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Chemical identity of carbon substrates drives differences in denitrification and N_2O reduction within agricultural soils

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Abstract

Rates of nitrous oxide (N_2O) production from agricultural soils are highly variable across space and time. Improving predictions of N_2O emissions will require improving our understanding of the drivers of denitrification and the sources of variability in the rates of N_2O production between soils and over time. While the amount of available carbon (C) is a known control on denitrification and N_2O reduction, relatively little attention has been paid to the effect of the chemical identity of C substrates on rates of denitrification and N_2O reduction. We investigated the effects of twelve different C-substrate additions on the production and reduction of N_2O in five soils taken from two distinct agricultural locations in Michigan under multiple land uses. We provided additions of glucose, cellulose, N-acetyl-glucosamine, chitin, amino acids, protein, vanillyl alcohol, lignin, citrate, succinate, methanol, and water in laboratory denitrification potential assays to determine the effects of denitrifier C preference on denitrification rates. We found that amino acids, protein, and organic acids stimulated the greatest rates of denitrification potential across all land uses. Similarly, we found these same substrates caused the most N_2O reduction, resulting in the lowest net concentrations of N_2O. Soils from agricultural rotations without cover crops had overall lower rates of denitrifier activity, leading to less net N_2O production compared to soils from other land uses. In general, C-utilization patterns were similar among all soils, and C-substrate identity had a much stronger effect than land use. Here, we demonstrate that the chemical identity of available C gives rise to wide variability in rates of denitrification and N_2O reduction.

1. Introduction

Nitrous oxide (N_2O) is a greenhouse gas with a global warming potential nearly 300 times greater than that of CO_2 (IPCC, 2014). Nearly half of global N_2O emissions are anthropogenic, with agriculture accounting for the largest share (Tian et al., 2020). Net emissions of N_2O are the result of multiple microbially mediated processes, but denitrification is thought to be the predominant N_2O

42 generation pathway in agricultural systems (Opdyke et al., 2009; Liang and Robertson, 2021).
43 Denitrification is an anaerobic, respiratory metabolism where inorganic N species are reduced in a
44 stepwise manner within the electron transport chain to generate ATP through oxidative
45 phosphorylation, resulting in the production of N_2O and/or N_2. The balance of these two end products
46 determines the contribution of denitrification to greenhouse gas emissions.

47 There are multiple controls on the process of denitrification in general and on the end-product
48 ratio in particular (Firestone and Davidson, 1989). Even though the main drivers of denitrification are
49 known to include carbon (C), nitrogen (N), and O_2 availability, denitrification rates and net N_2O
50 emissions remain difficult to predict in the field and are subject to large spatial and temporal
51 variation. Much of this variation is due to the spatial distribution of the main drivers of denitrification
52 throughout the soil profile, giving rise to micro-scale variation and episodic fluxes in denitrification
53 rates (Groffman et al., 2009; Kuzyakov and Blagodatskaya, 2015). For example, anaerobic microsites
54 can exist within and between soil aggregates, promoting denitrification even within well-aerated soils
55 (Hojberg and Sorensen, 1993; Kravchenko et al., 2017; Schlüter et al., 2018). In addition, the
56 distribution of particulate C substrates throughout the soil creates zones of high N_2O production
57 (Parkin, 1987; K. Kim et al., 2020), but the effect of the chemical identity of these heterogeneously
58 distributed forms of C on rates of N_2O production and reduction has not been well described.

59 The importance of the quantity of available C seems obvious, with more available C yielding
60 more electrons and driving denitrification, but there are complex interactions that control how C
61 availability affects the balance of N_2O production and consumption. For instance, when C is limiting,
62 net N_2O production tends to be higher, due to a lower demand for terminal electron acceptors
63 (Pidello et al., 1996). Likewise, N_2O reduction has been shown to be inversely related to the
64 availability of alternative electron acceptors, such as O_2 or nitrate (NO_3^-) (Firestone et al., 1980;
65 Miller et al., 2008; Senbayram et al., 2012). Therefore, by driving the consumption of terminal

electron acceptors, higher rates of C availability should stimulate more N_2O reduction and a lower N_2O:N_2 ratio.

In addition to C quantity, one of the most important yet least explored factors determining denitrification rates and N_2O production or consumption is the biochemistry of available C compounds. Although the idea that the chemical identity of available C is a driver of denitrification has been recognized for decades (e.g., de Catanzaro and Beauchamp, 1985), we still lack a clear understanding of how substrate identity is tied to rates of denitrification, such as which chemical characteristics are most important for denitrifiers. Multiple studies have come to widely different conclusions on the effects of particular C substrates on denitrification. Some studies have described glucose and other simple carbohydrates stimulating more denitrification than organic acids and amino acids (Smith and Tiedje, 1979; Dendooven et al., 1996; Miller et al., 2008; Morley and Baggs, 2010), while others have found the opposite (Morley and Baggs, 2010; Morley et al., 2014). Likewise, denitrifiers respond differently to whole plant residues compared to low-molecular-weight C additions (de Catanzaro and Beauchamp, 1985; Senbayram et al., 2012; Giles et al., 2017). Much of this inconsistency between studies comes from comparing experiments that were performed under a variety of conditions, making it difficult to differentiate the effects of C chemistry from soil characteristics and environmental or experimental factors, such as N availability and anaerobicity. By comparing denitrifier C utilization between different soils within the same study, we can identify the particular characteristics of C substrates that affect denitrification and whether C-based effects are consistent across different soils and microbial communities.

The C compounds available in the soil possess various inherent characteristics that may increase or decrease denitrification. For instance, the accessibility of C differs between monomeric and polymeric compounds, with the degradation of polymeric residues by extracellular enzymes often thought to be the rate-limiting step in the mineralization of complex C residues (Sinsabaugh, 1994). If depolymerization is limiting N_2O consumption by restricting the availability of C, then

91 additions of monomeric forms of C should result in lower net N_2O emissions than polymeric forms
92 because of the extra step involved in making C polymers accessible to denitrifiers. Moreover, the
93 C:N ratio of C substrates plays an important role in influencing denitrification and is also a good
94 predictor of N_2O production. As the C:N ratio narrows and the relative amount of N increases, N_2O
95 production tends to also increase (Huang et al., 2004; Millar and Baggs, 2004; Toma and Hatano,
96 2007). This is likely the result of low-C:N-ratio residues being a labile source of C since these
97 residues also tend to be easier to decompose. In addition to being a substrate for denitrification, N is
98 also a critical nutrient for microbial growth. Finally, the redox state of compounds can influence
99 denitrification, with more electron-rich, highly reduced substrates able to provide more electrons to
100 reduce more units of NO_3^- and therefore drive greater rates of denitrification. On the other hand,
101 more highly reduced substrates—such as simple carbohydrates—will often be available to organisms
102 with other anaerobic metabolisms, such as fermentation (Reddy et al., 1982; Pidello et al., 1996),
103 leading to competition that may reduce denitrifier access to such C sources but provide an ecological
104 opportunity for denitrifiers to specialize on more highly oxidized compounds such as organic acids.
105 Indeed, the organic acid succinate has been used as the C source in denitrifier isolation media
106 (Heylen et al., 2006). If competition with fermenters has influenced denitrifier C preference, then
107 compounds available more exclusively to denitrifiers, such as succinate or other organic acids,
108 should increase denitrification rates relative to simple sugars.
109 Denitrifier community composition and how different denitrifier species respond to different
110 C substrates is also likely to be a critical factor controlling N_2O production and reduction. Previous
111 research has demonstrated that individual denitrifier isolates possess their own C preferences and that
112 synthetic communities composed of denitrifiers with complementary C preferences can denitrify at
113 faster rates than other synthetic communities with overlapping resource niches (Salles et al., 2009,
114 2012). In addition, denitrifiers have differing capacities to carry out denitrification and N_2O
115 reduction. Community composition can be an important driver of N_2O emissions because denitrifier

communities vary across land uses, even within the same landscape (Cavigelli and Robertson, 2001; Juhanson et al., 2017; Maul et al., 2019), and possess unique rates of denitrification and distinct sensitivities to environmental factors (Cavigelli and Robertson, 2000; Krause et al., 2017; Maul et al., 2019). These variations could extend to their use of C. In general, soil microbial communities from separate land uses often differ in terms of C utilization. This is the basis for community-level physiological profiling and popular techniques such as the Biolog plate assay (Garland and Mills, 1991). Underlying these patterns, differing legacies of C inputs between land-use histories can dictate the C preference of soil microbial communities, giving rise to a home-field advantage where microbes more quickly mineralize the C types they have historically been exposed to (Ayres et al., 2009). In addition, extracellular enzyme activity has been linked to the quality and diversity of C-input legacies, with rotational diversity and intercropping both increasing extracellular enzyme activity (McDaniel et al., 2014; Curtright and Tiemann, 2021). Such land-use effects on enzyme activity could potentially lead to differences in the amount of denitrification stimulated by polymeric forms of C. Therefore, the C utilization profiles of denitrifiers may differ between land uses, with higher overall rates of denitrification in soils with greater aboveground diversity. Such land use effects could potentially account for discrepancies in C-utilization studies between denitrifiers from different soils. However, how land use influences the C preference of denitrifiers has not yet been examined.

