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Article

Determining Chemical Factors Controlling Abiotic Codenitrification

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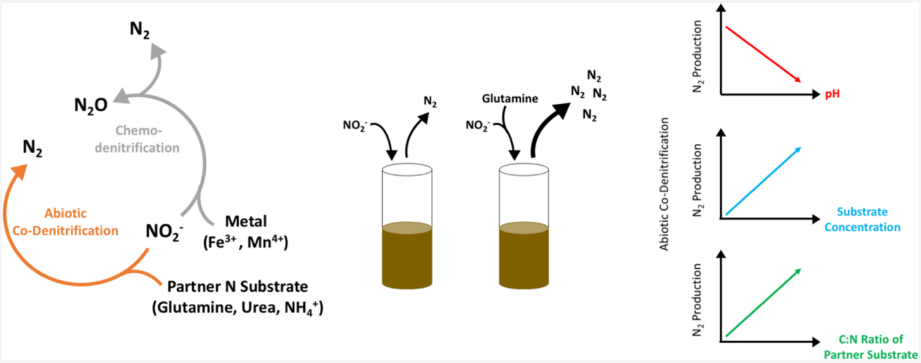

ABSTRACT: Codenitrification is a reactive nitrogen (N) removal pathway producing hybrid dinitrogen (N_2) by combining nitrite (NO_2^-) and a partner-N substrate. Abiotic codenitrification also produces hybrid N_2 through nitrosation of organic N by NO_2^-, but it is poorly constrained in soil N cycles. We determined the importance of abiotic codenitrification in soils and examined factors controlling abiotic codenitrification using live soils, sterile soils, and sterile solutions. Abiotic codenitrification in sterile soils ranged from 0.12 ± 0.001 to 0.60 ± 0.08 nmoles $^{29}N_2$-N g^{-1} day^{-1}, which accounts for 2.3 to 8.2% of total N_2 production measured in live soils. Increased abiotic N_2 production was observed in soils with the addition of an organic N partner (glutamine). Consistent with previous work, higher rates were observed in lower-pH soils, but the highest rate was found in the soil with the highest carbon:nitrogen (C:N) ratio. We further investigated a range of organic N partners and the influence of concentration and pH on abiotic codenitrification in solution. Similar to sterile soil incubations, abiotic $^{29}N_2$ production was negatively correlated with increasing pH in solution. Greater rates of abiotic $^{29}N_2$ production were measured as the substrate concentration increased and pH decreased. Solution experiments also showed that addition of organic N partners increased abiotic codenitrification rates, which are positively correlated with the C:N ratios of organic N partners. This is the first study demonstrating the importance of N removal through abiotic codenitrification in acidic soils and the C:N ratio of organic N partners as a controlling factor in abiotic codenitrification.

KEYWORDS: *codenitrification, nitrosation, nitrite, soil, nitrogen removal*

1. INTRODUCTION

Excess nitrogen (N) in the environment has widespread effects on ecosystems, biodiversity, human health, and climate, yet pragmatic solutions for removing excess N remain elusive.[1] The different oxidation states of N facilitate its participation in a variety of enzymatically and chemically mediated reactions, thus making the N cycle extremely complex. N removal pathways, which we define here as transformations of reactive N to inert dinitrogen (N_2) gas, are important components of the N cycle in soils and waterways.[2] Current N cycle paradigms focus on enzymatically mediated N removal processes such as denitrification, codenitrification, and anammox. These microbial processes can transform reactive forms of N, such as ammonium (NH_4^+), nitrite (NO_2^-), or nitrate (NO_3^-), to inert N_2 gas. Analogous to these biotic processes are abiotic reactions that also produce N_2, including chemodenitrification and abiotic codenitrification. Chemodenitrification couples the reduction of NO_2^- to the oxidation of metals, thus forming the greenhouse gas nitrous oxide (N_2O)[3−5] or inert N_2 from a single N source.[6] Abiotic production of hybrid N_2, referred to here as abiotic codenitrification, occurs through nitrosation of organic nitrogenous molecules (organic Ns) by NO_2^-, thus forming N_2 from two independent N sources.[7] The contribution of abiotic codenitrification to N removal and release of N_2 gas is relatively unknown in both terrestrial and aquatic ecosystems.

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NO_2^- is the well-known precursor or 'gateway' to biotic denitrification, anammox, and codenitrification processes;[8] this is also true for abiotic codenitrification and chemodenitrification.[3,7] Abiotic codenitrification is more likely to occur in acidic environments, where nitrous acid (HNO_2^-) is in equilibrium with NO and nitric acid (HNO_3).[9] Ions of NO, such as nitrosonium (NO^+),[10] are strong nitrosation agents.[11,12] The oxidized form of nitric oxide (NO^+) is a key species in N-nitrosation reactions, where the NO^+ is transferred to a nucleophilic aryl or alkyl amine that is then deprotonated and dehydrated.[7,10] The incorporation of NO^+ into the organic molecule may then form an unstable intermediate diazonium ion, which disassociates to a positively charged organic molecule (R^+) and N_2.[13] Some fraction of the immobilized N remains in organic N, and some fraction is released as hybrid N_2.[11] Controls on the production of abiotically formed N_2 are not clearly defined, but may be linked to pH, substrate concentration, or the specific nucleophile, which we refer to here as the organic N partner.[14]

While abiotic N_2 production is fundamentally controlled by pH,[15] it has also been shown to increase with NO_2^- concentration[6] and organic *N* concentration.[14,16] Lim et al. (2018) reported that abiotic NO_2^- decomposition accounted for 10−20% of the NO_2^-−N conversion to nitroso-compounds in acidic soils. Nelson and Bremner (1969) reported higher N_2 production and NO_2^- decomposition for acidic soils, as compared to neutral soils. These studies point out the importance of pH in abiotic codenitrification. However, it is difficult to tease out a single factor contributing to abiotic N_2 production in complex soil matrices. While the abiotic decomposition of NO_2^- at low pH is clear, other factors, such as the presence of metals, substrate concentration, or organic N partners, may also influence observed abiotic N_2 production.[17,18]

A review of NO_2^- accumulation in soils indicated that up to 40% of added NO_2^- may react abiotically with organic compounds,[19] resulting in release of gaseous N,[11,13,14,20] but it remains unclear what is driving abiotic conversion of reactive N to inert N_2. This abiotic reaction requires an organic, nitrogenous partner, which raises questions about the extent to which these partner-N compounds control rates of abiotic codenitrification. Defining the factors controlling abiotic codenitrification is imperative to determining (a) the environmental relevance of this process, (b) implications for reported reactive N removal data, and (c) how to assess abiotic processes in the complex N cycle.

The objectives of this study were to examine the factors controlling abiotic codenitrification and its importance in soil N removal by comparing it to biotic N_2 production mediated by anammox, codenitrification, and denitrification. We investigated if abiotic codenitrification is stimulated by the addition of an organic partner-N (glutamine) for a wide range of sterile soils—from a New Zealand volcanic to a North Dakota, US silty loess[21,22] and compared abiotic N_2 and N_2O production. We also conducted sterile solution experiments with different pH conditions, NO_2^- concentrations, and partner-N substrates to identify the chemical factors controlling abiotic codenitrification.

