Emissions of nitrous oxide, dinitrogen and carbon dioxide from three soils amended with carbon substrates under varying soil matric potentials

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Funding information
China Scholarship Council, Grant/Award Number: 201606180104; New Zealand Agricultural Greenhouse Gas Research Centre

Abstract
Carbon (C) substrates are critical for regulating denitrification, a process that results in nitrous oxide (N₂O) and dinitrogen (N₂) emissions from soil. However, the impacts of C substrates on concomitant soil emissions of carbon dioxide (CO₂) and N₂O under varying soil types and soil water contents are not well studied. Three repacked Pallic grassland soils, varying in texture and phosphorus (P) status, containing NO₃⁻/C0₂-15N were held at three levels of matric potential (ψ, −3, −5 and −7 kPa), while receiving daily substrate additions (acetate, glucose and water control) for 14 days. The CO₂ and N₂O emissions were measured daily. Additionally, the N₂O/(N₂ + N₂O) ratios were determined using ¹⁵N on days 3 and 14. Results showed that N₂O emissions increased exponentially as soil gas diffusivity declined, and N₂O peak emissions were higher with glucose than with acetate addition, with a range (± standard deviation) of 0.1 ± 0.0 to 42.7 ± 2.1 mg N m⁻² h⁻¹. The highest cumulative N₂O emission (2.5 ± 0.2 g N m⁻²) was measured following glucose addition with a soil ψ of −3 kPa. In comparison with added glucose, acetate resulted in a twofold increase in N₂ emissions in soils with relatively low gas diffusivities. The N₂O/(N₂ + N₂O) ratios varied with substrate (glucose, 0.91; acetate, 0.81) on day 3, and had declined by day 14 under substrate addition (≤0.10). Cumulative CO₂ emissions were enhanced with increasing soil gas diffusivity and were higher for soils amended with glucose (ranging from 22.5 ± 1.3 to 36.6 ± 1.8, g C m⁻²) than for those amended with acetate. Collectively, the results demonstrate that the increase of N₂O, N₂ and CO₂ emissions and changes in the N₂O/(N₂ + N₂O) ratio vary over time in response to C substrate type and soil gas diffusivity.

Highlights
- Co-regulation of CO₂ and N₂O emissions was assessed for varying soil types and C substrates.
- Soil diffusivity explained concurrently cumulative N₂O and CO₂ emissions.
- Acetate enhanced N₂O reduction to N₂ in three grassland soils more than glucose.
1 | INTRODUCTION

Nitrous oxide (N₂O) is a potent greenhouse gas and N₂O emissions from agricultural sources and synthetic fertilizers account for 6% of total anthropogenic radiative forcing (Davidson, 2009). In agricultural soils, N₂O is produced from nitrification under aerobic conditions by ammonia oxidizing bacteria (AOB) and archaea (AOA) that ultimately convert ammonia, via nitrite (NO₂⁻), to nitrate (NO₃⁻) (Firestone & Davidson, 1989). During hypoxic conditions AOB switch to “nitrifier denitrification”, producing N₂O via NO₂⁻ reduction, whereas under anaerobic conditions, AOB may also produce N₂O via the anaerobic oxidation of hydroxylamine (Stein, 2019). In addition, the nitrification intermediaries (hydroxylamine, nitric oxide (NO) and NO₂⁻) may undergo abiotic or biotic processes to produce N₂O (Stein, 2019). Under anaerobic conditions, denitrification sequentially reduces NO₃⁻ to the environmentally benign dinitrogen (N₂), with N₂O an obligate intermediary (Zumft, 1997). Hence, both the microbial production of N₂O, and its reduction to N₂ depend on the soil’s oxygen (O₂) status, which is affected by the interaction between O₂ supply and consumption.

Soil pores may be filled with water or gas and so a soil’s O₂ status is strongly influenced by water content, such that increasing soil water content results in a decreasing soil gas volume. Consequently, soil water-filled pore space (WFPS) has long been regarded as a measure of the potential for a soil to denitrify (e.g., Linn & Doran, 1984). However, Farquharson and Baldock (2007) cautioned against the use of WFPS to predict N₂O emissions, as the relationship varies with soil bulk density. This was demonstrated clearly by Balaine et al. (2013), who showed that peak emissions of N₂O did not occur at constant values of WFPS when soil varied across a range of bulk densities and matric potentials. The relationship was best described as a function of the soil’s relative gas diffusivity (Dp/Do, where Dp is the gas diffusion coefficient in the soil and Do is the gas diffusion coefficient of the same gas in free air). Nitrous oxide reductase is also sensitive to the soil O₂ concentration and Balaine, Clough, Beare, Thomas, and Meenken (2016) went on to show that the ratio of N₂O:N₂ could also be explained by changes in soil Dp/Do. This variable is indicative of the soil’s O₂ supply.