Using lab incubations of soils from two agricultural field experiments with varying management practices, we explore the interacting effects of land-use history, denitrifier community structure, and C-compound quality and accessibility on N_2O production and consumption due to denitrification. We hypothesized that in the same soils, the chemical identity of C inputs would result in different levels of denitrification and N_2O reduction, specifically: (1) compounds with lower C:N ratios will stimulate more denitrification, because of their ease of degradation; (2) monomeric compounds will stimulate more gross N_2O production but lower net N_2O production than their paired

polymers, due to greater bioavailability of monomers; and (3) organic acids will stimulate more gross N_2O production than reduced sugars, such as glucose, because organic acids will be preferentially available to denitrifiers. Moreover, we hypothesized that (4) different land uses would lead to denitrifier communities with distinct C-utilization profiles.

2. Materials and Methods

2.1. Land Uses and Sampling

We sampled soils from two locations in Michigan, USA. The first location was the W. K. Kellogg Biological Station (KBS) (Hickory Corners, 42° 24′ N, 85° 24′ W), where we utilized field treatments from the KBS Main Cropping System Experiment, a Long-Term Ecological Research site established in 1989. Soils at this location are Typic Hapludalfs (fine-loamy, mixed, mesic; Table 1). We sampled from three field treatments from this site: conventional agriculture, reduced-input agricultural, and perennial switchgrass. The conventional and reduced-input agriculture treatments are in a corn (*Zea mays*)-soybean (*Glycine max*)-wheat (*Triticum aestivum*) rotation with conventional tillage and synthetic fertilizer inputs at locally recommended rates. The reduced-input treatment receives a portion of its N inputs through winter leguminous cover crops after wheat (*Trifolium pratense* L.). The reduced-input treatment also receives an annual ryegrass (*Lolium multiflorum* Lam.) cover crop following corn. Both the conventional and reduced input treatments receive herbicide applications to manage weeds. The perennial treatment previously contained continuous alfalfa but was switched to continuous switchgrass (*Panicum virgatum* L.) in 2019. Additional details relating to these soils can be found in Robertson and Hamilton (2015). Treatments are organized in a randomized-block design, from which we sampled soils from four blocks. Soil cores (1.9 cm diameter) were taken to a depth of 10 cm in April 2021, following a corn rotation and prior to soybean planting.

The second set of soils was sampled from the Montcalm Research Center (MRC) (Montcalm, 43° 3′ N, 85° 1′ W). These soils are Oxyaquic Glossudalfs (fine, mixed, frigid; Table 1). We sampled soils from two treatments of a field experiment established in 2015. Both treatments are in a potato (*Solanum tuberosum*)-corn rotation and received the same conventional fertilizer and herbicide applications, and they were both irrigated during the potato phase of the rotation. The two treatments differed only in the use of cover crops; one treatment is seeded with a mixture of annual ryegrass and hairy vetch (*Vicia villosa*), while the other treatment has no cover crops. Field treatments are organized in a randomized-block design. We took soil cores to a depth of 10 cm in October 2020, one week following potato harvest.

For each set of soil samples, soil cores were kept on ice in the field and brought back to the lab for processing. Soils were sieved through a 2 mm mesh, and soil moisture content was assessed gravimetrically. Soils were kept at 4°C until utilized in denitrification assays, within two weeks of sampling.

2.2. Denitrification Assays

To assess denitrifier response to various C substrate additions, we modified a standard denitrification enzyme activity assay protocol (Groffman et al., 1999). Specifically, 5 g of soil was measured into 60 mL serum bottles. To each bottle, 5 mL of H_2O was added. After one hour, 10 mL of KNO_3 solution (0.1 mg N mL^{-1}; bringing total solution volume to 15 mL) was added together with one of 12 different C-substrate treatments to a final concentration of 4.4 mg C g^{-1} soil (Table 2). The different C substrates used were glucose, cellulose, N-acetyl-glucosamine (NAG), chitin, casamino acid mix, soy protein isolate, vanillyl alcohol, lignin, citrate, succinate, and methanol. In addition, a twelfth treatment contained no C addition. Casamino acid are a mixture of free amino acids produced from an acid hydrolysis of casein protein; the acid hydrolysis removes most cystine and tryptophan. Soluble forms of C were provided dissolved in the KNO_3 solution. Insoluble C treatments were

188 added in powder form together with an equivalent amount of KNO_3 solution to match the dissolved-
189 C additions. Jars were crimp capped with butyl-rubber septa. We then evacuated and flushed each jar
190 with UHP N_2 three times to atmospheric pressure to create an anaerobic atmosphere. Jars were
191 divided into two sets; one set received acetylene (C_2H_2) at 10% v/v and the other set received an
192 equivalent amount of N_2. C_2H_2 inhibits the enzyme responsible for the reduction of N_2O to N_2,
193 allowing for an estimation of gross versus net N_2O production (Yoshinari and Knowles, 1976).
194 Jars were kept on an orbital shaker at 150 rpm between gas sampling times, which were
195 determined based on earlier optimization studies that demonstrated nitrous oxide reductase (the
196 enzyme responsible for N_2O reduction) was fully induced after ~24 hours and that soil microbes had
197 not yet reached an exponential growth phase (Fig. S1). Headspace gas samples (3 mL) were injected
198 into pre-evacuated 12 mL vials at 24, 26, and 28 hours. The remaining volume of the gas tight vials
199 was filled with N_2. Following each gas sampling, the headspace removed from each jar was replaced
200 using either N_2 or a 90:10 mixture of N_2:C_2H_2. Gas samples were analyzed for N_2O and CO_2
201 concentrations on a TRACE 1310 (Thermo Fisher, USA) gas chromatograph equipped with an ECD
202 and TCD.
203 *2.3. Statistics*
204 Concentrations of N_2O in the jars that contained C_2H_2 were used for estimates of gross N_2O
205 production. Nitrous oxide concentrations in jars that did not receive C_2H_2 were used to estimate net
206 N_2O production. The proportional difference between gross and net N_2O production is often used to
207 scale N_2O emissions to total denitrification. When denitrification ends in the production of N_2O,
208 dN_2O will be high. Conversely, when N_2O is reduced fully to N_2, dN_2O will be low. We calculated
209 this value as dN_2O according to the following equation.
210
$$dN_2O = \frac{N_2O_{net}}{N_2O_{gross}}$$

211 Statistical analyses were performed in R. Data from each study site, KBS and MRC, were
212 analyzed separately. For analysis, all concentration data were log transformed to achieve normality.
213 The *lme4* package (Bates et al., 2015) was utilized to create mixed-effects models with land-use,
214 substrate addition, and their interaction as fixed effects and field-treatment block as a random effect.
215 Marginal means were calculated using the *emmeans* package (Lenth et al., 2019). Using the *lmerTest*
216 package (Kuznetsova et al., 2017), type III tests for fixed effects were performed using the Kenward-
217 Roger method for calculating the denominator degrees of freedom. When fixed effects were found to
218 be significant, mean comparisons between substrate treatments within fields and between fields
219 within substrate were performed using Fisher's LSD at a Type I error rate of 0.05.

220 **3. Results**

221 *3.1. C-Substrate Effects on Potential Gross N_2O Production*

222 C substrate treatments had large effects on gross N_2O production in soils from all land uses
223 across both locations (Table 3). We found that amino acids and protein stimulated the most gross
224 N_2O production in each soil, regardless of land management, with up to 42 μg N_2O-N g^{-1} soil d^{-1}
225 being produced from the amino-acid-amended soils from the KBS reduced input treatment (Fig. 1A).
226 Organic acids also consistently stimulated some of the largest amounts of gross N_2O production
227 (citrate produced 20 μg N_2O-N g^{-1} soil d^{-1} in reduced-input soils), followed by glucose (13.4 μg N_2O-
228 N g^{-1} soil d^{-1}) and NAG (8.7 μg N_2O-N g^{-1} soil d^{-1}). Vanillyl alcohol, lignin, and methanol had the
229 lowest rates of gross N_2O production. However, these substrates still stimulated significantly more
230 gross N_2O production than the no-C additions, which resulted in between 2.3 and 4.8 μg N_2O-N g^{-1}
231 soil d^{-1} among the land uses at KBS and 2.4 and 2.9 μg N_2O-N g^{-1} soil d^{-1} in the MRC soils.

232 The monomeric compounds inconsistently stimulated more gross N_2O production than their
233 polymeric counterparts. Within each land use at KBS, glucose and amino acids stimulated about
234 twice as much gross N_2O production compared to cellulose and protein, respectively (Fig. 1A).