2. MATERIALS AND METHODS

2.1. Soil Sample Collection and Sterilization. Soil samples were collected from five grassland research sites located in New Zealand (NZ) and the United States (U.S.). The three NZ grassland sites described by van der Weerden (2016) represent the Waikato (NZ6 Volcanic), Manawatu (NZ5 Fluvial), and Canterbury (NZ1 Stony) regions. One U.S. grassland site is located in the Northern Great Plains in the state of North Dakota (US2 Silty Loess), and the other is located in the southeastern U.S. Coastal Plains region in the state of North Carolina (US3 Clayey).[22] During the spring of 2018, four sample points were randomly selected at each site within a 10 m × 10 m plot. Within each plot, two small soil cores (3 cm diameter × 10 cm depth) were collected and composited to form one sample. A separate larger soil core (7 cm diameter × 10 cm depth) was collected in each plot to determine bulk density. Each of the five composite soil samples was sieved (2.0-mm) and separated into two parts: one was stored at 4 °C for soil measurement of physiochemical properties, and the other was sterilized for use in incubation experiments. Sterilization was performed by applying a dose of 27.8 kGy (60Co) γ-irradiation (Sterigenics, Haw River facility, North Carolina, US). The γ-irradiated soil was stored for approximately 1 month at 4 °C before use. Gamma irradiation was chosen because this method is reportedly highly effective at killing microorganisms in soil and applicable to this type of research.[12] Furthermore, γ-irradiation imposes less severe effects on relevant physical and chemical properties, as compared to autoclaving, which is known to induce quite profound changes in both the structure and chemistry.[23] Sterility of γ-irradiated soil was assessed in the laboratory by incubating 1 g of γ-irradiated soil representing each site in nutrient broth media (BD DifcoTM) for comparison with a live soil control from the same site. Sample turbidity was monitored daily for 1 week with a Milton Roy Spectronic 401 (Spectronic Instruments Rochester, NY). Turbidity remained unchanged for all γ-irradiated samples, but live controls (unirradiated soil) did show growth.

In addition to testing for sterility, we measured extractable mineral N, soil pH, total organic carbon (TOC), and total nitrogen (TN). Mineral N extraction was performed by amending soil samples with 2 M KCl and then shaking for 1 h prior to filtration with a Whatman 0.45 μm pore size filter (GE Healthcare Life Sciences). Extracts were analyzed for nitrate (NO_3^-), nitrite (NO_2^-), and ammonium (NH_4^+) with a Lachat QuikChem 8000 automated ion analyzer (Lachat Instruments, Milwaukee, WI, USA). Soil pH was measured using a Corning pH/ion meter 450 with an Accumet probe using a 1:2 ratio of air-dried soil to deionized water mixture. Soil TOC and TN were measured using a CHN 4010 Elemental Combustion System on air-dried soil (Costech Analytical, Valencia, CA, USA).

2.2. Measurement of Abiotic Codenitrification in Soils. Approximately 1 g of sterilized soil was placed in a 12 ml exetainer tube amended with one of the following solutions (1 mL volume): (a) 100 nmoles N as $Na^{15}NO_2$ (Cambridge Isotope, 98%atm) only, (b) 100 nmoles N as $Na^{15}NO_2$ and 100 nmoles glutamine, or (c) 100 nmoles glutamine. In previous studies, abiotic codenitrification was performed using a robotized gas chromatography system, which required high levels of NO_2^- (>250 μmoles) to overcome detection limit constraints.[24] However, high concentrations of soil NO_2^- tend to be rare or transient in nature. Here, we used concentrations more closely aligned with natural occurrence. Concentrations of NO_2^- in unfertilized soil typically vary from 0.01 to 4 μmoles per g soil,[19] and fertilizers tend to cause elevated accumulations of NO_2^-, such as anhydrous ammonia[25,26] and

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urea.[27,28] The isotopic composition of $^{29,30}N_2$ was measured by isotope ratio mass spectrometry (IRMS) on a gas bench isotopic ratio mass spectrometer (Delta V Plus, Thermo Fisher Scientific, Waltham, MA) that could reliably detect nanomolar changes in $^{29,30}N_2$. The unlabeled glutamine-only vials served as a background N_2 control. The amounts of $^{29,30}N_2$ produced were calculated using the method described by Song and Tobias.[29] Gas-tight vials were prepared for each N amendment (3 reps), sample collection site (5), and time point (2), for a total of 90 samples, plus calibration checks. After flushing the headspace of the exetainer tubes with helium (He) gas for 5 min, the first time point (T0) was immediately analyzed using IRMS, and the remaining samples were incubated in the dark for 24 h. The 24 −h-incubated samples (Tfinal) were analyzed by IRMS to measure rates of $^{29,30}N_2$ production. Similar incubation and measurement protocols were performed for the buffer solution experiments described below.

Live soil incubation experiments were conducted with the NZ soils (NZ1, NZ5, and NZ6) to measure the rates of biotic N_2 production. Approximately 1 g of soil was placed in a 12 ml exetainer tube amended with 100 nmoles N as $Na^{15}NO_3^-$ (Cambridge Isotope, 99%atm). Nitrate (NO_3^-) was used to measure total N_2 production mediated by abiotic and biotic pathways in soil samples similar to the methods described by Lim et al. (2018). Gas-tight vials were prepared for each sample collection site (3) and time point (2) in duplicate, for a total of 12 samples, plus calibration checks. The exetainer tubes were flushed with He gas for 5 min and preincubated for 24 h at room temperature. The preincubated tubes were flushed again for 5 min with He gas and then amended with 0.1 mL of ^{15}N-NO_3^- (1 mM and 99%atm). The initial time point samples (T0) were treated with 50% zinc chloride (ZnCl) immediately following $^{15}NO_3^-$ addition to terminate biotic N_2 production. Remaining samples were incubated for 1 h (T1) and then treated with 50% ZnCl. The production of $^{29,30}N_2$ from live soil incubations was measured using IRMS, and rate calculations were conducted as described by Song and Tobias.[29] Extractable NO_2^- and NO_3^- of live soils were measured as described above and used to calculate the diluted ^{15}N ratio (atm %) in each soil incubation experiment.

2.3. Measurements of Abiotic Codenitrification in Buffer Solution under Varying Conditions of pH and N Substrates. Phosphate buffer (0.1 M K_2HPO_4 and 0.1 M KH_2PO_4) was prepared with autoclaved MilliQ water and used to determine the effects of the N substrate and pH conditions on abiotic codenitrification. To test the effects of NO_2^- concentrations on N_2 production by abiotic codenitrification, the pH of the phosphate buffer was adjusted to pH 6 with a hydrochloric acid (5%) solution. This pH (6) was selected because the pH for all soil samples used in incubation was < 7 (Table 2). The pH-adjusted buffer was filter-sterilized with a Whatman 0.45 μm filter. The sterilized buffer (1 mL) was pipetted into 12 ml exetainer tubes and amended with 1 mM ^{14}N-glycine, an unlabeled organic partner-N substrate. Each tube with buffer plus glycine was amended with varying concentrations of $Na^{15}NO_2^-$ (0, 5, 10, 50, 100, 500, and 1000 μM, Cambridge Isotope, 98% atm). Three replicates were prepared for each $^{15}NO_2^-$ concentration. After flushing the headspace of the tubes with helium gas, the tubes were incubated for 24 h (Tfinal) to measure $^{29,30}N_2$ using IRMS. Controls with $^{15}NO_2^-$ only and the partner substrate (unlabeled glycine) only were also prepared in triplicate and used to determine background $^{29,30}N_2$ after 24 h.

