrA, 30 s at 55 °C and 30 s at 72 °C, and (iii) for nxrB, 80 s at 60 °C, and, for all genes, following a dissociation phase from 60 to 95 °C to verify the melting curve of all samples. Gene copy numbers were determined according to the standard curve method using gBlocks gene Fragments (Integrated DNA technology, USA). The R² values for all standard curves were >0.99 and primer efficiencies ranged from 80 to 95%. Gene copy number was expressed per gram of dry soil normalized to extraction yield of DNA (i.e., gene copies ng⁻¹ DNA g⁻¹ soil). In addition, the copy numbers of nxrA and nxrB were normalized to amoA copy numbers and are referred to here as the nxrA:amoA and nxrB:amoA ratios, respectively.

2.4. Ammonium sorption capacity (ASC) and ammonia determination

In parallel with the microcosm experiments, ASC was determined for each soil using batch isotherm methods. Preliminary trials were performed to determine optimum soil-to-solution ratios, solution concentrations and mixing times. Several solutions containing NH₄⁻N over the range of 0–500 μg N mL⁻¹ were prepared using NH₄Cl in 0.01 M CaCl₂. Each solution (20 mL) was added to triplicate 50-mL polyethylene tubes containing 0.75 g of soil, which were then equilibrated on a reciprocating shaker at 100 rpm for 18 h followed by filtration and NH₄⁺ analysis as described above. Sorbed ammonium (sNH₄⁺) was plotted as the ordinate vs. the equilibrium solution-phase ammonium concentration (sNH₄⁺) (Liu et al., 2008; Vogeler et al., 2011). The relationships for all soils were well described by a previously used ASC model (Venterea et al., 2015) (Supplementary Fig. S1):

\[ s\text{NH}_4^+ = \frac{\mu \times s\text{NH}_4^+}{K + s\text{NH}_4^+} \]  

(1)

The model parameters \( \mu \) and \( K \) (Table 1) obtained by regression for each soil were used together with measured tNH₄ and pH to calculate corresponding solution-phase NH₄⁺ concentrations in the microcosm experiment using equations developed by Venterea et al. (2015).

2.5. Data analysis

The microcosm experiments generated approximately 1000 values for each of 11 variables (H⁺, NO₂⁻, tNH₄⁺, NH₃, N₂O, NO₃⁻, amoA, nxrA, nxrB, nxrA:amoA and nxrB:amoA) producing ~12,000 total values. These variables are referred to as ‘point-in-time’ values to distinguish them from time-integrated, or ‘cumulative,’ values which were calculated by trapezoidal integration vs. time for each individual replicate microcosm (Burton et al., 2008; Venterea et al., 2015). This resulted in \( n = 120 \) for each cumulative variable. Cumulative variables are indicated by the prefix ‘c-.’ All variables, except c-H⁺, were log₁₀ transformed prior to analysis to meet the requirements of normality and homogeneity of variance, based on scatterplots of residuals vs. predicted values (Kutner et al., 2004) and the UNIVARIATE procedure of SAS (version 9.2, Cary, NC). Point-in-time and cumulative variables were each subjected to correlation analyses to determine if NO₂⁻ and N₂O were correlated with other variables, and single and multiple regression analyses with NO₂⁻ and N₂O as dependent variables and all other variables as independent variables using Statistix (version 9, Tallahassee, FL). Selected cumulative variables (c-NO₂⁻, c-N₂O, c-NH₃, c-amoA, c-nxrA, c-nxrB) were analyzed by non-linear regression using individual replicates (\( n = 15 \)) values for each soil type. Relationships between each variable and urea addition rate were evaluated for 10 regression models using the non-linear regression module in SigmaPlot (version 12.5, San Jose, CA), and two additional models (i.e., linear rise to maximum and linear decay to minimum) using the NLIN procedure of SAS. Cumulative variables were also analyzed at \( P < 0.05 \) using the MIXED procedure of SAS, with soil type and urea addition rate considered as fixed effects and replication and interactions with replication considered as random effects. Means were compared with pairwise \( t \) tests using the PDIF option of the MIXED procedure of SAS.