Most denitrifiers are aerobic heterotrophs that use a carbon (C) source as an electron donor to reduce N oxides under anaerobic conditions (Zumft, 1997). The quantity and quality of soil C can also affect the rate of denitrification and the N₂O:N₂ ratio (Firestone & Davidson, 1989; Gillam, Zebarth, & Burton, 2008; Senbayram, Chen, Budai, Bakken, & Dittert, 2012). As the quantity of C available to denitrifiers increases, the rate of denitrification increases if sufficient NO₃⁻ substrate and anaerobic conditions are present (Senbayram et al., 2012). In pasture soils, C substrates are derived from a wide range of sources that include the mineralization of soil organic matter, plant root exudation, and the deposition of manures and slurries (Henry et al., 2008; Laughlin & Stevens, 2002). Increasing availability of labile C in the soil can enhance O₂ consumption due to increased respiration, potentially enhancing anaerobic conditions that favour denitrification and the production of N₂O (Friedl et al., 2018; Petersen, Ambus, Elsgaard, Schjenning, & Olesen, 2013).

Dual regulation of N₂O emissions due to variations in C form and O₂ concentration has been demonstrated: addition of butyrate and glutamic acid to soil slurries caused greater N₂O production relative to glucose and mannitol after 110 h in the presence of NO₃⁻ at 21% O₂ but not at ~2% O₂ (Morley & Baggs, 2010). The higher N₂O production at 21% O₂ was thought to result from the increased availability of labile C lowering O₂ concentrations (Morley & Baggs, 2010) but this was not tested. Previously, it was also shown that the efficiency of N₂O reduction to N₂ was substrate dependent and it was proposed that this effect may vary with soil type (Morley, Richardson, & Baggs, 2014; Paul, Beauchamp, & Trevors, 1989).

Furthermore, the potential for an enhanced C substrate supply to increase soil respiration and thus modify O₂ supply (soil Dp/Do) has not been investigated with respect to N₂O and/or N₂ production under controlled conditions. Thus, the objective of this study was to vary both soil matric potential, in order to modify O₂ supply (Dp/Do), and C substrate supply, in order to modify O₂ consumption (increased respiration), and to determine their combined effects on the production of N₂O and N₂. For a given soil, we hypothesized that (i) C substrate addition would enhance soil respiration, and thus denitrification, when Dp/Do was suboptimal for denitrification, (ii) increasing soil matric potential (reducing Dp/Do) would increase the rate of denitrification but decrease the N₂O: (N₂O + N₂) ratio, regardless of C substrate type, and (iii) acetate substrate would enhance N₂O reduction to N₂ relative to glucose regardless of the soil.

C substrate effects on soil N₂O, N₂ and CO₂ emissions were soil type specific.

KEYWORDS
acetate, glucose, greenhouse gas emissions, matric potential, soil diffusivity
2 | MATERIALS AND METHODS