To test the effect of partner-N concentration (glycine) on $^{29,30}N_2$ production, we spiked the phosphate buffer (pH 6), amended with 1 mM $^{14}NO_2^-$ with varying concentrations of ^{15}N glycine (0, 1, 10, 100, and 1000 μM, Cambridge Isotope, 98%atm). The effect of the specific partner-N substrate on abiotic codenitrification was further tested with different inorganic and organic Ns including ammonium (NH_4^+), urea (Ure), alanine (Ala), arginine (Arg), glycine (Gly), glutamine (Glu), histidine (His), lysine (Lys), ornithine (Orn), and tryptophan (Trp). The partner-N substrates were selected based on their relevant presence in soils.[30] The phosphate buffer (pH 6) with $^{15}NO_2^-$ (1 mM N) was prepared, and 1 mL of the buffer solution was aliquoted in 12 mL exetainer tubes. Different partner-N substrates (1 mM N) listed above were added to the tubes in triplicate of each N substrate. The production of $^{29,30}N_2$ was measured after 24 h incubation with IRMS as described above.

The effect of pH on abiotic codenitrification was tested along a pH gradient (pH 3, 4, 5, 6, 7, and 8), which covers a wide variety of environmental pH values.[31] The phosphate buffer was adjusted to each pH with hydrochloric acid (5%) and/or potassium hydroxide (10%). The pH-adjusted solutions (1 mL) were aliquoted into 12 mL exetainer tubes and amended with 1 mM N of both $^{15}NO_2^-$ and partner ^{14}N substrates. The partner ^{14}N substrates include NH_4^+, Ure, Gly, Glu, and Orn. After substrate amendment, triplicate reactions were incubated for 24 h prior to measurement of $^{29,30}N_2$ production by IRMS.

2.4. Comparison of Abiotic N_2 and N_2O Production. To compare the abiotic production of N_2 and N_2O, incubation experiments similar to those described above were conducted using sterile solution and soils. Phosphate buffer adjusted to pH 5 was aliquoted into gas-tight vials prepared with one of the following treatments: (a) no N amendment or (b) ^{15}N-NO_2^- and Glu (1 mM N each). In triplicate, each treatment was prepared for both products (N_2 or N_2O) for a total of 12 samples, plus calibration checks. Samples were incubated at room temperature for 24 h prior to measurement of $^{29,30}N_2$ by IRMS, and N_2O production was measured using a gas chromatograph fitted with an electron capture detector (GC-ECD: Shimadzu). Production of $^{29}N_2$ was detected, but N_2O production was negligible for both solution treatments.

Sterile soils from North Carolina (NC), North Dakota (ND), and New Zealand (NZ1) were used for comparison of abiotic N_2 and N_2O production. Gas-tight vials were prepared for each N amendment (3 reps), sample collection site (3), and product (2), for a total of 54 samples, plus calibration checks. The three N amendment treatments included: (a) Control (no amendment), (b) $^{15}NO_2^-$ (1 mM N and 1 μmoles N g^{-1}), and (c) ^{15}N-NO_2^- and Glu (1 mM N each and 1 μmoles N g^{-1}). The concentration of 1 mM NO_2^- was required to measure N_2O production above atmospheric N_2O concentrations. Soil samples were incubated for 24 h prior to analysis for $^{29,30}N_2$ by IRMS and N_2O with a GC-ECD.

2.5. Statistical Analyses. The $^{29}N_2$ produced for sterile soils incubated for 24 h was analyzed by a two-way analysis of variance (ANOVA). We evaluated (1) if soils amended with both $^{15}NO_2^-$ and Glu produce more $^{29}N_2$ than soils amended with $^{15}NO_2^-$ only and (2) how $^{29}N_2$ production varied among the five grassland sites. The ANOVA included the fixed effect of site (NZ1, NZ5, NZ6, US2, and US3), treatment ($^{15}NO_2^-$

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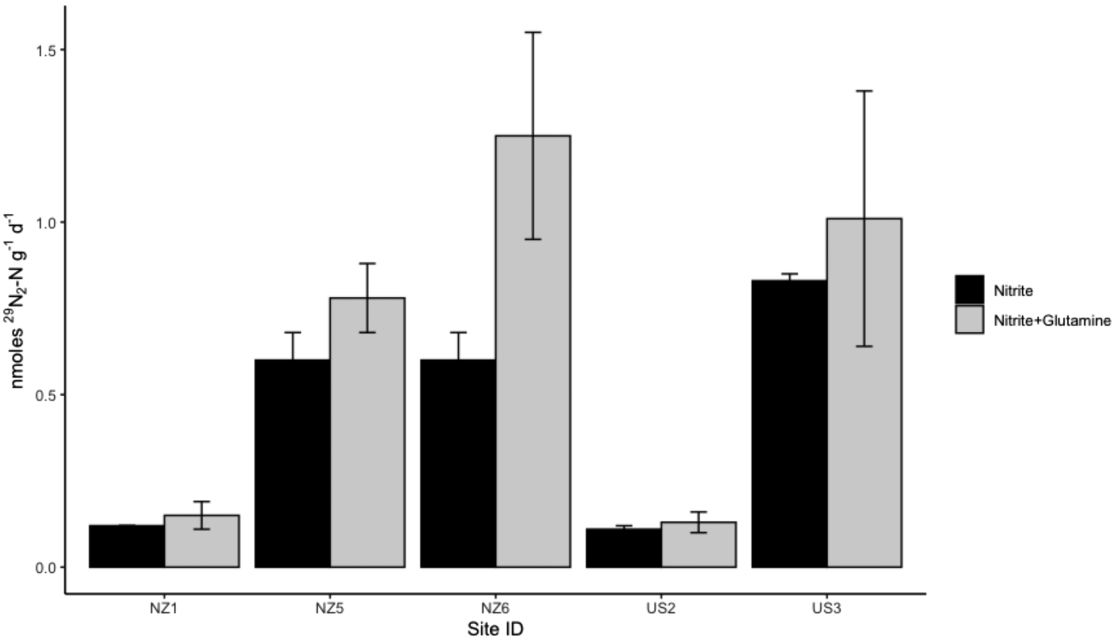

Figure 1. Abiotic codenitrification rates (nmoles $^{29}N_2$-N g^{-1} day^{-1}) in each soil sample (NZ1, NZ5, NZ6, US2, and US3) for each treatment (nitrite and nitrite + glutamine). Error bars represent one standard deviation in each direction. ANOVA results indicate statistically significant effects of treatment (p-value <0.01), site (p-value <0.001), and the interaction site x treatment (p-value <0.05).