However, the denitrifier response to amino acids was stronger than to protein only in the KBS soils; in the MRC soil with no cover crops, protein stimulated approximately 75% more gross N_2O production than amino acids (Fig. 1B). Vanillyl alcohol never stimulated significantly more gross N_2O production than lignin.

3.2. C-Substrate Effects on Potential Net N_2O Production

In each of the KBS soils, amino acid and protein additions resulted in the lowest rates of net potential N_2O production (between 0.31 and 1.0 μg N_2O-N g^{-1} soil d^{-1}), indicating the greatest N_2O reduction (Fig. 2A). Organic acids also tended to have lower net N_2O production, but only the succinate addition in the reduced-input treatment (0.59 μg N_2O-N g^{-1} soil d^{-1}) was significantly different from the no-C treatment (between 1.9 and 3.4 μg N_2O-N g^{-1} soil d^{-1}). Meanwhile, in none of the soils did any of the C additions result in net N_2O production significantly greater than that of water alone, indicating that N_2O reduction kept pace with the N_2O production stimulated by each C substrate.

Within MRC soils, proteins and citrate had the lowest net N_2O production with only 0.05 and 0.08 μg N_2O-N g^{-1} soil d^{-1} being produced by soils amended with protein in the cover cropped and no-cover treatments, respectively (Fig. 2B). In contrast to the KBS soils, amino acids (~2.6 μg N_2O-N g^{-1} soil d^{-1}) did not result in lower net N_2O production than other substrates. In fact, amino acids had significantly higher net N_2O production than water in the no-cover treatment (2.8 versus 1.4 μg N_2O-N g^{-1} soil d^{-1}**)**. In the no-cover treatment at MRC, glucose had the highest amount of net N_2O production (4.7 μg N_2O-N g^{-1} soil d^{-1}), while cellulose, NAG, chitin, amino acids, and lignin also had significantly higher net N_2O production compared to the water-only addition. In the cover crop treatment, only glucose, cellulose, and lignin had significantly greater net N_2O production than the water-only treatment (1.9 μg N_2O-N g^{-1} soil d^{-1}).

The proportional difference between net and gross N_2O production , dN_2O, describes the portion of total N_2O production that remains as N_2O following N_2O reduction. Across the KBS land uses, proteins and amino acids had the lowest dN_2O (3.4–14%; Fig. 3A). Organic acids (7–24%) also had a significantly lower dN_2O than other substrates in each of the KBS soils. Glucose and NAG had an intermediate dN_2O. Within the conventional treatment, lignin had a low dN_2O comparable to that of glucose and NAG. In addition, methanol-induced N_2O reduction was significantly greater than that of water, but only in the conventional treatment. All other substrates had dN_2O values not significantly different from that of water (~7%) in any land-use treatment at KBS. Protein and citrate stimulated denitrification with the lowest dN_2O values in both treatments at MRC (Fig. 3B). Amino acids and succinate also had low dN_2O values. In the cover crop treatment, NAG and chitin had dN_2O values significantly lower than that of water. Within the conventional treatment, glucose, vanillyl alcohol, and lignin had dN_2O values significantly higher than water.

3.3. Land-Use Effects on Gross N_2O Production

Among the KBS treatments, we observed that the conventional treatment had significantly less potential N_2O production across most C substrate additions, with almost half as much N_2O production in some additions (Fig. 1A). Interestingly, the reduced-input treatment tended to be more similar in denitrifier C preferences to the perennial system than the conventional system, despite the latter sharing a corn-soy-wheat rotation. Land-use differences were similar across most substrates. Amino acids were the only substrate that did not have a significant land-use effect. At MRC, land-use effects were not significant on gross N_2O production (Table 3).

3.4. Land-Use Effects on Net N_2O Production

At KBS, land use had a significant effect on net N_2O production, but this depended on the substrate addition treatment (Table 3). The conventional-agriculture soils had significantly lower net N_2O production than the other land uses in response to glucose, vanillyl alcohol, lignin, succinate,

and methanol (Fig. 2A). Protein resulted in higher net N_2O levels in the conventional-agriculture soils compared to the other two treatments. The reduced-input and perennial systems tended to have similar levels of net N_2O production across most substrates, but the reduced-input land use had significantly lower net N_2O levels in response to succinate. When comparing dN_2O, a significantly greater portion of N_2O remained when soils from the perennial management were amended with glucose compared to soils under conventional management (Fig. 3A). Lignin stimulated a significantly lower dN_2O in the conventional treatment compared to the other two land managements. dN_2O was higher in the conventional treatment following protein addition and in the perennial treatment following succinate, but these differences were not significant.

Among the MRC treatments, the cover cropped soils had higher net N_2O levels than the no-cover-crop treatment and this effect did not depend on substrate additions (Table 3). Comparing dN_2O, there were no significant land-use effects.

4. Discussion

Our results support the general hypothesis that the chemical identity of available C affects rates of N_2O production and reduction. Although preliminary data indicated that exponential growth did not occur after 24 hours in unamended soils, it appears that microbial growth may have been stimulated with the addition of some of the substrates in the KBS soils (Fig. S2). In the glucose, amino acid, protein, citrate, and succinate treatments, the rate of CO_2 production over 26 hours was significantly greater than the rate of CO_2 production in the first 6 hours. The denitrification stimulated by these substrates could therefore be caused by an increase in the number of denitrifiers as well as, or instead of, greater activity of the extant denitrifier community. Stimulating growth is one potential mechanism behind the substrate differences we observed; however, it is unlikely to account for all the substrate differences. For instance, although substrate addition did not appear to stimulate growth in MRC soils after 26 hours, denitrifiers from KBS and MRC soils still

306 demonstrated similar C-utilization characteristics with respect to N_2O production and reduction. In
307 addition, differences in the N_2O end-product ratio do not fully match the patterns in apparent
308 stimulated growth. Specifically, while glucose addition appeared to stimulate microbial growth in
309 KBS soils, it did not reduce dN_2O as much as other substrates.

310 By providing forms of C with particular chemical characteristics, we tested specific
311 hypotheses designed to help identify the various physiological and ecological mechanisms that may
312 account for the wide differences in denitrification rates that we observed between substrates. Below,
313 we discuss a handful of such mechanisms and whether they are supported by our data. We note at the
314 outset that the conditions of these assays were not representative of those in the field, and the
315 conclusions are therefore somewhat limited in their scope.

316 *4.1. C-to-N Ratio of Available Substrates*

317 Amino acids and proteins tended to stimulate the most N_2O production, with nearly ten times
318 more N_2O production than the no-C addition and a five-fold increase compared to glucose in the soils
319 from KBS (Fig. 1). In addition to C, amino acids supply varying levels of N, and in the field this
320 additional N could ultimately lead to increased denitrification since N availability is known to be a
321 primary control on denitrification (Wallenstein et al., 2006). For instance, among different plant-
322 residue additions, those with greater amounts of N tend to stimulate more N_2O production by
323 alleviating N limitation and providing the requirements for denitrification (de Catanzaro and
324 Beauchamp, 1985; Aulakh et al., 1991; Huang et al., 2004). However, in our experiments, all C
325 substrate additions also received non-limiting quantities of NO_3^-, ensuring adequate amounts of N to
326 serve as electron acceptors. Further, the organic N of amino acids is in a reduced form and would
327 first need to be removed from the amino acid and then oxidized through either autotrophic or
328 heterotrophic nitrification to NO_3^- before being used as an electron acceptor in denitrification (Tiedje
329 et al., 1983). Since most nitrification pathways utilize ammonium monooxygenase, which requires

oxygen, the oxidation of amino-acid N to NO_3^- seems unlikely in our anaerobic assays (Martikainen, 2022). Moreover, among the C substrates we added was NAG, which also contains additional organic N. But despite this additional N, we never observed NAG to stimulate more denitrification than glucose. Therefore, the increased denitrification from amino acids and proteins is not due simply to the provision of additional N to fuel denitrification.

While N availability *per se* might not explain the differences we observe among substrates, compounds with more N tend to be more easily incorporated into biomass. Amino acids are the building blocks of proteins, and environmental amino acids are rapidly recycled into new biomass (Geisseler et al., 2009, 2010). Similarly, proteins are broken down into peptides and individual amino acids, which are quickly utilized by soil microbes (Payne, 1976; Hill et al., 2011). A high availability of these biochemical building blocks could stimulate growth as well as result in faster production of denitrification enzymes and other metabolic machinery, leading to an increase in microbial activity. The degree of C substrate incorporation into biomass—so called, C-use efficiency—can be measured using stable-isotope-labelled C additions. However, when directly compared to glucose and other C compounds, amino acids have tended to show lower C-use efficiencies (Brant et al., 2006; Frey et al., 2013), suggesting they are not typically preferred for biomass any more than other substrates. Nevertheless, past experiments measuring the relative C-use efficiency of amino acids have been carried out under aerobic conditions, quite distinct from the anaerobic conditions in our experiment. Since we did not track the C-use efficiency of denitrifiers in this study, we are unable to determine whether direct incorporation of C substrates in new biomass is driving substrate differences in denitrification rates.