2.1 | Experimental design and site characterization

A few kilograms of soil were collected from three randomly selected locations (0–150 mm depth) in three grazed grassland sites (nine samples in total) all dominated by perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.). The sites were located within a 5-km distance with the same climatic conditions but with different soil types. The soils were collected from the Ashley Dene Research & Development Station (AD, latitude 43° 65′ S, longitude 172° 35′ E, elevation above sea level 34 m, Mottled Argillic Pallic Soil (Hewitt, 1998), Udic Ustochrept (Soil Survey Staff, 1999)), the Lincoln University Dairy Farm (LU, 43° 65′ S, 172° 48′ E, Typic Immature Pallic soil (Hewitt, 1998), Typic Haplustept (Soil Survey Staff, 1999)), and the Lincoln University Demonstration Farm (LD, 43° 65′ S, 172° 44′ E, Typic Immature Pallic soil (Hewitt, 1998), Typic Haplustept (Soil Survey Staff, 1999)). Soil samples were air-dried and then sieved (≤2 mm), with any visible plant material removed, and stored at 4°C. A subset of each sample was used to characterize soil properties at each site, with three replicates (Table 1). Soil total C and total nitrogen (N) concentrations were analysed on an Elementar Vario-Max CN Elemental Analyser (Elementar GmbH, Hanau, Germany) (Table 1). Total soil phosphorus (P) was determined after sulphuric digestion with concentrated H$_2$SO$_4$ + H$_2$O$_2$ 30% v v$^{-1}$ (Olsen, Sommers, & Page, 1982). Total extractable organic P (Olsen P) was measured after extraction with NaOH 0.25 M + EDTA 0.05 M (Olsen et al., 1982). Texture analyses were performed using a laser diffraction particle analyser (Mastersizer 3000, Malvern Panalytical, UK). Soil pH was measured on deionized water extracts (Rowell, 2014). The remainder of each sample was used for the experiment. The three samples from each site were amalgamated and then packed into stainless steel rings (73 mm internal diameter, 74 mm depth) to a depth of 41 mm, to achieve a soil bulk density ($\rho_b$) of 1.1 Mg m$^{-3}$. The base of each soil core was covered with a fine nylon mesh (25 μm) to prevent any soil loss. The water holding capacity of each soil was determined by immersing the soil cores in water for 2 h and then draining for 24 h (Priha & Smolander, 1999).

For each soil (AD, LU and LD), a factorial experiment consisted of four replicates of two factors: matric potential and C substrate, comprising three levels each of soil matric potential ($\psi$; −3, −5 and −7 kPa), and C substrate (acetate, glucose, or water as a control). Glucose was selected because it is used commonly as a C source for
soil organic matter (SOM) priming (Kuzyakov, Friedl, & Stahr, 2000) and to determine C substrate limitation when determining soil denitrification potential (Morley et al., 2014). Acetate, applied as sodium acetate, was selected because it has been shown to increase N2O reductase efficiency compared to carbohydrates (e.g., glucose) (Morley et al., 2014) and because low-molecular-weight organic acids, such as acetate, occur in the soil due to plant litter degradation, root exudation and organic C decomposition under anaerobic conditions (Castaldelli, Colombani, Vincenzi, & Mastrocicco, 2013). Soil ψ levels were based on those previously observed to give a range of denitrification rates and products (Balaine et al., 2016) and where denitrification rates were observed and organic C decomposition under anaerobic conditions (Castaldelli, Colombani, Vincenzi, & Mastrocicco, 2013). Soil ψ levels were based on those previously observed to give a range of denitrification rates and products (Balaine et al., 2016) and where denitrification rates were observed to be higher between 0 and −6 kPa. In total, 72 soil cores were packed for each soil and this allowed for the destructive analyses of a fully replicated set of treatments on day 3 of the experiment and at the end of the experiment on day 14, which also aligned with the 15N gas emission sampling undertaken on days 3 and 14 as described below.

Soils were maintained at the set soil ψ values by placing the cores on tension tables after they had been saturated with distilled water and allowed to drain for 4 days (Romano, Hopmans, & Dane, 2002). Then 1 mL of a KNO₃, 15N-enriched solution (300 μg N g⁻¹ soil or 27.6 mg N mL⁻¹; 40 atom% excess 15N; Cambridge Isotope Laboratories Inc., USA) was applied. The day of KNO₃ addition was defined as day 1 of the experiment. Subsequently, a total of 0.9 mL of C solution was added daily for 14 days (80 μg C g⁻¹ soil or 16.4 mg C mL⁻¹) by injecting 0.18 mL of the C soil at five evenly spaced points, to a depth of 20 mm, using a syringe. Tension tables and soil cores were maintained at an average temperature of 20°C.