3. Results

3.1. Point-in-time data

Point-in-time variables varied widely by soil type, urea addition rate, and over time (Supplementary Figs. S1 and S2). The time courses of the chemical variables during the first 10 d of incubation exhibited increases in pH and tNH₄⁺, and thereafter exhibited decreases in pH and tNH₄⁺, continual increases in NO₂⁻, and transient increases in NO₃⁻ and N₂O. The time courses of gene abundances exhibited a variety of temporal patterns, tending to increase initially over the first 10–20 d, and then decrease, although there was substantial variation in these patterns by soil and urea addition rate. Compared to the treatments receiving urea, the control treatments exhibited little to no change in chemical variables except for some apparent increases in NO₃⁻. Several significant correlations were evident among the point-in-time variables (Table 2). When accounting only for chemical variables, the strongest correlation was between NO₂⁻ and N₂O (\( r = 0.78 \)), followed by NO₂⁻ and NH₃ (\( r = 0.70 \)). With respect to gene abundances, NO₂⁻ and N₂O were both positively correlated with amoA gene copy number (\( r = 0.66 \) and 0.62, respectively), but were more strongly and negatively correlated with the nxrA:amoA ratio (\( r = -0.79 \) and -0.68, respectively).

The multiple linear regression model that explained the greatest amount of variance in NO₂⁻ included NH₃, and amoA and nxrA gene copy number as explanatory variables and accounted for 70% of the total variance (Fig. 1a). A model of the same structure, which included NH₃ together with the nxrA:amoA ratio as explanatory variables, also explained 70% of the variance. Substituting tNH₄⁺ instead of NH₃ in these models resulted in a lower \( R^2 \) value (0.66).
A multiple regression model using a combination of chemical and genetic explanatory variables including NO₂⁻/CO₂, H⁺, and amoA and nxrA gene copy numbers accounted for 66–72% of the variance in N₂O (Fig. 1b). Analysis of residuals indicated that observed N₂O values < 0.5 ng N g⁻¹ h⁻¹ were consistently over-predicted by this model. When these low values (n = 74 or 7.6% of the data) were excluded, residuals were more normally distributed and the $R^2$ increased from 0.66 to 0.72. Separate analysis of the data for which N₂O ≤ 0.5 ng N g⁻¹ h⁻¹ found no significant correlation with any chemical or genetic variables.

There were some significant ($P < 0.001$) correlations between basic soil properties and NO₂⁻, but the relationships were weak, including pH ($r = 0.25$), organic matter ($r = -0.19$), clay ($r = -0.16$) and sand ($r = 0.15$) content. Silt ($r = -0.08$) and sand content ($r = 0.08$) were weakly correlated with N₂O ($P < 0.05$). Incubated soil water content was weakly correlated with NO₂⁻ ($r = 0.23$, $P < 0.001$) but not with N₂O. Including any of the basic soil properties with chemical and/or genetic variables did not improve the

![Figure 1](image-url)

**Table 2**

Pearson correlation coefficients ($r$) for chemical and genetic variables in microcosm experiments.²

<table>
<thead>
<tr>
<th>Dependent variables</th>
<th>Independent variables</th>
<th>Chemical (concentrations)</th>
<th>Genetic (gene copy numbers)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Point-in-time (n = 963)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NO₂⁻</td>
<td>-0.38</td>
<td>0.68</td>
<td>0.70</td>
</tr>
<tr>
<td>N₂O</td>
<td>-0.10</td>
<td>0.59</td>
<td>0.47</td>
</tr>
<tr>
<td>c-NO₂⁻</td>
<td>-0.36²</td>
<td>0.80</td>
<td>0.92</td>
</tr>
<tr>
<td>c-N₂O</td>
<td>-0.20</td>
<td>0.81</td>
<td>0.81</td>
</tr>
<tr>
<td>c-N₂O</td>
<td>-0.20</td>
<td>0.81</td>
<td>0.81</td>
</tr>
</tbody>
</table>

ns, not significant.

² All variables except c-H⁺ were log₁₀ transformed prior to analysis.

³ Relationships for all $r$ values shown are significant at $P < 0.001$, except when indicated by * ($P < 0.05$) or ** ($P < 0.01$).
amount of variance explained by the regression models for NO₂ or N₂O.