Interestingly, although amino acids and protein stimulated the greatest amount of denitrification, they also induced the most reduction of N_2O, resulting in the lowest dN_2O values. While somewhat paradoxical, this indicates that these substrates stimulated N_2O reduction just as much, or more than, N_2O production. Previous studies have reported on the effect of the amino acids

cysteine (Morley et al., 2014) and glutamate (Giles et al., 2017) on the denitrification end-product ratio, with the former resulting in less N_2O reduction than glucose and the latter having no significant difference. Our study is notable insofar as it demonstrates such high denitrification efficiency with an amino acid mixture and proteins. The availability of substrates, such as amino acids and proteins, to be incorporated into new biomass may be particularly important for N_2O reduction. If these substrates also stimulated the greatest amount of microbial growth (Fig. S2), it could suggest that fast growth may favor N_2O reduction as a trait of copiotrophic denitrifiers. When comparing different denitrifying taxa, others have also found that denitrifiers with the greatest affinities for N_2O reduction also happened to have the highest growth rates (Conthe et al., 2018; Qi et al., 2022). Nevertheless, the available research linking N_2O reduction to other microbial traits such as growth rate is quite limited, and we are hesitant to draw any premature conclusions regarding the ecological significance of N_2O reduction to life-history strategies without additional study.

4.2. Competition and Niche Partitioning

We hypothesized that resource partitioning would result in some C compounds, namely organic acids, being utilized more readily by denitrifiers than reduced sugars, which would be the object of competition with other anaerobes. Evidence for niche partitioning between denitrifiers and fermenters is hard to observe directly (Stevens et al., 1998). Paul and Beauchamp (1989) observed the production and consumption of fermentation byproducts and suggested that syntrophies between denitrifiers and fermenters improved the energy yield of available substrates. We found that citrate did stimulate more denitrification than glucose and NAG (Fig. 1), and it also resulted in a greater portion of N_2O being reduced (Fig. 3), supporting the possibility of niche differentiation between denitrifiers and fermenters. However, succinate did not result in more denitrification or N_2O reduction. Although denitrifiers did appear to prefer citrate over glucose, this did not reflect a

universal preference for organic acids. We are thus unable to conclude whether this C preference emerged via resource partitioning with fermenters or by other ecological mechanisms.

Resource partitioning among denitrifier populations could also account for the particularly high rates of denitrification following the addition of amino acids and protein. Rather than being a single source of C, these additions were a mixture of different amino acids, each potentially available to a different set of denitrifiers. These different forms of C can allow for niche partitioning and complementarity in resource utilization, allowing more microbes to be metabolically active at the same time (Goldfarb et al., 2011; Baran et al., 2015). Niche partitioning has been shown to increase overall denitrification rates in synthetic communities of denitrifiers with complementary substrate usage (Salles et al., 2009). Comparing the effects of C substrates within mixtures is, of course, more representative of the soil environment (Henry et al., 2008) and demonstrates an important aspect of the effect that C substrate identity has on denitrification rates. The single-substrate additions of our assays were of course not fully representative of how denitrifiers would encounter C substrates in the field.

4.3. Substrate Bioavailability

The bioavailability of C substrates will determine the rate of their utilization, with polymeric forms of C being relatively less bioavailable than dissolved monomeric forms (Sinsabaugh, 1994). To determine how this affected denitrification rates, we included pairs of polymers and their composite monomers among our C addition treatments. At KBS, glucose and NAG tended to stimulate more denitrification than their polymeric counterparts, cellulose and chitin, although the difference between NAG and chitin was only statistically significant in one soil (Fig. 1A). The effect of monomers on dN_2O values was more consistent, with monomers stimulating more N_2O reduction relative to total denitrification (Fig. 3A). A greater supply of ready electron donors (monomers) increases demand for terminal electron acceptors, thereby driving the reduction of N_2O to N_2 and

reducing dN_2O (Senbayram et al., 2012). While previous studies have demonstrated dN_2O to be inversely related to C availability (Beauchamp et al., 1989; Weier et al., 1993; Miller et al., 2008; Qin et al., 2017), they did not address the role of depolymerization in making substrates available. Even though we provided all substrates in the same C-normalized quantity, monomeric substrates were immediately available for rapid uptake and metabolism, while the bioavailability of polymeric substrates was determined by the rate of extracellular depolymerization. In our study, this resulted in a greater amount of N_2O being consumed when more biologically available monomers were supplied.

The difference between monomers and polymers was not universal across all substrates and land uses. Vanillyl alcohol never stimulated more denitrification or N_2O reduction than lignin. The constituent monomers of lignin, such as vanillyl alcohol, are phenolic compounds that require highly specialized biochemical pathways for their anaerobic degradation (Rabus, 2005). Although some denitrifiers are capable of utilizing phenolic compounds as their sole C source, these microbes are often fastidious and slow growing (van Schie and Young, 1998), so the lack of a large response of denitrifiers to lignin and vanillyl alcohol in our study is not wholly unexpected. Among the soils from KBS, the difference between monomers and polymers was greatest with amino acids and proteins (Fig. 1A), but interestingly, proteins stimulated more gross N_2O production than amino acids at MRC (Fig. 1B). Peptidase degradation of proteins was clearly not a rate limiting step in these soils, and it was interesting to observe proteins stimulating more denitrification than amino acids. Proteins can be broken down into peptide fragments, which may be more efficiently taken into cells than individual amino acids (Matthews and Payne, 1980; Geisseler et al., 2010), potentially resulting in a more rapid supply of C. Accordingly, within the rumen, peptides have been found to be utilized by microbes more readily than amino acids (Wallace, 1996). The apparent preference for proteins over amino acids could also be due to the specific composition of the two mixtures. The amino acid addition was derived from a digestion of casein protein, whereas the protein addition was an isolation

427 of soy protein. While these contained similar levels of total amino acids overall, the specific
428 distribution and amounts of each can differ between the two. For example, tryptophan and cystine are
429 often lost in the acid hydrolysis step of prepared amino acid mixtures. The specific composition of
430 the protein mixture may have contained essential amino acids that were limiting to the denitrifiers in
431 the MRC soils or for which these microbes had a strong affinity (Wallace, 1996; Kajikawa et al.,
432 2002; Liu et al., 2020).

433 *4.4. Land Use and Soil Microbial Communities*

434 To determine the effect of land-use legacies on denitrifier C utilization we utilized different
435 field treatments from two locations in Michigan. Previous research has demonstrated dissimilar
436 denitrifier communities among soils at the KBS site that were functionally distinct with respect to the
437 sensitivity of nitrous oxide reductase to oxygen (Cavigelli and Robertson, 2001) (though only one of
438 the treatments utilized here was included in that research). Across both locations, we found that the
439 land uses that did not have cover crops tended to have the least potential for N_2O production across
440 most C substrate additions (Fig. 1). In general, cover cropping has been shown to increase overall
441 microbial activity (Tiemann et al., 2015; N. Kim et al., 2020). Therefore, the lower denitrification
442 potential in the land uses that did not include cover crops may be due to an overall reduction in
443 microbial activity, rather than an altered response of denitrifiers to substrate additions. It was also
444 interesting to note that, at KBS, denitrifier C utilization in the reduced-input treatment tended to be
445 more similar to C-utilization patterns in the perennial system rather than the conventional treatment,
446 which had the same crop rotation as reduced-input but without cover crops. This suggests that the
447 amount of time that plants cover the soil may be a stronger determinant of land-use effects rather
448 than specific plant composition or diversity, *per se* (Garland et al., 2021). Moreover, the N dynamics
449 of these soils have been extensively characterized previously (Millar and Robertson, 2015); despite
450 fewer fertilizer inputs, the reduced-input and switchgrass treatments have similar inorganic N levels

to the conventional treatment throughout the year, due to mineralization of cover crop residues and soil organic matter. While the overall amount of available N remains similar, the form and rate of release of this N may produce land-use differences between the treatments.

Among the treatments at each location, we did not find a strong effect of land use on the C-utilization profiles of denitrifiers. The denitrifier communities from the soils we used in our experiment appear to be functionally equivalent in their C preference. While representing distinct management strategies, each of the land uses in this experiment were agricultural systems. Other agricultural management factors, such as weed management or aboveground biomass removal, may be more influential in determining denitrifier C-utilization profiles than the chemical composition of plant residue inputs. For instance, previous studies have found that soil type is more important than plant identity in determining denitrification rates (Graf et al., 2016). In another comparison of land use on denitrifier communities, edaphic factors, such as pH and soil organic C, were identified as primary drivers of differences in denitrification rates (Krause et al., 2017). While these land uses at KBS have previously been shown to have differences in C content (Grandy and Robertson, 2007), these may be too slight to affect the C preference of denitrifiers. Similar studies on a broader range of soils and land uses will be required before drawing general conclusions as to the universality of denitrifier C preference. It is also possible, and likely, that differentiating C preferences between denitrifier communities manifest in the utilization of C compounds that were not included in this study.