### 2.2 Soil analyses on day 3 and day 14

On day 3 and day 14, pH at the soil surface was measured with a flat surface pH meter (Mettler Toledo, Part No. 51343180) prior to destructive sampling. Soil cores extruded from the stainless-steel rings were homogenized manually and subsampled to determine gravimetric water content (θₑ₆) by drying at 105°C for 24 h. Soil WFPS was calculated using θₑ₆, ρₑ and, for all soils, an assumed particle density of 2.65 Mg m⁻³ (Nimmo, 2004). Dissolved organic carbon (DOC) concentrations were determined after extracting homogenized soil subsamples with deionized water for 1 h and then centrifuging (3,500 rpm, 2862 g) the extracts for 20 min before filtering through 0.45-μm cellulose nitrate membrane filters (Ghani, Dexter, & Perrott, 2003). The DOC concentrations were determined on a Shimadzu TOC analyser (Shimadzu Oceania Ltd, Sydney, Australia). Soil inorganic-N was determined by extracting subsamples of the homogenized soil with 2 M KCl for 1 h (1:10 ratio of soil:KCl), centrifuging (3,500 rpm, 2862 g) and filtering (Whatman grade 42 paper). The NO₃⁻N and NH₄⁺-N concentrations of the KCl extracts were determined using flow injection analysis (Blakemore, Searle, & Daly, 1987).

### 2.3 Emissions of N₂O, N₂ and CO₂, and measurement of relative gas diffusivity

Measurements of gaseous emissions were made daily until day 7 and then on days 9, 11 and 14 by placing each soil core into a glass jar (1 L) equipped with a gas-tight lid fitted with a rubber septum. A syringe fitted with a two-way stopcock and a 25G hypodermic needle was used to remove gas samples (10 mL) for measurement of N₂O concentrations, at 30 and 60 min after the jar was sealed. These samples were injected into previously evacuated 6-mL Exetainer® vials (Labco Ltd, High Wycombe, UK) for analysis on a gas chromatograph (SRI-8610, Torrance, CA) equipped with a 63Ni electron capture detector. Increases in N₂O concentration over time (0, 30 and 60 min) were used to calculate rates of N₂O emissions according to Hutchinson & Mosier (1981). Additional gas samples (15 mL) were taken on days 3 and 14, after 180 min, for determination of the 15N enrichment of the N₂O and N₂ evolved using the 15N gas-flux method (Mulvaney & Boast, 1986). These samples were injected into pre-evacuated 12-mL Exetainer® vials. A continuous flow isotope ratio mass spectrometer (CFIRMS, Sercon 20–22; Sercon, Cheshire, UK) interfaced to a TGII cryofocusing unit (Sercon, Cheshire, UK) was used to measure the ion currents 44, 45 and 46 for N₂O, 15N enrichment (Stevens & Laughlin, 1998) and for calculating the N₂ emissions (Mulvaney & Boast, 1986). Standard deviations of repeated measures of ambient air samples (n = 10) resulted in Δ²⁹R and Δ³⁰R values of 1.4E-6 and 1.1E-6, respectively, and a detection limit of 0.1 mg m⁻² h⁻¹ for N₂. Days 3 and 14 were selected for determining the N₂O and N₂ emissions because, at approximately day 3, Samad et al. (2016a) found that N₂O emissions from pasture soils approached their peak, whereas at day 14 it was expected that soil N₂O emissions would be relatively low because there had been sufficient time for expression and function of N₂O reductase (Liu, Zhang, Bakken, Snipen, & Frostegård, 2019) and utilization of C amendments would have reached steady state.

Soil CO₂ emissions were measured by placing a static chamber on top of the soil core, which was connected to
an automatic soil respiration system (Model LI-8100, Li-Cor Inc., Lincoln, Nebraska, USA).

For both CO₂ and N₂O, daily emissions were calculated and integrated over time to give cumulative emissions over 14 days. In the absence of measurements on days 8, 10, 12 and 13, when soil CO₂ emissions had reached steady state, and soil N₂O emissions had dramatically declined, the Loess model (Cleveland & Moldrup, 2002). Briefly, a chamber containing a calibrated oxygen (O₂) sensor (KE-25, Figaro Engineering Inc., Osaka, Japan) was purged with O₂-free air (90% Ar and 10% N₂) while the base of the soil core was isolated from the chamber. Once the chamber O₂ concentration fell to zero, the base of the soil core was exposed to the O₂-free chamber atmosphere and subsequently the elevated O₂ concentration in the chamber, resulting from O₂ diffusing through the soil core into the chamber, was measured after 120 to 180 min. The technique assumes that any error in the calculated value of Dₚ (O₂ diffusion coefficient in soil) due to O₂ consumption was negligible (Masis-Melendez, de Jonge, Deepagoda, Tuller, & Moldrup, 2015; Moldrup et al., 2000). Dₚ was calculated from the rate of O₂ increase in the chamber using regression analysis (Rolston & Moldrup, 2002). All diffusivity measurements were made at 20°C and the value of Dₒ at this temperature was 0.072 m² h⁻¹ (Currie, 1960).