3.2. Cumulative data

There was a significant \((P < 0.001)\) soil-by-urea rate interaction for all cumulative variables (means separations in Tables S1–S3).

Individual soil responses of c-NO₂, c-N₂O and c-NH₃ to urea addition rate were well described \((R^2 = 0.89\text{--}0.99)\) by linear, exponential rise to maximum (ERM), exponential growth, sigmoidal and Gaussian peak models (Fig. 2, Table 3, Supplementary Table S4). For all soils except B, the same model type accurately described both the c-NO₂ and c-N₂O responses. For soil B, the ERM model, which described the c-NO₂ response, also described the c-N₂O response.

Fig. 2. Time-integrated (a) NO₂, (b) N₂O, (c) NH₃, (d) amoA, (e) nrrA and (f) nrrB at varying rates of urea addition to eight soils. Vertical axis variables are log₁₀ transformed. Symbols are means with standard errors \((\ell = 3)\) and lines are regression models (Table 3). In (e) and (f), values in parentheses are critical urea rates \((U_c)\) corresponding to maximum nrrA or nrrB values for peak models.
copy number exhibited a maximum at intermediate urea addition. For genetic variables, c-NO2 (Table 2). Whereas c-NH3 (Fig. 2f, Table 3). For four soils (W, R-CT, R-NT and S-C), c-NO2 was positively correlated with c-amoA (Fig. 3). Correlations among the time-integrated variables were similar to those observed for point-in-time variables, except that the relationships were stronger. Among the chemical variables, c-NO2 was strongly correlated with c-NH3 (r = 0.92) and c-N2O (r = 0.90) (Table 2). For genetic variables, c-NH3 and c-N2O were positively correlated with c-amoA (r = 0.75 and 0.76, respectively), but were more strongly and negatively correlated with c-nxrAc-amoA (r = –0.88 and –0.89, respectively). Considered as an explanatory variable, the c-nxrAc-amoA ratio accounted for 78 and 79% of the variance in c-NO2 and c-N2O, respectively (Fig. 3).

The multiple regression model that explained the greatest amount of variation in c-NO2 (88%) included c-NH3, and the relative abundances of c-amoA and c-nxrA as explanatory variables (Fig. 1c), consistent with the results obtained for NO2. A model of the same structure, which included c-NH3 together with the c-nxrA:c-amoA ratio instead of c-nxrA and c-amoA gene copy numbers separately, explained 88% of the variance. Substituting c-amoN2 instead of c-NH3 in these models resulted in a lower R² value (0.79).

The multiple regression model that explained the greatest amount of variation in c-N2O (87%) included NO2, and abundances of c-amoA, c-nxrA and nxrB as explanatory variables (Fig. 1d). The form of this model was similar to that for N2O (Fig. 1b), with the exceptions that c-amoA gene copy number was also a significant (P < 0.001) explanatory variable and that including c-H was not able to explain any additional variable in c-N2O. Unlike the model for NO2, there were no trends in residuals that varied with observed c-N2O values.

There were some significant correlations between basic soil properties and c-NO2, but the relationships were weak; e.g., pH (r = 0.38, P = 0.001), organic matter (r = –0.29, P = 0.0012), clay (r = –0.22, P = 0.015) and sand (r = 0.21, P = 0.019) content. Sand (r = 0.22) and silt content (r = –0.19) were weakly correlated with c-N2O (P < 0.05). Incubated soil water content was weakly correlated with c-N2O (r = –0.34, P = 0.001) and c-N2O (r = –0.19, P = 0.02). Including any of the basic soil properties with chemical and/or genetic variables did not improve the multiple regression models for c-NO2 or c-N2O.