Table 1. Comparison of Abiotic and Total N_2 Production Rates from Soils NZ1, NZ5, and NZ6[a]

sample	abiotic N_2 rate (nmoles $^{29}N_2$-N g^{-1} d^{-1})	total N_2 rate (nmoles $^{29}N_2$-N g^{-1} d^{-1})	% abiotic N_2 production	^{15}N ratio (atm %)
NZ1	0.12 ± 0.00	15.09 ± 2.57	0.80	8.18
NZ5	0.6 ± 0.08	31.54 ± 9.81	1.90	2.87
NZ6	0.6 ± 0.08	7.44 ± 2.05	8.07	2.34

[a]Abiotic rate determined with sterile soil incubations amended with $^{15}NO_2^-$. Total N_2 production rates determined with live soil incubations amended with $^{15}NO_3^-$. Percent of abiotic N_2 production = (abiotic N_2 production / total N_2 production) × 100. ^{15}The N ratio is the $^{15}NO_3^-$:$^{14}NO_2^-$ ratio (atm %) calculated by $^{15}NO_3^-$ (0.1 μmoles N g^{-1}) added into the live soils (1 g) divided by a sum of added $^{15}NO_3^-$ and extractable NO_3^- in the live soils (NZ1 = 1.12 μmoles N g^{-1}, NZ5 = 3.38 μmoles N g^{-1}, and NZ6 = 4.16 μmoles N g^{-1}).

only vs glutamine +$^{15}NO_2^-$), and interactions. A posthoc Tukey test for multiple comparisons of means was used to indicate how $^{29}N_2$ production varied among the five grassland soils with 95% confidence intervals.

The $^{29}N_2$ production observed with increasing $^{15}NO_2^-$ or ^{15}N-Gly concentrations in buffer solution experiments was statistically analyzed with a linear model ($y = \alpha x + \beta$). Data were log-transformed to meet the model assumption of normality. Modeled and observed $^{29}N_2$ production data were compared, and R^2 values were reported. The effects of partner-N substrates on abiotic codenitrification were statistically evaluated based on the $^{29}N_2$ production as compared to the control ($^{15}NO_2^-$ only) with a simple one-way ANOVA. The ANOVA was followed by posthoc analysis with a Tukey test for multiple comparisons of means with a 95% family-wise confidence level. Finally, the influence of pH on $^{29}N_2$ production was tested for each of the tested partner-N substrates using a linear model ($y = \alpha x + \beta$). Modeled and observed $^{29}N_2$ production data for each partner-N substrate were compared and the R^2 value reported. Data were log-transformed to meet the model assumption of normality. Significance for all tests was determined at $\alpha = 0.05$.

3. RESULTS

3.1. Abiotic Codenitrification in Soils. Production of $^{29}N_2$ was clearly measured in soil samples, while $^{30}N_2$ production was negligible. Mean values for $^{29}N_2$ (nmoles g^{-1} d^{-1}) by site for each time point (± std. dev.) are shown for $^{15}NO_2^-$ only and Glu plus $^{15}NO_2^-$ (Figure 1). While all soils responded positively to inclusion of the Glu, as compared to $^{15}NO_2^-$ only, the magnitude of this effect varied with site (site x treatment, p-value <0.05). NZ6 volcanic soils had a stronger response to the inclusion of glutamine (Glu) than soils collected at other sites. Abiotic $^{29}N_2$ production increased by 100%, as compared to $^{15}NO_2^-$ only for NZ6 (Figure 1). Inclusion of Glu for the remaining four sites increased $^{29}N_2$ production by 20−30%. The average rates of $^{29}N_2$ production (and std. dev.) by treatment and site are shown in Table S1. Soils from sites NZ1 and US2 produced less $^{29}N_2$ than the others (US3, NZ5, and NZ6), and these two sites were not significantly different from each other (Table S2).

Three NZ soils were used to measure and compare the rates of abiotic and total N_2 production. $^{29}N_2$ was the major N_2 gas product while $^{30}N_2$ production was negligible in the three live soils. Considering that the $^{15}N-NO_3^-$ enrichment ratios were 2.34 to 8.18 atm%, the observed $^{29}N_2$ production can be attributed to high amounts of residual NO_3^- in the soils ranging from 1.12 to 4.16 μmoles g^{-1} (Table 1). Observed

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$^{29}N_2$ production in live soils could have resulted from both abiotic and biotic pathways, including abiotic codenitrification, anammox, codenitrification, and denitrification. Total N_2 production rates ranged from 7.44 ± 2.05 to 31.54 ± 9.81 nmoles $^{29}N_2$−N g^{-1} h^{-1}. whereas the rates of abiotic codenitrification ranged from 0.12 ± 0.001 to 0.6 ± 0.08 $^{29}N_2$-N g^{-1} h^{-1}. (Table 1 and Figure S1). Despite abiotic rates being lower than the total N_2 production rates, the abiotic production accounted for 0.8 to 8.2% of the total production (Table 1).

3.2. Comparing Abiotic N_2 and N_2O Production in Soils. The N_2 production rates were higher than N_2O production rates in the soils amended with both $^{15}N{-}NO_2^-$ or $^{15}N{-}NO_2^-$ and Glu (Figure S2). Control soils did not show any N_2 or N_2O production. The rates of $^{29}N_2$−N production in the soils amended with $^{15}N{-}NO_2^-$ (1 μmole N) ranged from 31.47 ± 2.25 to 52.12 ± 20.11 nmoles N_2−N g^{-1} d^{-1}, whereas N_2O production rates ranged from 0.51 ± 0.10 to 4.41 ± 0.85 nmoles N_2O−N g^{-1} d^{-1} (Figure S2). Soils amended with ^{15}N-NO_2^- and Glu (1 μmole N each) had rates of N_2−N production ranging from 61.97 ± 5.17 to 71.47 ± 5.66 nmoles N_2−N g^{-1} d^{-1} and N_2O−N production rates ranging from 1.72 ± 0.21 to 3.99 ± 0.21 nmoles N_2O−N g^{-1} d^{-1} (Figure S2). The rates of N_2 production were higher in all the soils amended with ^{15}N-NO_2^- plus Glu than $^{15}NO_2^-$ only. The N_2O production rates in NC and NZ soils showed no difference between substrate conditions as compared to the ND soil, which had higher N_2O production with $^{15}NO_2^-$ and Glu addition. Overall, the abiotic N_2O production in soil incubation was only 1−12% of the N_2 production by abiotic codenitrification in soils.