2.4 | Data analyses

Differences in the soil properties at the three sites were analysed using ANOVA and are presented in Table 1.

For each soil separately, the effects of the treatments on the temporal evolution of soil CO₂ emissions were tested for significance using a non-linear mixed-effect (NLME) model using the nlme package of R (Pinheiro, Bates, DebRoy, Sarkar, & Team, 2017). Each CO₂ emission measurement was treated as a sample, with soil ψ, and substrates set as fixed effect factors. To account for non-independence of repeated measurements over time, the replicate number was included as a random effect in each model. A three-parameter rectangular hyperbola (Crawley, 2007) was fitted to the data as:

\[ R_s = a - b \, e^{-ct} \]

where \( R_s \) is the CO₂ emission rate, \( t \) is time, \( a \) is the value for steady-state CO₂ emissions, \( b \) is the difference between the value of CO₂ emissions on a given day and the value of CO₂ emissions on day 0, and the parameter \( c \) describes the shape of the curve. Model comparisons were based on Akaike's information criterion (AIC). The model with the lowest AIC indicated the best-fitting model (Anderson & Burnham, 2002) and analyses of residuals were undertaken to ensure that residuals were independent, normally distributed and homoscedastic. Parameter values were compared using Tukey's honest significant difference (HSD) test in the ‘agricolae’ package of R (Mendiburu, 2013).

For each soil separately, the effects of C substrate, soil ψ, and their interactions on soil pH, DOC, NO₃⁻-N, NH₄⁺-N concentrations, the N₂O:(N₂ + N₂O) ratio, and cumulative values of CO₂-C emissions and N₂O-N emissions were tested using ANOVA in the ‘agricolae’ package of R version 1.3.1 (Mendiburu, 2013). In addition, cumulative values of CO₂-C emissions and N₂O-N emissions were compared using Tukey's HSD test in the ‘agricolae’ package of R (Mendiburu, 2013).

3 | RESULTS

3.1 | Effect of the treatments on soil physical and chemical properties

For each soil, the soil pH increased with either acetate or glucose addition compared to the water treatment. On day 3, soil pH values under the acetate treatment (range, 6.3–7.2) were higher than those under glucose (5.9–6.5), which in turn were higher than those for the control (5.4–5.7) \( (p < 0.001; \text{ Table S1}) \). Similar findings were observed on day 14, with soil pH values under the acetate, glucose and water treatments ranging from 8.7 to 8.8, 7.1 to 8.3, and 5.3 to 6.0, respectively (Table S2). There was no effect of soil ψ on the soil pH on either day 3 or 14.

As expected, on both days 3 and 14, soil water content was lower in treatments with lower soil ψ. Values of WFPS in the AD, LU and LD soils ranged from 71 to 55%, 90 to 83%, and 94 to 90%, respectively, as soil ψ treatment decreased from −3 to −7 kPa. For the LD soil, WFPS declined as soil ψ decreased from −3 kPa (94%) to −5 kPa (90%) but not from −5 to −7 kPa. When averaged across all soil ψ treatments, soil water content was higher \( (p < 0.001) \) for the LU and LD soils than for the AD soil. There was no effect of C substrate addition on soil water content.

Values (mean ± standard deviation) of relative soil gas diffusivity \( (D_p/D_o) \) in the AD soil were \( 0.0040 ± 0.0023, 0.0110 ± 0.0019 \) and \( 0.0154 ± 0.0028 \) at \( −3, −5 \) and \( −7 \) kPa, respectively, whereas for the LU soil the respective values were \( 0.0026 ± 0.0023, 0.0043 ± 0.0010 \) and \( 0.0037 ± 0.0018 \). In the LD soil the respective \( D_p/D_o \)
values at −3, −5 and −7 kPa were 0.0045 ± 0.0024, 0.0048 ± 0.0022 and 0.0058 ± 0.0025. Values of $D_p/D_o$ did not vary with C substrate treatment ($p = 0.817$).