4. Discussion

4.1. Variation in NO2 responses among soils

The soils examined here exhibited a wide range of NO2 responses to urea addition, and thus four different model types were required to describe them. Some soils showed much larger

\[ y = y_0 + a \exp(-b x) \]
responses at low to moderate urea addition rates. For example, following addition of 250 μg N g⁻¹ urea, soils B and C exhibited 200- to 800-fold increases in c-NO₂ compared to when no urea was added, while soils L, W and R-NT exhibited <10-fold increases in c-NO₂. In spite of the wide variation in individual soil responses, linear models with the same structure were able to describe NO₂ and c-NO₂ as a function of NH₃, and amoA and nxrA gene copy numbers across all soils and urea addition rates. These models are consistent with our understanding of the processes that affect NO₂ accumulation under aerobic conditions, including urea hydrolysis, pH, and pH buffering capacity, NH₃ oxidation and ASC. Urea hydrolysis releases NH₃, which acts both as the primary substrate for AOB (amoA) that produce NO₂ (Suzuki et al., 1974) and as an inhibitor of NOB (nxrA and nxrB) that utilize NO₂ (Park and Bae, 2009). Thus, positive model coefficients for NH₃ and amoA gene copy number and the negative model coefficient for nxrA gene copy number are consistent with this description of key processes, as illustrated in Fig. 4.

Fig. 3. Regression models describing (a) c-NO₂ and (b) c-NO₂ as a function of the c-nxrA:amoA ratio (P < 0.001). All variables were log₁₀ transformed prior to analysis and transformed variables are plotted.

Fig. 4. Processes regulating NO₂ accumulation and associated N₂O production following urea addition to soil. Urea hydrolysis (1) releases NH₃ which consumes H⁺ in its equilibrium with NH₄ (2) while solution-phase NH₃ equilibrates (3) with exchangeable NH₄. NH₃ remaining in solution is oxidized (4) by AOB (amoA) to NO₂ which also produces H⁺. NO₂ can be oxidized (5) by NOB (nxrA and nxrB) to NO₃; NH₃ can also inhibit (6) NOB resulting in accumulation of NO₂ which promotes reduction (7) to N₂O via nitriﬁer-denitriﬁcation carried out by AOB, and chemo-denitriﬁcation (8) which may be enhanced by H⁺. Also included is the possibility of complete nitriﬁcation from NH₃ to NO₂ (9) carried out by some NOB within the genus Nitrospira (Daims et al., 2015; van Kessel et al., 2015).

given the central role of free NH₃ concentrations which are inﬂuenced by multiple processes and properties. It is unlikely that all of these processes would be regulated by, or correlated with, any single soil property. Moreover, these ﬁndings demonstrate how it is possible for similar NH₃ and NO₂ levels to evolve under contrasting initial soil conditions. This is illustrated by comparing the responses of soils B and C. Even though these two soils had contrasting pH, texture, CEC, and ASC (Table 1), they both accumulated greater NH₃ than all other soils (Fig. 2c), and had very similar NO₂ responses to urea addition rate (Fig. 2a). The elevated NH₃ response in soil B was likely due to its low ACS, which was weakest of all soils. Weak ASC minimizes the removal of NH₄ from solution and thereby favors NH₃ formation during urea hydrolysis (Venterea et al., 2015). Whereas the ACS of soil C was approximately twice as strong as soil B, soil C also had the greatest initial pH and a strong buffering capacity as evidenced by relatively small changes in pH during incubation. Thus, even though soil C had lower solution-phase NH₄ than did soil B due to its greater ASC, the greater pH in soil C induced a greater fraction of the solution-phase NH₄ to disassociate to NH₃. Differences in nitrifier activity likely also contributed to the patterns in NH₃ and NO₂ responses; e.g., soil B had greater c-amoA abundances than all soils except R-CT. These multiple differences and effects highlight the challenge of predicting NH₃, NO₂ and N₂O based on static soil properties in a system driven by several interacting processes. Reports of NH₃ in soil N cycling studies are infrequent, and few studies have addressed its role in N₂O production. When it is quantiﬁed, NH₃ is typically determined based on measurements of NH₄ and soil pH, together with the Kₛ value (e.g., Smith et al., 1997). While this simpler approach is valid in aqueous systems, in soils it ignores the role of sorption in regulating solution-phase NH₄ and NH₃.