Microbial community effects were strongest in the qualitative differences in N_2O reduction between the two sites. At KBS, amino acids and proteins resulted in significantly less net N_2O production compared to the other substrates. On the other hand, at MRC amino acids did not result in any less net N_2O production, while proteins stimulated almost complete removal of N_2O from the microcosms. Microbial communities have widely different capacities for N_2O reduction depending on the abundance and type of N_2O reducers present. In particular, communities that have more

organisms with *nosZ-II* may have a greater ability to consume N_2O than those with *nosZ-I* (Graf et al., 2014; Jones et al., 2014; Yoon et al., 2016). Therefore, the addition of substrates stimulating *nosZ-II* organisms could result in lower net production of N_2O. At least one study has shown that *nosZ-II* organisms are more responsive to changes in C availability than those with *nosZ-I* (Assémien et al., 2019); however, other studies have shown that both clades of *nosZ* are equally responsive to C availability (Domeignoz-Horta et al., 2015, 2018; Juhanson et al., 2017). How these two groups of organisms respond to different forms of C deserves further study.

5. Conclusion

In this study, we demonstrate how the chemical identity of C inputs influences N_2O production and reduction in different agricultural soils. To resolve apparently divergent patterns in denitrifier C utilization between different studies, we compared a set of twelve C-addition treatments over five soils under identical assay conditions. We found that amino acids, proteins, and organic acids consistently stimulated the most denitrification and N_2O reduction among the substrates examined here. While soils from distinct land uses had differing overall rates of denitrification, C-utilization profiles were largely similar between soils, suggesting denitrifier C preferences may be widely held between microbial communities, at least within agricultural soils. The bioavailability of C substrates appears to be a significant driver in denitrification and N_2O reduction, with labile substrates stimulating greater activity than polymeric and recalcitrant C additions. Nevertheless, this pattern was not universal across all the substrates tested. Given the heterogeneous distribution of different forms of C throughout the soil profile, substrate effects likely contribute to the spatial and temporal variability of N_2O production within soils. Determining whether these denitrifier C preferences also occur under field conditions may provide opportunities to reduce emissions of N_2O from soils by controlling soil C inputs.

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Tables

Table 1: Edaphic factors of study sites

	Total C (%)[c]	pH	CEC (cmol kg^{-1})
Kellogg Biological Station[a]			
Conventional Row Crop	0.91 ± 0.08	6.12 ± 0.03	8.13 ± 0.23
Reduced Input Row Crop	1.09 ± 0.05	6.28 ± 0.01	7.95 ± 0.11
Perennial Switchgrass	1.42 ± 0.06	6.38 ± 0.02	7.95 ± 0.15
Montcalm Research Center[b]		6.5	10
Maize-Potato with No Cover	0.87 ± 0.03		
Maize-Potato with Vetch and Rye	1.15 ± 0.19		

Total soil carbon, pH, and cation exchange capacity of the soil utilized in the study. [a] Soil data for Kellogg Biological Station was obtained from Robertson and Simmons (2020). [b] Cation exchange capacity and pH for soils sampled from Montcalm Research Center were obtained from the Web Soil Survey (USDA NRCS). [c] Soil carbon data for KBS was obtained from Grandy and Robertson (2007).

Table 2: Characteristics of added carbon substrates

Substrate	Formula	C:N Ratio	NOSC	Utilization	Degradation
Glucose	$C_6H_{12}O_6$	No N	0	Short-term energy storage. Monomer of cellulose	Widely used substrate in cellular respiration and fermentation reactions
Cellulose	$(C_6H_{10}O_5)_n$	No N	0	Polymer of glucose. Structural component of plant cell walls	Requires specialized enzymes to depolymerize extracellularly
N-acetylglucosamine (NAG)	$C_8H_{15}NO_6$	8:1	0	Monomeric subunit composing the cell walls of bacteria (peptidoglycan) and fungi (chitin)	Initial degradation steps require specialized enzymes to remove the acetyl and amino groups
Chitin	$(C_8H_{13}O_5N)_n$	8:1	0	Polymer of NAG. Structural component of fungal cell walls and insect exoskeletons	Requires specialized enzymes to depolymerize extracellularly
Amino acids		~4:1 (3:2 to 9:1)	-0.1	Monomeric subunit of proteins	Can be recycled into new proteins or catabolized via cellular respiration and fermentation depending on the specific microbe and amino acid
Protein		~4:1	-0.1	Polymer of amino acids. Multiple uses in cellular functioning and structure	Requires specialized enzymes to depolymerize extracellularly
Vanillyl alcohol	$C_8H_{10}O_3$	No N	-0.5	Monomeric subunit of lignin	Aromatic ring structure requires specialized pathways to break down. Often requires an oxidase
Lignin		No N	-0.5	Highly stable aromatic polymer, structural component of plant cell walls	Requires specialized enzymes to depolymerize extracellularly
Citrate	$C_6H_8O_7$	No N	+1	Intermediate of central metabolism	Intermediate in central metabolism for nearly all microbes, but use as a C source is restricted by the ability to transport into the cell. Not a typical fermentation substrate but there are some specialized pathways
Succinate	$C_4H_6O_4$	No N	+0.5	Intermediate of central metabolism	Intermediate in central metabolism for nearly all microbes, but use as a C source is restricted by the ability to transport into the cell. Not a typical fermentation substrate but there are some specialized pathways

Methanol	CH_3OH	No N	-2	Waste product	Energy dense but requires specialized pathways to be utilized

Characteristics of added C substrates, including the C:N molar ratio, the nominal oxidation state of C (NOSC), how the compounds are commonly utilized by microorganisms, and how they can be degraded. NOSC is related to the amount of energy that is attainable from a C substrate. More energy-dense substrates will be more reduced and have a more negative NOSC, while more oxidized compounds with have a more positive NOSC.

Table 3: Type III ANOVA table of fixed effects

	Gross N_2O		Net N_2O		dN_2O	
	F	P	F	P	F	P
Kellogg Biological Station						
Substrate	93.9	< 0.001	5.5	< 0.001	33.4	< 0.001
Land Use	89.1	< 0.001	3.5	0.100	0.7	0.546
Substrate × Land Use	2.0	0.013	3.5	< 0.001	1.4	0.140
Montcalm Research Center						
Substrate	207.0	< 0.001	85.3	< 0.001	70.7	< 0.001
Land Use	6.0	0.092	12.1	0.040	0.5	0.520
Substrate × Land Use	1.9	0.081	1.9	0.074	2.1	0.048

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Figure 1: Differences in gross N_2O production between substrate additions at KBS (A) and MRC (B)