On day 3, DOC concentrations in the acetate (66–254 µg C g$^{-1}$ soil) and glucose (50–254 µg C g$^{-1}$ soil) treatments were higher than those under the control treatment (40–105 µg C g$^{-1}$ soil) in both the AD and LU soils ($p < 0.05$; Table S1). For the LD soil on day 3, the DOC concentrations in the acetate treatment (183–289 µg C g$^{-1}$ soil) were higher than those for the control treatment ($p < 0.05$) but the glucose treatment DOC concentrations were not (138–244 µg C g$^{-1}$ soil; Table S1). On day 14, for all soils, the DOC concentrations in the acetate (180–789 µg C g$^{-1}$ soil) and glucose (68–520 µg C g$^{-1}$ soil) treatments were, when averaged across soil ψ treatments, higher ($p < 0.05$) than those in the control treatment (24–188 µg C g$^{-1}$ soil; Table S2).

On day 3, soil NO$_3$–N concentrations were unaffected by treatments, with values ranging from 218 to 361 µg NO$_3$–N g$^{-1}$ soil (Table S1). On day 14, in the AD soil NO$_3$–N concentrations were lower ($p < 0.05$) at a soil ψ of −3 kPa, in both the acetate (88 µg NO$_3$–N g$^{-1}$ soil) and glucose (79 µg NO$_3$–N g$^{-1}$ soil) treatments, when compared to the control treatment (242 µg NO$_3$–N g$^{-1}$ soil), but this was not the case at −5 and −7 kPa (Table S2). Regardless of soil ψ and substrate treatment the NO$_3$–N concentrations, on day 14, in the LU and LD soils ($≤60$ µg NO$_3$–N g$^{-1}$ soil) were consistently an order of magnitude lower ($p < 0.001$) than those in the control ($≥130$ µg NO$_3$–N g$^{-1}$ soil) treatment (Table S2).

Soil NH$_4^+$–N concentrations did not differ with C substrate on either day 3 or day 14 (Tables S1, S2). At a soil ψ of −3 kPa, the NH$_4^+$–N concentrations were higher than those at a soil ψ of −7 kPa, with the exception of the AD soil on day 14 where no such effect occurred ($p < 0.05$; Table S2).

### 3.2 N$_2$O and N$_2$ emissions

For all treatments, N$_2$O emissions generally peaked between days 3 and 5 (Figure 1). An exception was the less sensitive response to substrate addition, in terms of N$_2$O emissions, for the AD soil at −7 kPa (Figure 1; Table S3). During this time the N$_2$O peak emissions were generally highest when glucose was applied ($p < 0.05$; Figure 1). Over the first 7 days, the highest N$_2$O emissions occurred in the LU soil at −3 kPa when glucose substrate was applied (Figure 1). From day 8, N$_2$O emissions from the LU and LD control treatments were higher than those from the acetate and glucose treatments ($p < 0.05$; Figure 1). In the AD soil, N$_2$O emissions were close to zero after day 8, regardless of soil ψ or substrate treatment.

After 14 days, glucose addition resulted in higher cumulative N$_2$O emissions ($p < 0.05$) when averaged across soil ψ treatments (Table S4). However, soil ψ treatment did not affect cumulative N$_2$O emissions when averaged across substrate treatments.

On day 3, for all soils, regardless of soil ψ treatment, N$_2$ emissions were higher following glucose and acetate substrate addition than with water addition, with the exception of the AD soil at −7 kPa where no N$_2$ flux was measured ($p < 0.05$; Figure 2). N$_2$ emissions were also higher for the −3 kPa treatment as compared to the −7 kPa treatment ($p < 0.05$), with neither of these treatments differing from the values for the −5 kPa treatment.

On day 14, higher N$_2$ emissions occurred with acetate than glucose addition in the LU and LD soils ($p < 0.01$) but no such difference occurred in the AD soil ($p = 0.15$). In the LU and LD soils there was no effect of soil ψ on N$_2$ emissions when averaged across substrate treatment ($p > 0.16$) but N$_2$ emissions declined ($p < 0.01$) with increasing drainage in the AD soil (Figure 2). The N$_2$ emissions from glucose-treated LU and LD soils were higher than those from water-treated soils ($p < 0.05$; Figure 2). Averaged across soil ψ potential the ratios of N$_2$ emissions from the acetate and glucose treatments were $2.56 ± 0.75$ (standard deviation), $2.35 ± 0.81$ and $0.83 ± 0.31$ for the LD, LU and AD soils, respectively.