4.2. Nitrite-mediated N₂O production

Given the oxic conditions maintained in the microcosms and the relatively weak correlation (r = 0.29–0.46) between NO₂ and N₂O, heterotrophic denitriﬁcation was likely not an important process in this system. In contrast, the strong overall correlation (r = 0.78–0.90) between NO₂ and N₂O indicate that nitrifier-
denitrification, and possibly chemo-denitrification, were the primary sources of N2O given that these processes can reduce NO2 to N2O under ambient as well as sub-ambient O2 (Goreau et al., 1980; Wrage et al., 2001; Venterea, 2007). The positive coefficients for NO2 and amoA copy number, and negative coefficients for nxr and/or nxrA copy number, in the regression models (Fig. 1b, d) are consistent with our understanding of N2O production via nitritivdenitrification; i.e., NO2 is the main substrate for AOB to produce N2O, and when NOB abundances are reduced (e.g., due to NH3 toxicity), NO2 becomes more readily available as a substrate for other reactions, among them the reduction to N2O by AOB (Fig. 4). Although the amoA gene does not encode for N2O production, its abundance is likely to be positively correlated with NOB abundances. The current results are also consistent with chemo-denitrification reactions between NO2 and soil organic matter that can produce N2O under slightly acidic conditions (Stevenson et al., 1970; Thorn and Mikita, 2000). This mechanism is supported by the positive coefficient for H+ in the model for N2O (Fig. 1b), and by the finding that rate coefficients determined from the linear slope of the relationship between c-NO2 and c-N2O for each soil were positively correlated (r2 = 0.63, P < 0.001) with soil organic matter, consistent with Venterea (2007). However, when N2O production was below 0.5 ng g−1 h−1 (<8% of the data) the regression model (Fig. 1b) over-estimated observed values, and there were no correlation between N2O production and any measured variable. The majority of these data occurred in the control, where NH4 was <5 pg N g−1. It is possible that AOA were important in producing N2O under these low-substrate conditions, where AOA have been found to be more competitive than AOB (Prosser and Nicol, 2012). This hypothesis is consistent with a recent study that implicated the role of AOA in producing N2O under low substrate conditions (Giguere et al., 2017).

4.3. Gene abundances

The c-nxrA abundances exhibited a consistent pattern of increasing and then decreasing below and above critical urea addition rates, respectively. This pattern is consistent with the hypothesis that Nitrobacter-associated NOB were inhibited by increasing levels of NH3 (Figs. 2c and 3b). Under this hypothesis, soils with lower Uc values accumulate NO2 at lower urea addition rates. Indeed, for the majority of soils (six of eight), the contrasting NO2 responses shown in Fig. 2a were consistent with, and can be explained by, the differences in c-nxrA responses and Uc values. For example, soil S-C showed a steep increase in c-NO2 when urea was added at 500 µg N g−1 (Fig. 2a), and this coincided with a decline in nxrA (Fig. 2e). Also, the two soils (B and C) displaying the steepest increases in c-N2O at urea addition rates ≤ 250 µg N g−1 also had Uc values ≤ 250 µg N g−1, while the three soils (L, W and R-NT) with the least pronounced NO2 responses had Uc values ≥ 340 µg N g−1. The c-NH4 results are also consistent with these trends; soils B and C had greater c-NH4 levels while soils L, W, and R-NT tended to have lower c-NH4 (Fig. 2c). The c-N2O results for the two soils (R-CT and S-S) that exhibited intermediate NO2 responses were not necessarily consistent with the above trends, which may have been due to inaccurate estimation of Uc as determined by the regression model. It is not surprising that nxrA by itself did not fully explain the NO2 responses, since NO2 must first be produced by AOB before it can accumulate, and it is logical that the abundances of NOB relative to AOB would be a better predictor of NO2 responses. In this sense, the nxrA:amoA gene copy ratio is actually a NO2 sink:source ratio, and as such, NO2 would be expected to increase as this ratio decreases. The nxrA:amoA ratio was the single best predictor of NO2, a strong single predictor of N2O and c-N2O, and a significant predictor of NO2 and c-NO2 in the multiple regression models.