3.3. Soil Mineral N, pH, TOC, and TN. Extractable NO_3^- concentrations in the sterile soils ranged from 0 to 3.06 μmoles g^{-1} (Table 2). The soils with the lowest and highest NO_3^- concentrations were NZ5 and US2, respectively. The extractable NH_4^+ concentrations ranged from 2.02 to 3.44 μmoles g^{-1}. NZ1 had the lowest NH_4^+ concentration while NZ6 had the highest NH_4^+ concentration (Table 2). The pH in sterile soils ranged from 5.0 to 5.9 (Table 2). The lowest pH was observed in NZ5 and NZ6 samples (5.0), and the US2 had the highest pH (5.9). The soils (NZ5 and NZ6) with the highest $^{29}N_2$ production had the lowest soil pH. The soils (US2) with the highest pH had the lowest $^{29}N_2$ production. Table 2 lists soil TOC, TN, and carbon:nitrogen (C:N) ratio, where NZ6 and US3 stand out as substantively higher in TOC. The C:N ratio measured in soils ranged from 9 to 16 with the highest in US3, which also exhibited the highest N_2 production in the $^{15}NO_2^-$ only treatment. C:N ratios for soils from NZ6 and NZ5 were higher and produced more $^{29}N_2$ than soils from sites with a lower C:N ratio (NZ1 and US2).

3.4. Abiotic Codenitrification in Buffer Solution under Varying N Conditions. Higher production of $^{29}N_2$ was observed as the concentration of $^{15}NO_2^-$ increased in buffer solution experiments (Figure 2 and Table S1). $^{29}N_2$−N production was <10 nmoles N L^{-1} Day^{-1} when $^{15}NO_2^-$ concentration was below 100 μM, whereas the highest rate was 30 nmoles N L^{-1} day^{-1} when $^{15}NO_2^-$ concentration was 1000 μM. There was a statistically significant linear relationship between the concentration of $^{15}NO_2^-$ and $^{29}N_2$ production (p-value <0.001 and R^2 = 0.9734). Similarly, $^{29}N_2$ production increased as the concentration of ^{15}N-Gly increased (Figure 2). The $^{29}N_2$ production ranged from 3.84 nmoles N L^{-1} Day^{-1} to 31.82 nmoles N L^{-1} Day^{-1} with concentrations

Table 2. Soil Sample Characteristics Including the Sampling Site, Bulk Density, Percent Sand, Percent Silt, Percent Clay, Sterile Soil pH, Extractable NO_3^- and NH_4^+ Concentrations, TOC, Total Organic Nitrogen, and C:N Ratio[a]

sample ID	type	soil classification	soil series	location	reference	bulk density	% sand	% silt	% clay	sterile soil pH	extractable NO_3^- (μmoles per g^{-1})	extractable NH_4^+ (μmoles per g^{-1})	sterile soil TOC (% dw)		sterile soil TN (% dw)		C:N ratio
													average	std. dev	average	std. dev	
NZ1	stony	pallic orthic brown soil, typic dystustept	Lismore	43.644 S, 172.426 E	van der Weerden (2016); Carrick, unpublished (2016)	1.16	46	30	24	5.6	1.06	2.02	3.38	0.33	0.31	0.01	10.90
US2	silty loess	frigid typic haplustols	Tombk	46.406 N, 100.388 W	Semedo et al. (2018)	1.19	65	23	12	5.9	3.06	3.04	1.45	0.11	0.15	0.01	9.67
US3	clayey	clayey, mixed, thermic, typic umbraquult	Cape Fear	35.849 N, 76.651 W	Tare (1981); Phillips et al (2015)	1.15	37	39	24	5.3	1.91	3.15	5.43	1.5	0.33	0.04	16.45
NZ5	fluvial	dystric fluvcntic eutrochrep	Karapoti	40.383 S, 175610 E	van der Weerden (2016)	1.08	34	47	19	5.0	0.00	2.33	2.79	0.31	0.30	0.04	9.30
NZ6	volcanic	orthic allopharic, typic udivitrand	Horotlu	40.777 S, 175.313 E	van der Weerden (2016)	1.10	34	48	17	5.0	0.65	3.44	11.7	0.06	1.03	0.01	11.36

[a]Extractable NO_2^- concentrations were negligible or below detection limits.

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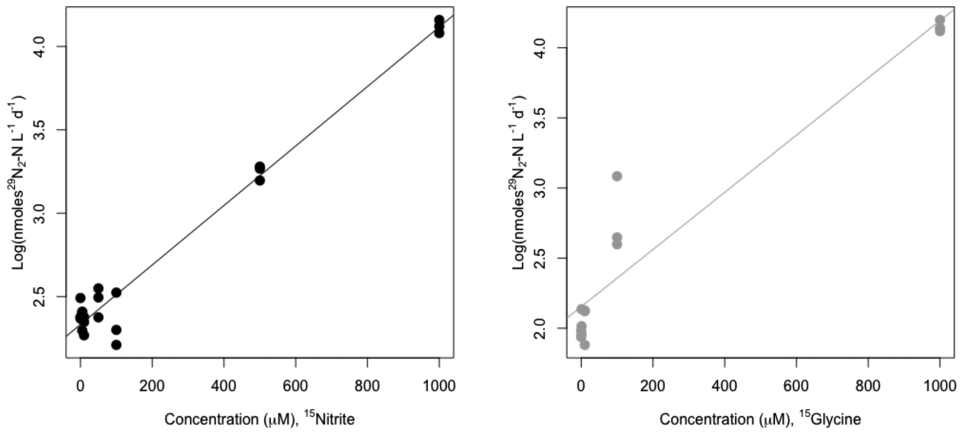

Figure 2. Log-transformed concentration gradient data (log (nmoles $^{29}N_2$–N L^{-1} d^{-1})) (points) and linear models (lines). NO_2^- concentration gradient linear model (black line), p-value <0.001, and R^2 = 0.97. Glycine concentration gradient linear model (gray line), p-value <0.001, and R^2 = 0.9.