Carbon substrate type affected the N$_2$O:(N$_2$O + N$_2$) emission ratio on day 3 (Figure 3), with higher ($p < 0.05$) values under glucose and water (0.91 and 0.90, respectively) than those for soils treated with acetate (0.81). On day 14, the N$_2$O:(N$_2$O + N$_2$) emission ratios for soils treated with acetate (0.10) or glucose (0.07) were lower than those for the water-treated soil (0.86, $p < 0.05$). The N$_2$O:(N$_2$O + N$_2$) emission ratio was highest under water-treated LU and LD soils and lowest under glucose-treated LU and LD soils on day 14 ($p < 0.05$; Figure 3). The AD soil N$_2$O:(N$_2$O + N$_2$) emission ratio did not vary as a result of glucose or acetate treatment at this time. Soil ψ had no effect on the N$_2$O:(N$_2$O + N$_2$) emission ratio on either day 3 or day 14.

### 3.3 Soil CO$_2$ emissions

Based on the model (Equation 1), the response of CO$_2$ emissions to glucose or acetate addition was best fitted by an exponential curve (Figure 4). An exception to this was the AD soil treated with acetate at a soil ψ of −3 kPa where the steady state was not reached. Steady-state CO$_2$ emissions in the other treatments at −3 kPa did not differ with substrate treatment (Table S5). With the exception of the AD soil treated with glucose (2.6 ± 0.2 µmol m$^{-2}$ s$^{-1}$, $p < 0.05$), where maximum steady-
state CO₂ emissions occurred at −5 kPa, and the LU soil treated with acetate (1.6 ± 0.0 μmol m⁻² s⁻¹, p < 0.05), where the minimum value of steady-state CO₂ emissions occurred at −5 kPa, there were no differences in the magnitude of steady-state CO₂ emissions at −5 kPa due to substrate (Table S5). Steady-state CO₂ emissions were highest in the AD soil treated with glucose at −7 kPa (Table S5, p < 0.05); otherwise, there were no other treatment effects on the magnitude of steady-state CO₂ emissions at −7 kPa.

The rate at which a steady state of CO₂ emissions was reached at −3 kPa generally did not differ with substrate treatment, the exception being the glucose-treated LU soil, which took longer to reach a steady state of CO₂ emissions than with acetate addition (p < 0.05; Table S5). At −5 kPa, the glucose-treated AD soils required more time to reach a steady state of CO₂ emissions than the acetate-treated soil (p < 0.05; Table S5). There was no difference in the time period required to reach a steady state of CO₂ emissions at −7 kPa as a result of substrate addition (Table S5). There was generally no effect of soil ψ on the time required to reach a steady state of CO₂ emissions in the AD or LU soils. In the LD soil, a higher steady-state value occurred at −7 kPa than at −3 kPa, in both glucose- and acetate-treated LU soil (p < 0.05; Table S5).
For all soils, cumulative CO₂ emissions from the water treatment (control) were lower than those in the acetate and glucose treatments regardless of soil ψ treatments (p < 0.05; Table S5). A C substrate by soil ψ treatment interaction resulted in higher (p < 0.05) cumulative CO₂ emissions occurring under glucose amendment and as soil ψ became more negative (increasing drainage) (Table S4).

3.4 | Comparisons of CO₂ and N₂O emissions with Dₚ/Dₒ

Pooling data showed cumulative N₂O emissions declined exponentially with increasing Dₚ/Dₒ, with 67% and 65% of the variation in cumulative N₂O losses explained by glucose and acetate application, respectively (Figure 5). In contrast, pooling the data in a similar manner showed a positive linear relationship between Dₚ/Dₒ and cumulative CO₂ emissions, with 47% and 21% of the variation explained by glucose and acetate applications, respectively (Figure 5).

4 | DISCUSSION

Soil WFPS and Dₚ/Dₒ showed that soil conditions were suitable for denitrification, with Dₚ/Dₒ < ~0.006 and WFPS > ca. 80% (Balaine et al., 2013; Linn & Doran, 1984; Owens, Clough, Laubach, Hunt, & Venterea, 2017), with the exception of the AD soil, which, due to its higher sand content, held less water at matric potentials of ~5 kPa and ~7 kPa. Increasing N₂O and N₂ production following application of NO₃⁻ and C substrates indicates denitrification was the dominant pathway responsible for N₂O and N₂ production. The dominant role of denitrification is also supported by the NO₃⁻-N concentrations being an order of magnitude lower in the presence of C substrate when soils were anaerobic (Dₚ/Dₒ values < ~0.006). Dissimilatory nitrate reduction to ammonia (DNRA) can also produce N₂O under anaerobic conditions in grassland soils (Friedl et al., 2018). Higher soil NH₄⁺-N concentrations at a soil ψ of ~3 kPa than those at ~7 kPa suggest only a minor contribution from DNRA given that the soil NH₄⁺-N concentrations were relatively low when compared with the
The magnitude of the decrease in the soil NO$_3^-$-N concentrations. The low level of NH$_4^+$ substrate available, a precursor to hydroxylamine, and the low O$_2$ levels (hypoxic conditions) also imply that anaerobic oxidation of hydroxylamine did not make a significant contribution to the observed N$_2$O emissions (Stein, 2019).