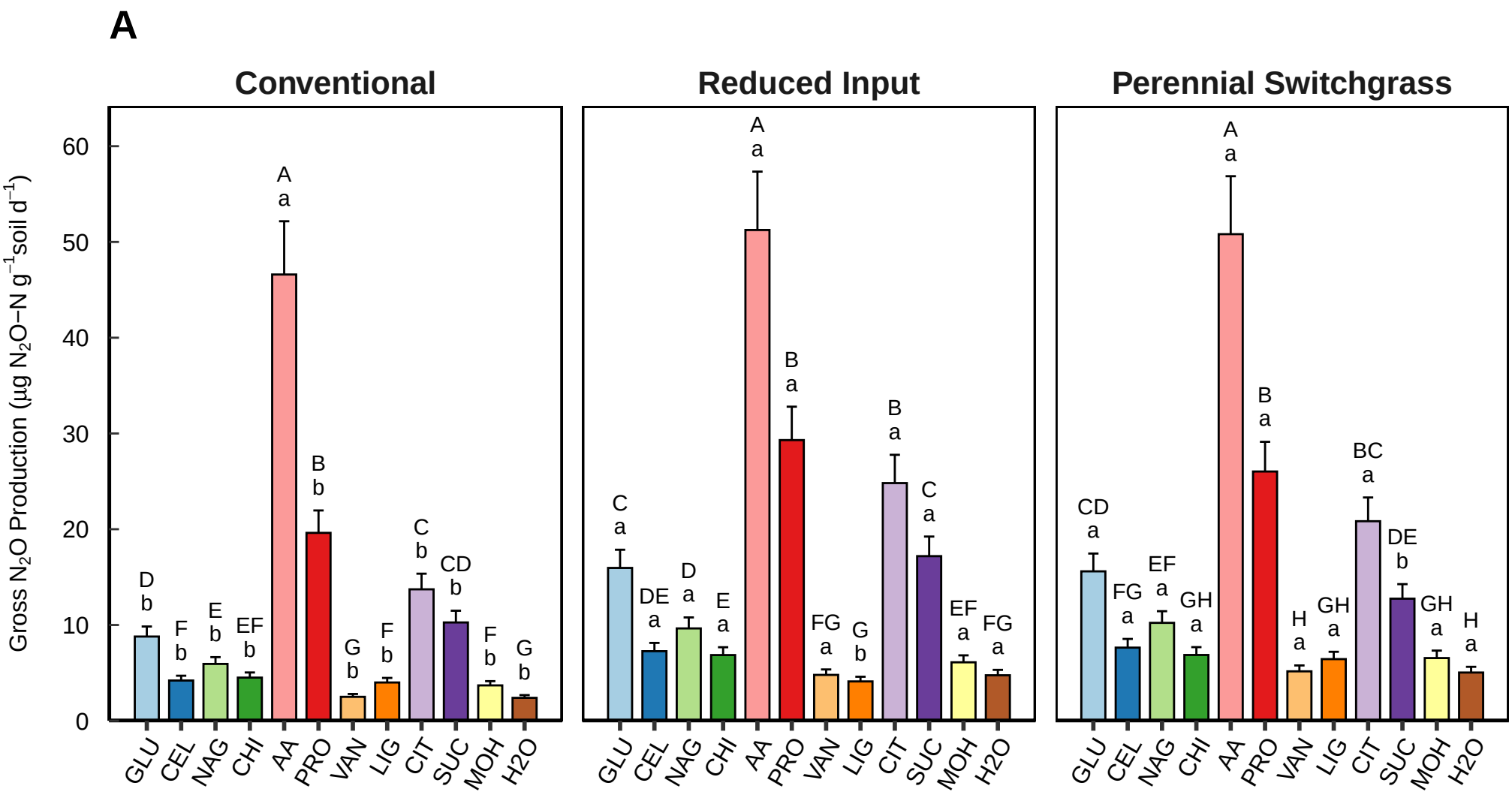
Cumulative production of N_2O in treatments receiving acetylene. Acetylene inhibits N_2O reduction; thus, N_2O concentrations represent total gross production of N_2O following the addition of glucose (GLU), cellulose (CEL), N-acetyl-glucosamine (NAG), chitin (CHI), casamino acids (AA), soy protein isolate (PRO), vanillyl alcohol (VAN), lignin (LIG), citrate (CIT), succinate (SUC), methanol (MOH), or no C addition (H2O). Means are shown with error bars representing one standard error (n = 4). Capital letters indicate significantly different means between substrates within each land use; lowercase letters indicate significantly different means between land uses for each substrate (α = 0.05).

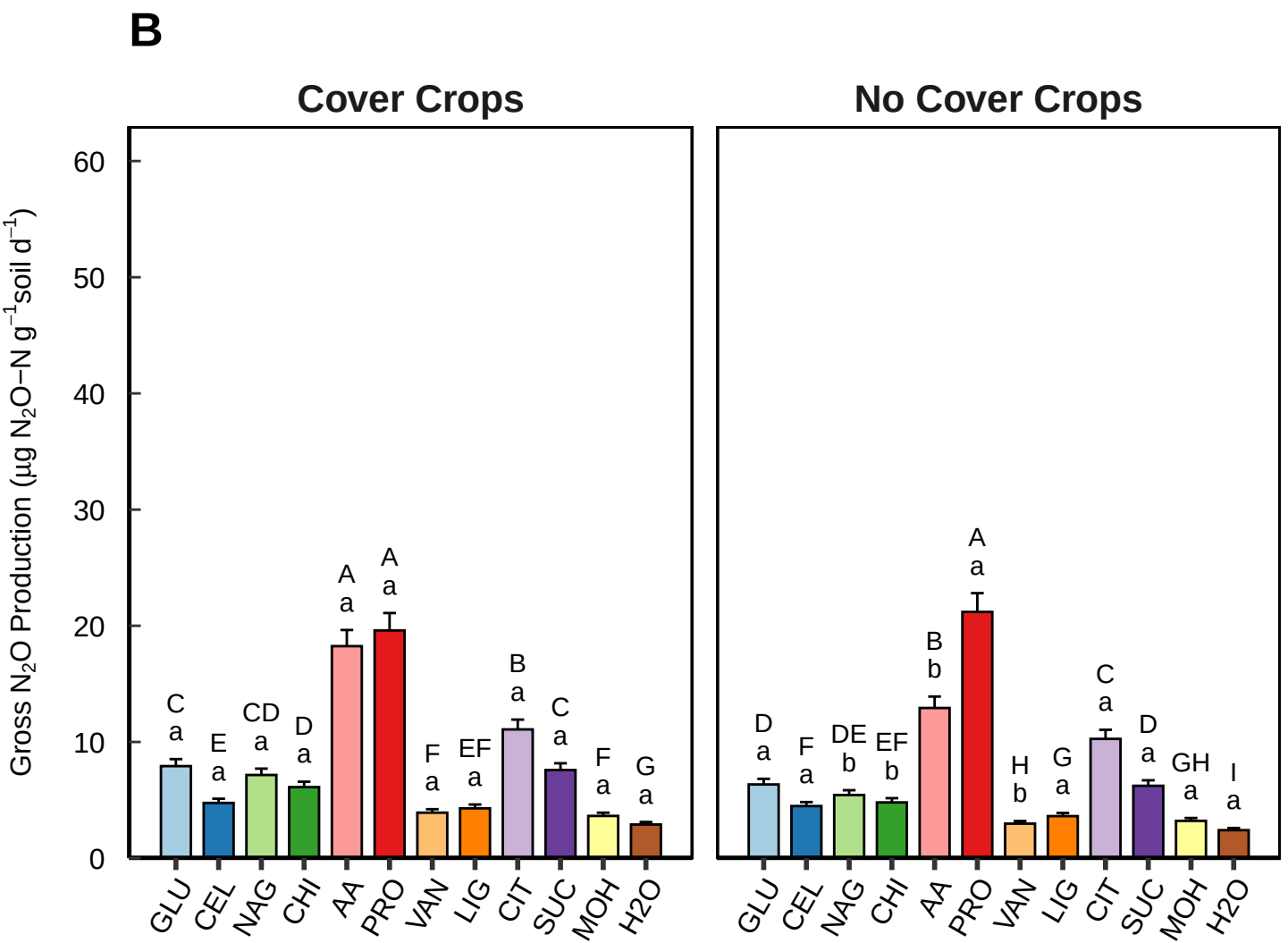
Figure 2: Differences in net N_2O production between substrate additions at KBS (A) and MRC (B)