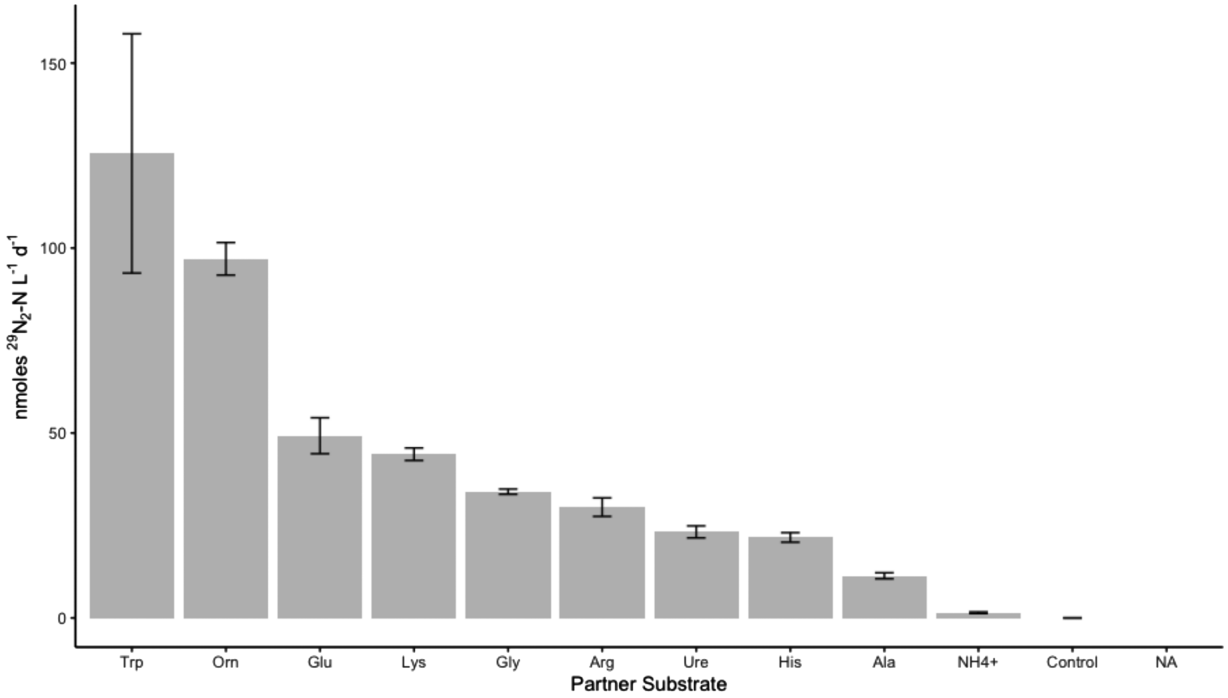

Figure 3. Abiotic $^{29}N_2$ production rates for different partner-N substrates in buffer solution (pH 6). Two included controls are also shown: no substrate control with nitrite only (control) and a buffer control with a no nitrite and no partner substrate (NA). Each partner-N substrate was shown to be significantly different from the control sample (p-value <0.05).

of 1 μM and 1000 μM ^{15}N-Gly, respectively. There was a statistically significant linear relationship between ^{15}N-Gly and $^{29}N_2$ production (p-value <0.001 and R^2 = 0.9041).

Partner-N substrates (NH_4^+, Ure, Ala, Arg, Gly, Glu, His, Lys, Orn, or Trp) produced different amounts of $^{29}N_2$ in the buffer solutions (pH 6) amended with $^{15}NO_2^-$ (Figure 3). The highest $^{29}N_2$ production was observed with Trp treatment; 125.57 ± 32.34 nmoles $^{29}N_2$ L^{-1} Day^{-1}. In comparison, the lowest N_2 production was observed when NH_4^+ was the partner substrate ammonium; 1.42 ± 0.21 nmoles $^{29}N_2$ L^{-1} Day^{-1}. A one-way ANOVA indicated that the substrate had a significant effect on the $^{29}N_2$ production (p-value <0.05). Posthoc analysis indicated statistically significant differences (p-value <0.05) between each partner substrate condition (substrate + $^{15}NO_2^-$) and the $^{15}NO_2^-$ only control.

3.5. Abiotic Codenitrification in Buffer Solution under Different pH and N Substrate Conditions. The effects of different pH values and N substrates on abiotic codenitrification were tested with six different pH values (3, 4, 5, 6, 7, and 8) and five N substrates (NH_4^+, Ure, Gly, Glu, and Orn). Regardless of the partner-N substrate, $^{29}N_2$ production decreased as pH increased (Figure 4, Table S2). The Orn treatment produced the highest amount of $^{29}N_2$, 19,267.90 ± 5898.65 nmoles N L^{-1} Day^{-1} at pH 3, but the $^{29}N_2$ production rate declined markedly to 30.00 ± 17.32 nmoles N L^{-1} Day^{-1} at pH 8. Ure produced the second highest amount of $^{29}N_2$, 15,241.89 ± 2953.69 nmoles N L^{-1} Day^{-1} at pH 3 and 90.97 ± 9.24 nmoles N L^{-1} Day^{-1} at pH 4 and also had a decline to 0.94 ± 0.81 nmoles N L^{-1} Day^{-1} at pH 8. The $^{29}N_2$ production was lowest in NH_4^+ treatment, which exhibited a rate of 18.22

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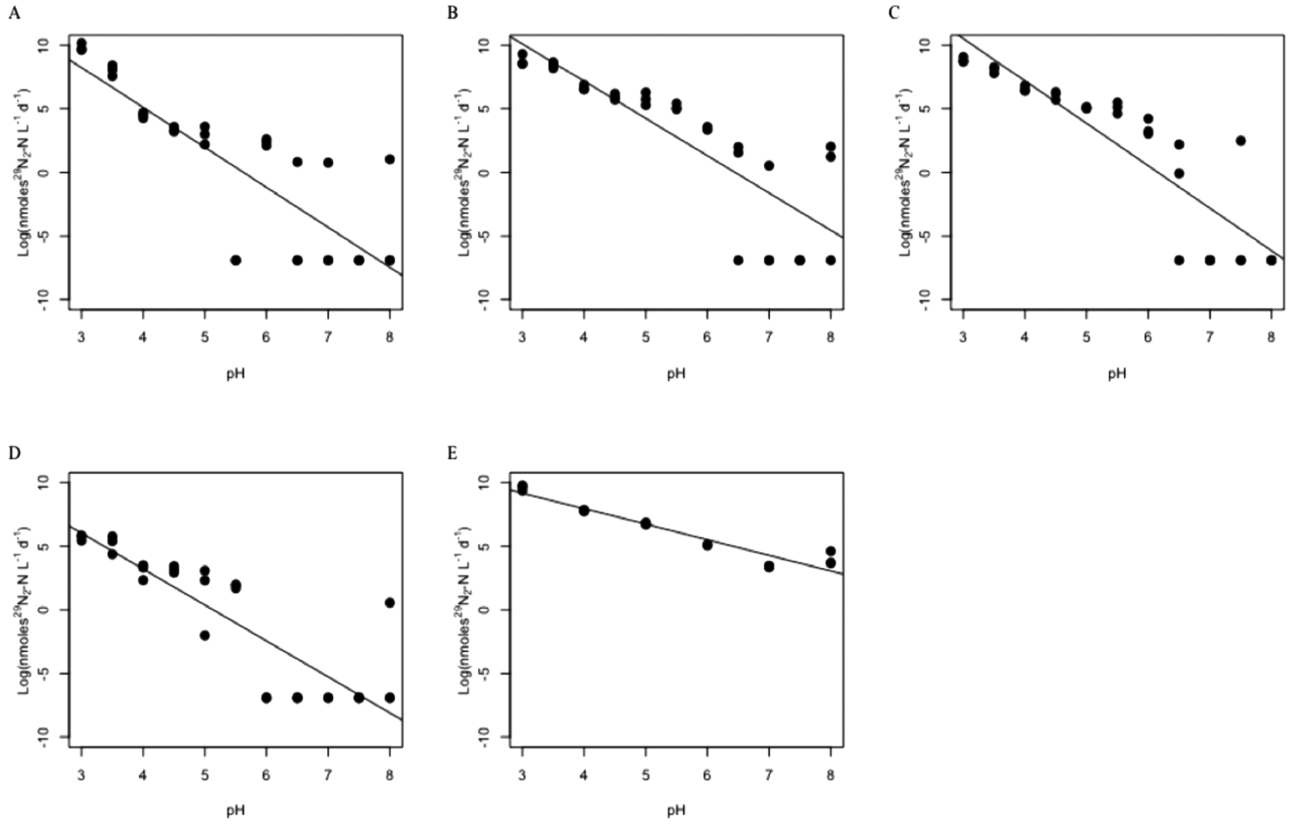