The contrasting responses of nxrA and nxrB abundances appear consistent with the greater affinity of Nitrospira (nxrB) relative to Nitrobacter (nxrA) for NO2 (Nowka et al., 2015). The abundances of nxrB were greater than nxrA in the control (no urea) treatments, where NO2 levels remained < 0.5 µg N g−1. This trend is consistent with Nitrospira acting as a K-strategist wherein high population densities can be achieved despite substrate limitation. In contrast, the greater and more consistent increases in nxrA compared with nxrB abundances following urea addition are consistent with Nitrobacter being a r-strategist (Daims et al., 2016). These results are also in agreement with greater responsiveness of Nitrobacter (nxrA) relative to Nitrospira (nxrB) observed following N additions to soil (Simoin et al., 2015).

The consistency in the functional responses of nxrA abundances across soils, and the strong explanatory power of the c-nxrA:amoA ratio, suggest that Nitrobacter exerted greater regulatory control, in general, over NO2 and N2O relative to Nitrospira. However, some differences in nxrB abundances among soils were observed, and may explain the corresponding differences in NO2 and N2O. Most notable were the differences in nxrB gene copy number for soils R-CT and R-NT, which were sampled from long-term conventional tillage (CT) and no-till (NT) research plots, respectively. While the abundance of nxrB in R-CT exhibited a peak-type response indicative of NH3 toxicity, abundances of this gene in R-NT showed no signs of suppression and were consistently greater than in R-CT except in the control (Fig. 2f). This finding suggests that greater activity of Nitrospira in R-NT was responsible for the significantly smaller NO2 and N2O responses at intermediate urea addition rates as compared to R-CT (Fig. 2a and b). Moreover, this suggests that long-term implementation of NT caused shifts in dominant NOB populations such that Nitrospira under NT were able to maintain NO2 oxidizing activity in spite of similar NH3 concentrations (Fig. 2c). It is possible that comammox capability within these Nitrospira populations resulted in a tighter coupling of the two steps of nitrification, due in large part to both processes being carried out by the same organism (Daims et al., 2015; van Kessel et al., 2015), although direct evidence of comammox occurring in agricultural soil has not been reported. In contrast to the greater abundance of nxrB in soil R-NT, a greater abundance of c-amoA was found in R-CT, except in the control (Table S2). The potential for shifts in dominant nitrifying populations due to tillage requires further investigation, but may be related to differences in soil organic matter, moisture retention and/or temperature (Venterea et al., 2006). Such shifts may also provide an explanation for the importance of long-term adoption of NT for effective N2O mitigation (van Kessel et al., 2013).

Studies in wastewater found evidence for NH3 inhibition of AOB (Park and Bae, 2009). Here, only soil B showed evidence of declining amoA abundances with increasing urea addition, and only at the highest addition rate (Fig. 2d). This decline in amoA abundances was consistent with NH3 inhibition in that this treatment (soil B + 1000 µg N g−1) had the greatest accumulation of NH3 of any soil (Fig. 2c).

4.4. Conclusions and ecological implications

The wide variation in soil responses observed here could not be explained by basic soil properties. However, coherent models that incorporated N substrate concentrations and nitrification gene copy numbers accounted for 70–89% of the total variance in NO2 and N2O. The time-integrated nxrA:amoA gene ratio was found to be a reliable sink:source ratio for NO2, and explained 78 and 78% of the variance in cumulative NO2 and N2O, respectively. In all soils, nxrB abundances declined above critical urea addition rates, indicating a consistent pattern of NH3 suppression of Nitrobacter-
associated NOB. In contrast, *Nitrospira*-associated *nxrB* abundances exhibited a broader range of responses, and suggested that long-term management practices (e.g., tillage) can induce shifts in dominant NOB populations with impacts on NO2− accumulation and N2O production. These results highlight the challenge of predicting NO2− and N2O responses based solely on static soil properties in a system driven by dynamic and interacting physical, chemical, and biological processes, and suggest that models that account for the underlying processes are needed. In the field, a range of additional processes including fluctuating water content and temperature, plant N uptake and transport via advection and diffusion would likely reduce soil chemical concentrations and dampen the responses observed in these soil microcosms. The relationships found here provide a basis for incorporating the relevant chemical and biological processes into N cycling and N2O emissions models that also account for these field-scale processes.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2017.04.007.

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