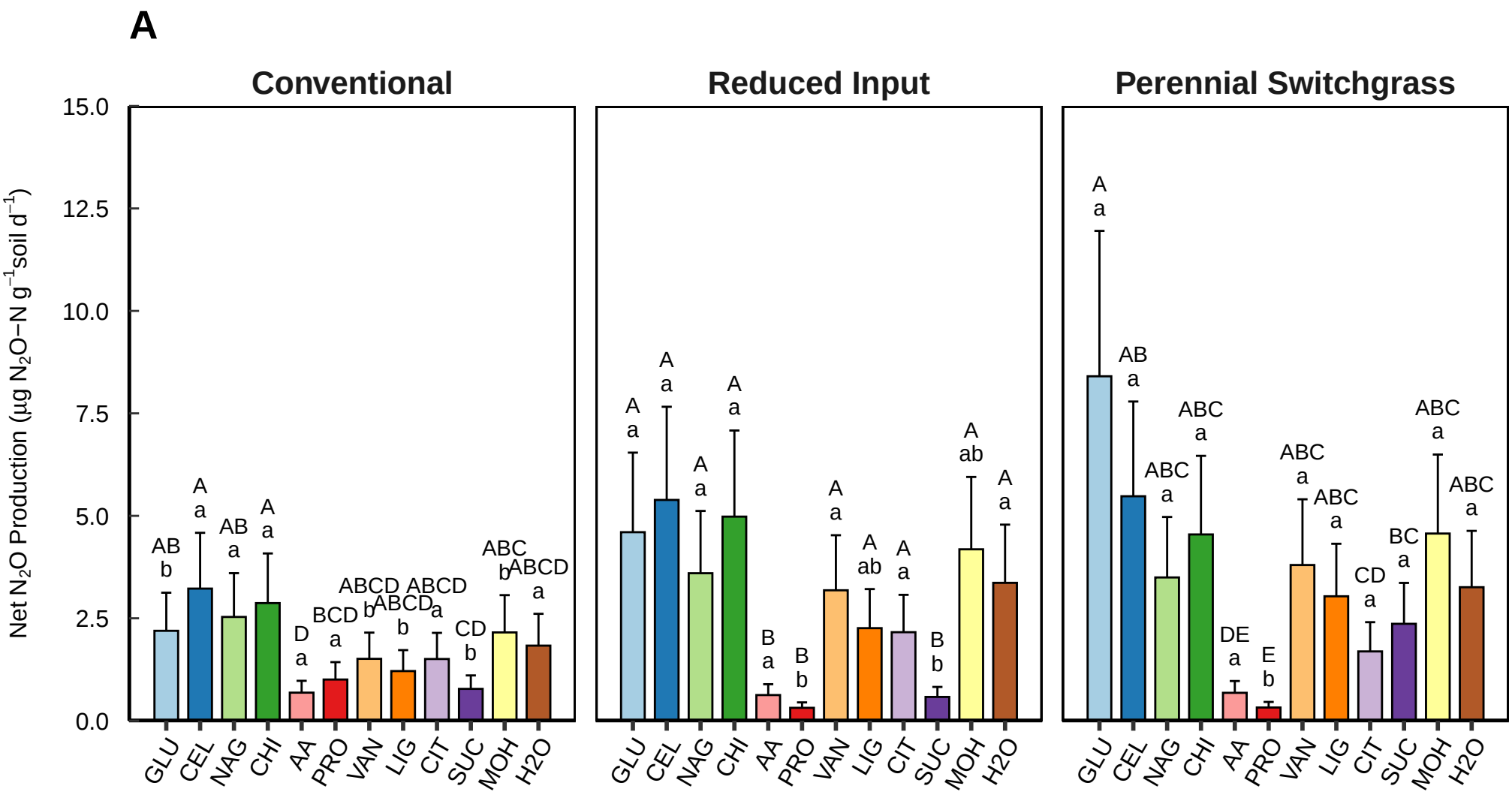
Cumulative production of N_2O in treatments that did not receive acetylene. N_2O values reflect the balance of N_2O production and N_2O reduction following the addition of glucose (GLU), cellulose (CEL), N-acetyl-glucosamine (NAG), chitin (CHI), casamino acids (AA), soy protein isolate (PRO), vanillyl alcohol (VAN), lignin (LIG), citrate (CIT), succinate (SUC), methanol (MOH), or no C addition (H2O). Means are shown with error bars representing one standard error (n = 4). Capital letters indicate significantly different means between substrates within each land use; lowercase letters indicate significantly different means between land uses for each substrate (α = 0.05).

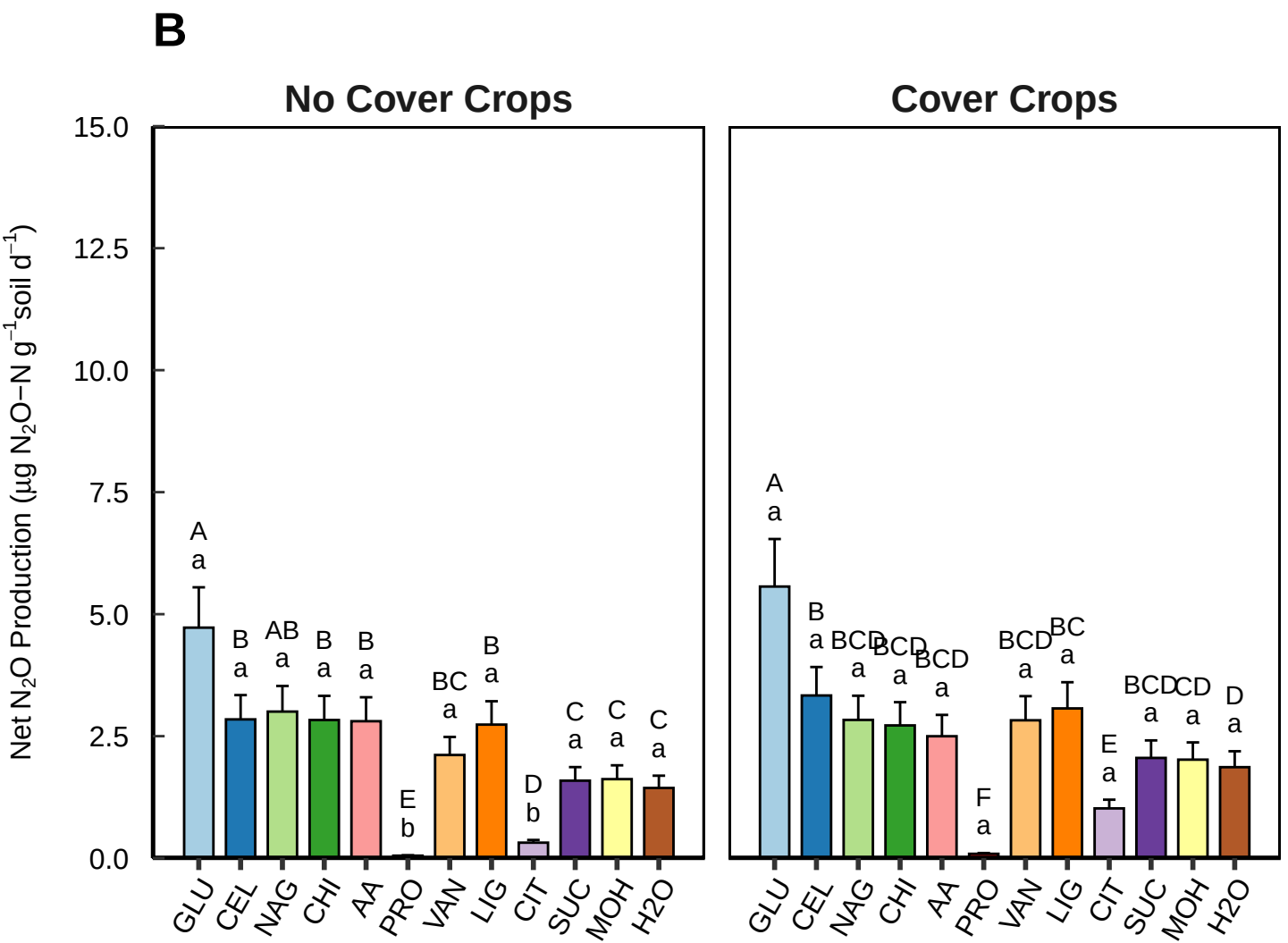
Figure 3: Portion of total N_2O production remaining as N_2O between substrate additions at KBS (A) and MRC (B)

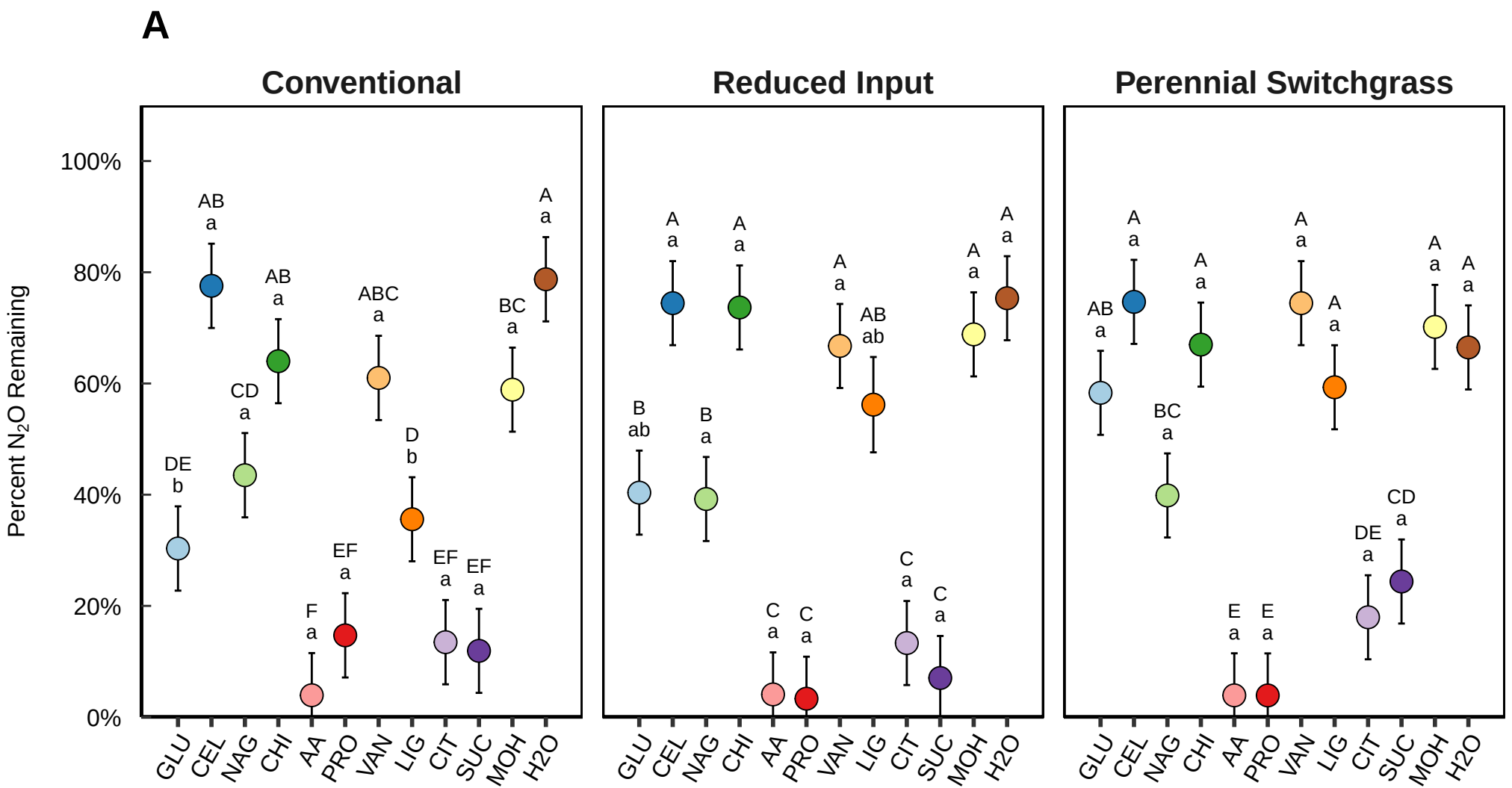
The portion of N_2O remaining (dN_2O) scales net production of N_2O to total gross production of N_2O. Higher values indicate less N_2O reduction occurred relative to total denitrification, while values close to zero indicate near complete reduction of all N_2O produced. Values are means of treatments receiving glucose (GLU), cellulose (CEL), N-acetyl-glucosamine (NAG), chitin (CHI), casamino acids (AA), soy protein isolate (PRO), vanillyl alcohol (VAN), lignin (LIG), citrate (CIT), succinate (SUC), methanol (MOH), or no C addition (H2O). Error bars represent one standard error (n = 4). Capital letters indicate significantly different means between substrates within each land use; lowercase letters indicate significantly different means between land uses at each location for each substrate (α = 0.05).