Figure 4. Abiotic $^{29}N_2$ production rates from different partner-N substrates along the pH gradient. Log of nmoles $^{29}N_2$−N L^{-1} d^{-1} along pH gradient data (observed data = points) and fitted models ($^{29}N_2$-N − m × pH + b) (linear model = line). (A) Urea pH gradient test (Urea + $^{15}NO_2^-$): R^2 = 0.67 and p-value = 3.272 × 10^{-09}. (B) Glutamine pH gradient test (Glu + $^{15}NO_2^-$): R^2 = 0.7 and p-value = 6.429 × 10^{-10}. (C) Glycine pH gradient test (Gly + $^{15}NO_2^-$): R^2 = 0.79 and p-value = 2.094 × 10^{-12}. (D) Ornithine pH gradient test (Orn + $^{15}NO_2^-$): R^2 = 0.92 and p-value = 3.059 × 10^{-10}.

± 2.83 nmoles N L^{-1} Day^{-1} at pH 4 and 0.54 ± 0.94 nmoles N L^{-1} Day^{-1} at pH 8. Although the magnitude of $^{29}N_2$ production at each pH varied with the partner-N substrates, the negative relationship between $^{29}N_2$ production and pH was consistent. There was a significant linear relationship between pH and the natural log of $^{29}N_2$ production rates for each substrate tested (all p-values <0.05, see Figure 4 and Table S2 for R^2 values). Orn had the strongest linear relationship between $^{29}N_2$ production rates and pH (R^2 = 0.858), whereas ammonium had the weakest linear relationship (R^2 = 0.401).

4. DISCUSSION

4.1. Abiotic and Biotic N_2 Production in Soils. The net rate of abiotic codenitrification in the environment depends upon competition for NO_2^- between biotic and abiotic processes. Soils are complex matrices with the capability of producing N_2 through abiotic codenitrification, codenitrification, anammox, and denitrification. Anammox and codenitrification are biotic pathways that produce hybrid N_2, the same product resulting from abiotic codenitrification. Here, we report that abiotic N_2 production contributed 0.8 to 8.2% to the total N_2 production in the acidic grassland soils. This is within the range reported by Lim et al. (2018), where abiotic NO_2^- decomposition converted a significant fraction (10−20%) of NO_2−N to nitroso-compounds. The importance of organic matter in nitrosation reactions was detailed by Williams[32] and is also noted here in this study. In our results, the highest relative contribution of abiotic to biotic N_2 production was found in the soil with the highest C:N ratio (NZ6). Higher C:N ratios are indicative of a less labile soil C pool compared to soils with lower C:N ratios.[33] Our data suggest that rates of abiotic codenitrification could be influenced by larger pools of C relative to N, particularly if the reaction introducing the NO^+ ion to partner-N C atoms increases when fewer N atoms are available.

4.2. Abiotic N_2 and N_2O Production in Soils. Although this study was focused on the N_2 production by abiotic codenitrification, it should be noted that N_2O can be produced by abiotic processes such as chemodenitrification and nitrosation.[7,24,34,35] When abiotic N_2 and N_2O production was compared in this study, we observed that N_2O production was a small fraction of the N_2 produced in all samples. Our results indicate that the major pathway of abiotic NO_2^- reduction is abiotic codenitrification to N_2. Nitrosation reactions require hydroxylamine or oxime compounds with an oxidation state of -I to produce N_2O[7]. The low production of N_2O in acidic soils could be explained by low availability of the compounds that react with NO_2^- to produce N_2O. However, this study clearly demonstrates that abiotic codenitrification producing hybrid N_2 is the major pathway of abiotic N removal in acidic soils and that abiotic N_2 production can be enhanced by the addition of amino acids commonly found in soils.[30]

4.3. Controlling Factors of Abiotic Codenitrification in Soils. The results of the incubation experiments with five different sterile soils revealed potential factors controlling abiotic codenitrification. Abiotic codenitrification was greater

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for sterile soils collected at the US3, NZ5, and NZ6 sites, where pH ranged between 5.0 and 5.3. Soil pH for the two soils with the lowest N_2 production (NZ1 and US2) was 5.6 and 5.9, respectively. The importance of pH and NO_2^- concentration has been corroborated by numerous studies where both NO_2^- and an organic-N nitrosation partner were available in soils.[12,27,36] Recently, a sterile peat reportedly produced N_2 at pH < 5, but not at pH > 7.[12] Results of a NO_2^- tracer experiment reported that abiotic production of N_2 and N_2O (the nitrosation agent NO derived from the decomposition of HNO_2) was elevated in acidic soils.[13] While pH was not manipulated in the soil experiments, more N_2 was produced from acidic soils, and the preponderance of the data in the literature points to pH as a controlling factor for abiotic codenitrification.

The soils amended with Glu and $^{15}NO_2^-$ had higher rates of abiotic codenitrification than the ones with $^{15}NO_2^-$ only (Figure 1). This finding is consistent with the previous studies reporting the importance of organic matter in the abiotic immobilization of NO_2^- in sediment[14] and soil[16] and the concomitant production of N_2 gas.[6] While all soils responded positively to Glu addition, this was most evident for NZ6 volcanic soil, which doubled N_2 production by abiotic codenitrification. NZ6 volcanic soil is an allophane soil, which is characteristically higher in TOC and specific surface area, as compared to nonallophanes.[37] The specific surface area of allophanes is reportedly[38] 700 $m^2\ g^{-1}$, which is similar to the specific surface area of organic matter,[39] ranging from 580 to 800 $m^2\ g^{-1}$. The allophane then provides more organic matter required for the nitrosation reaction and more surface area. Both organic matter and mineral particle surfaces are sites where the self-decomposition of HNO_2 to NO can occur.[40] NO_2^- is the most efficient species of DIN in terms of the reaction with soil organic matter to form organic N and N_2 precisely because of the NO spontaneously produced at low pH[16]. Chemical formation of NO occurs when NO_2^- is converted to nitrous acid at low pH, which then decomposes to NO and HNO_3 as described below:[9]

$$3NO_2^- + 3H^+ \leftrightarrow 3HNO_2 \rightarrow 2NO + HNO_3 + H_2O \quad (1)$$

It is important to note in this discussion that a full-throated investigation of how γ irradiation affected each of these disparate soils was outside the scope of the project, but worthy of future consideration, so sterile soil measurements may not directly align with live soil measurements for abiotic codenitrification. NO_2^- in a sterile acidic peat (pH < 5) with 45% TOC was more readily used by microbes for denitrification, although abiotic production of N_2 (2 to 14 nmoles $g^{-1}\ day^{-1}$) was observed.[12] Soils reported here are for grasslands, where TOC < 5% resulted in N_2 production of 1 nmole N $g^{-1}\ day^{-1}$ at pH >5. At higher levels of NO_2^- addition, a sterile NZ soil produced over 700 nmoles N g^{-1} over the initial 24 h of an incubation time series.[21] These NZ soils were similar to NZ1 Stony with respect to pH and organic matter, so higher NO_2^- addition likely explains the contrast in abiotic hybrid N_2 production rates.