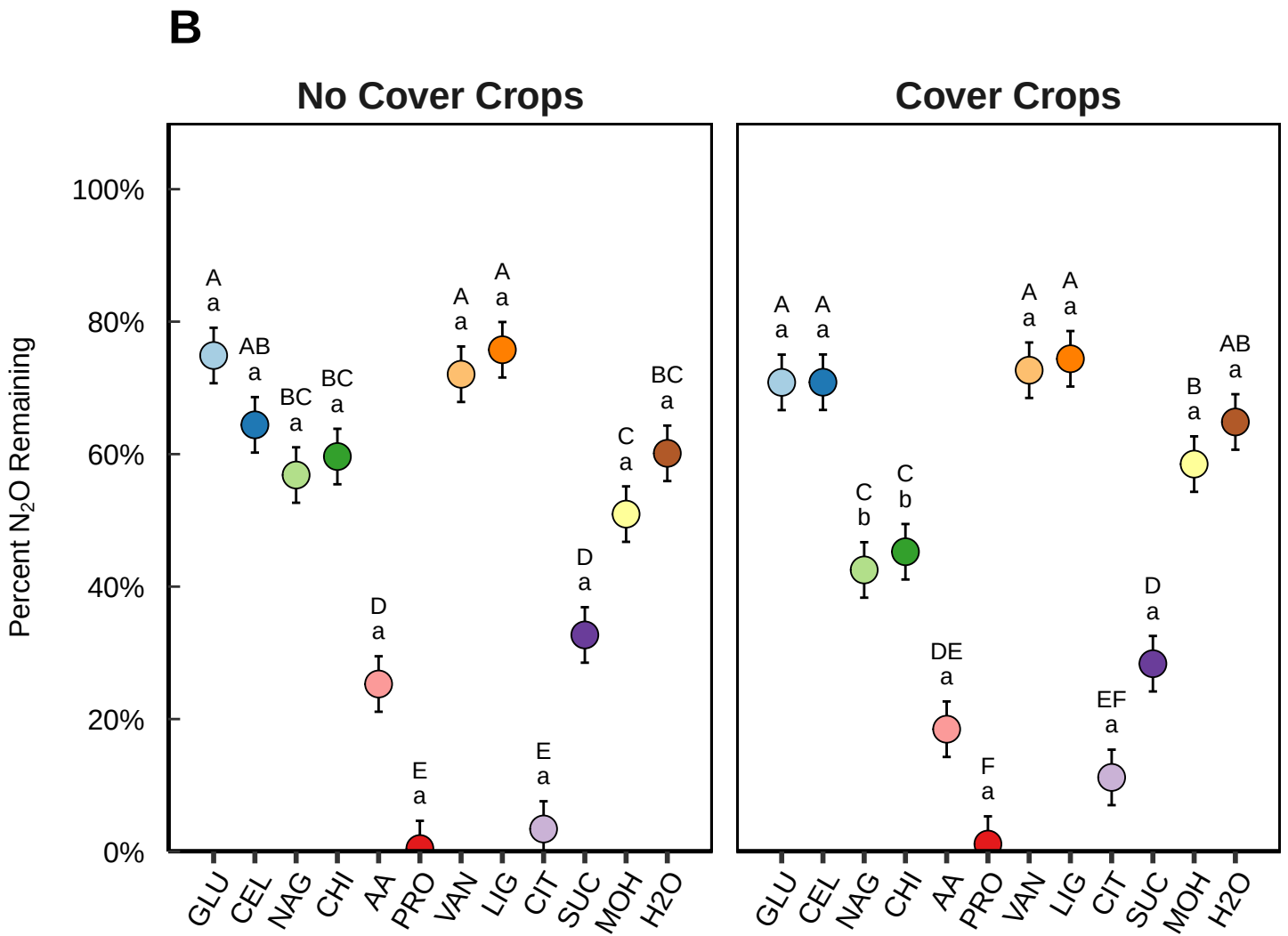

A
Conventional
Reduced Input
Perennial Switchgrass
Gross N2O Production (µg N2O–N g−1soil d−1)
0
10
20
30
40
50
60
GLU
CEL
NAG
CHI
AA
PRO
VAN
LIG
CIT
SUC
MOH
H2O
D b
F b
E b
EF b
A a
B b
G b
F b
C b
CD b
F b
G b
C a
DE a
D a
E a
A a
B a
FG a
G b
B a
C a
EF a
FG a
CD a
FG a
EF a
GH a
A a
B a
H a
GH a
BC a
DE b
GH a
H a


B
Cover Crops
No Cover Crops
Gross N2O Production (µg N2O–N g−1soil d−1)
0
10
20
30
40
50
60
GLU
CEL
NAG
CHI
AA
PRO
VAN
LIG
CIT
SUC
MOH
H2O
Ca
Ea
CDa
Da
Aa
Aa
Fa
EFa
Ba
Ca
Fa
Ga
Da
Fa
DEb
EFb
Bb
Aa
Hb
Ga
Ca
Da
GHa
Ia


A
Conventional
Reduced Input
Perennial Switchgrass
Net N2O Production (µg N2O–N g−1soil d−1)
15.0
12.5
10.0
7.5
5.0
2.5
0.0
GLU
CEL
NAG
CHI
AA
PRO
VAN
LIG
CIT
SUC
MOH
H2O


B
No Cover Crops
Cover Crops
Net N2O Production (µg N2O–N g−1soil d−1)
15.0
12.5
10.0
7.5
5.0
2.5
0.0
A a
B a
AB a
B a
B a
E b
BC a
B a
D b
C a
C a
C a
A a
B a
BCD a
BCD a
BCD a
F a
BCD a
BC a
E a
BCD a
BCD a
D a
GLU
CEL
NAG
CHI
AA
PRO
VAN
LIG
CIT
SUC
MOH
H2O


A
Conventional
Reduced Input
Perennial Switchgrass
Percent N2O Remaining
100%
80%
60%
40%
20%
0%
GLU
CEL
NAG
CHI
AA
PRO
VAN
LIG
CIT
SUC
MOH
H2O
DE b
AB a
CD a
AB a
F a
EF a
ABC a
D b
EF a
EF a
BC a
A a
B ab
A a
B a
A a
C a
C a
A a
AB ab
C a
C a
A a
A a
AB a
A a
BC a
A a
E a
E a
A a
A a
DE a
CD a
A a
A a


B
No Cover Crops
Cover Crops
Percent N_2O Remaining
100%
80%
60%
40%
20%
0%
GLU
CEL
NAG
CHI
AA
PRO
VAN
LIG
CIT
SUC
MOH
H2O
A a
AB a
BC a
BC a
D a
E a
A a
A a
E a
D a
C a
BC a
A a
A a
C b
C b
DE a
F a
A a
A a
EF a
D a
B a
AB a