4.4. Effect of N Concentration on Abiotic Codenitrification. Abiotic hybrid $^{29}N_2$ production increased linearly with $^{15}NO_2^-$ concentration. Similarly, abiotic hybrid $^{29}N_2$ production increased linearly with ^{15}N-Gly concentration. These results confirm that rates of abiotic codenitrification are dependent on both NO_2^- and partner-N concentrations. Van Cleemput et al. (1995) also reported abiotic gaseous N production at high NO_2^- concentrations, but they did not include low concentrations that may be observed in the environment.[19] Abiotic N_2 production above 10 nmoles $L^{-1}\ day^{-1}$ was not observed below a concentration of 100 μM for either substrate ($^{15}NO_2^-$ or ^{15}N-Gly). This could indicate either a substrate concentration threshold for N_2 formation by abiotic codenitrification or a detection limit for measuring small changes in $^{29}N_2$ production at lower concentrations.

4.5. Effects of Partner-N Substrates on Abiotic Codenitrification. We found specific N substrates commonly found in soils[30] varied in their effects on abiotic hybrid N_2 production in buffer solution experiments. Trp- and Orn-treated samples exhibited the highest N_2 production, whereas the lowest N_2 production was observed in samples with NH_4^+. Among various characteristics of each N substrate (Table 3), the C:N ratio had a significant linear relationship with N_2 production (p-value <0.05 and R^2 = 0.6411) as well as with molecular weight (p-value <0.05 and R^2 = 0.5335). Interestingly, when comparing the C:N ratio of each soil sample with N_2 production, the same pattern was observed in the soil incubation experiments. The C:N ratio may be an important factor for abiotic codenitrification occurring in recalcitrant organic matter or humic substances, which have high C:N ratios and may not be widely used by biotic processes.[30]

Table 3. Characteristics of Amino Acids Used as Partner-N Substrates in Buffer Solution Experiments Including Polarity, Acidic/Basic, Number of Amine Groups, Molecular Weight, and C:N Ratio

amino acid	polarity	acidic/basic	#of amine groups	molecular weight g/mol	C:N
ammonium	polar	neutral	1	53.49	
urea	nonpolar	neutral	2	60.06	1:2
alanine	nonpolar	neutral	1	89.09	3:1
arginine	polar	basic	3	174.20	4:4
glycine	nonpolar	neutral	1	75.07	2:1
glutamine	nonpolar	neutral	2	146.14	5:2
histidine	polar	basic	3	155.15	5:3
lysine	polar	basic	2	146.20	6:2
ornithine	polar	moderately acidic	2	168.6	5:2
tryptophan	slightly polar	neutral	2	204.23	11:2

4.6. Effects of pH and N Partner Substrates on Abiotic Codenitrification. Five N substrates, including NH_4^+, Ure, Gly, Glu, and Orn, were tested along a pH gradient (3−8) for N_2 production with $^{15}NO_2^-$. All partner-N substrates tested in solutions with $^{15}NO_2^-$ exhibited increased N_2 production at low pH. A significant negative linear relationship between pH and $^{29}N_2$ production was observed for each of the different substrates. This aligns with previous studies reporting increased abiotic hybrid N_2 production at low pH.[12] This also agrees with the soil incubations in which higher N_2 production from soils with lower pH was observed. These buffer solution experiments isolated pH from other, potentially confounding factors commonly present in environmental samples.

The results of the pH gradient testing indicate that there may be substantial N removal capacity by abiotic codeni-

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trification in low-pH environments. High reactivity of Ure to abiotic codenitrification at low pH values is of particular significance as Ure is commonly used as an agricultural fertilizer. According to the International Fertilizer Association, Ure accounts for >57% of the global fertilizer demand.[41] The widespread use of Ure and its high reactivity to abiotic codenitrification could lead to significant N losses by abiotic codenitrification, especially in acidic soils. Acidic environments may include but are not limited to acidic soils, cave systems, and acid mine drainage sites. Abiotic codenitrification may be an important N removal process under acidic conditions, which can be unfavorable for microbial N removal processes.[42,43] Glass and Silverstein showed that in batch reactors, denitrification was inhibited at pH < 7.0.[42] Waring and Gilliam confirmed that denitrification may be inhibited at low pH,[43] but the threshold of pH tolerance was unclear. N_2 production by anammox bacteria also decreases at low pH.[44] The potential inhibition of microbial N removal processes at low pH suggests that abiotic codenitrification could dominate over biotic codenitrification in low-pH environments. An ideal location where abiotic codenitrification may be a major pathway of N removal could be urine patches in agricultural soils; these areas are typically high in organic N compounds such as urea but also have high NO_2^- concentrations.[35,45,46]

5. CONCLUSIONS

Abiotic codenitrification is an important N removal process in acidic soils with low labile organic C to support biotic processes. We found that abiotic N_2 production is much greater than abiotic N_2O production and contributes up to 8.2% of total N_2 production in the acidic soils examined. Abiotic codenitrification is enhanced by the addition of organic N such as Glu. The magnitude of the effect of Glu addition, however, varies with soil properties, such as pH. Here, we show that substrate concentration, pH, and partner-N compounds are all important factors determining the magnitude of N_2 produced by abiotic codenitrification. Our results indicate that N_2 production by abiotic codenitrification increases with increasing substrate concentration and decreasing pH and shows differential activity with various compounds favoring those with a high molecular weight and C:N ratio. In situ, abiotic processes may persist where microbial pathways are inhibited or are unfavored, for example, in the environments enriched with recalcitrant organic matter and/or with low pH. This gives insight into the potential hotspots of abiotic codenitrification and the required conditions for this reaction such as in agricultural soils and ruminant urine patches. The contribution of abiotic codenitrification to N removal in the environment remains unconstrained because of difficulties discriminating from abiotic codenitrification from biotic processes. Further research is needed to fully understand the implications of abiotic codenitrification to the greater N cycle and budgets.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsearthspacechem.0c00225.

Comparison of abiotic and biotic nitrogen production rates from NZ1, NZ5, and NZ6 soils, $^{29}N_2$ production rates along concentration gradients in buffer solution experiments, abiotic $^{29}N_2$ production rates from different partner-N substrates along the pH gradient, and comparison of N_2 and N_2O production in soils amended from NC, ND, and NZ (PDF)

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Author Contributions

‖S.J.W., B.S., and R.P. contributed equally. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

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■ ABBREVIATIONS

N_2,dinitrogen; NO_2^-,nitrite; NO_3^-,nitrate; NH_4^+,ammonium; Ure,urea; Ala,alanine; Arg,arginine; Gly,glycine; Glu,glutamine; His,histidine; Lys,lysine; Orn,ornithine; Trp,tryptophan; IRMS,isotope ratio mass spectrometry.

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