The low rate of N$_2$O production in the AD soil (−5 kPa), or lack of both N$_2$O and N$_2$ production in the AD soil (−7 kPa), can be attributed to conditions being too aerobic for denitrification ($D_p/D_o > 0.006$), which in turn explains why NO$_3^-$ concentrations remained one or two orders of magnitude higher in the presence of C substrate at −5 and −7 kPa in the AD soil.

Peak N$_2$O emissions at ~3 days after substrate addition are consistent with the result of Samad, Bakken, et al. (2016a), who examined 13 grassland soils from Ireland and New Zealand that were wetted and amended with NO$_3^-$ before undergoing anaerobic incubation. Upon commencement of the anaerobic incubation, production of NO, N$_2$O and N$_2$ occurred, with N$_2$O production generally peaking at ca. 90 h and N$_2$ peaking after this time.

Petersen, Schjonning, Thomsen, and Christensen (2008) proposed that increased consumption of O$_2$, as a result of an enhanced bioavailable C supply, could increase the anoxic zone within a soil. However, the utilization of the applied C substrates in the AD soil as evident from the CO$_2$ emissions, which were comparable in magnitude to those from the LU and LD soils, was not sufficient to induce anaerobic conditions at −7 kPa in the AD soil based on relative N$_2$O emissions. Thus, for the data from the AD soil at −7 kPa ($D_p/D_o = 0.0154$), we must reject the hypothesis that enhanced soil respiration following substrate addition will promote denitrification when soil O$_2$ supply is suboptimal for denitrification ($D_p/D_o > 0.006$). Under these conditions the soil O$_2$ supply was sufficiently high to maintain aerobic conditions while respiration occurred. However, if we accept (i) the $D_p/D_o$ value of 0.006, shown by Balaine et al. (2013) to demarcate the hypoxic–anaerobic boundary where peak N$_2$O production and the onset of N$_2$ production occur (Zhu, Burger, Doaneb, & Howarth, 2013), and (ii) the fact that nitrifying bacteria lack nosZ for reducing N$_2$O to N$_2$ (Hallin, Philippot, Löfler, Sanford, & Jones, 2018), then the increase in
both N₂O and N₂ emissions in the presence of either of the C substrates, at −5 kPa ($D_p/D_o = 0.0110$), in the AD soil indicates C-induced respiration altered O₂ supply sufficiently to induce anaerobic conditions. Thus, we can accept the hypothesis that enhanced soil respiration following substrate addition will promote denitrification. Similar comparisons cannot be made for the LU and LD soils, where, despite the soil ψ treatments applied, the value of $D_p/D_o$ was constantly ≤0.006, and thus the LU and LD soils were predisposed to denitrify on the basis of these anaerobic conditions.

Although this current study used repacked soil cores, the results are consistent with an in-situ study on pasture soil that also observed denitrification being promoted when $D_p/D_o$ decreased to ≤0.006 (Owens et al., 2017). Chamindu Deepagoda, Jayarathne, Clough, and Thomas (2019) showed that N₂O fluxes from intact soil cores peaked within a narrow range of $D_p/D_o$ of 0.005–0.010 for soil cores from three soil depths taken from three perennial pastures that received nitrate.

The effect of soil texture on soil O₂ supply is further supported by the observed relationship between cumulative N₂O and diffusivity, where the trend for N₂O emissions to increase exponentially with declining $D_p/D_o$ (ca. < 0.006) aligns with the findings of Balaine

**FIGURE 4** CO₂ emissions over the 14 days. Soils were treated with three levels of soil matric potential (−3, −5 and −7 kPa) and three different substrates (acetate, glucose and water). Soils were sampled from three sites: Ashley Dene Research & Development Station (AD), Lincoln University dairy farm (LU) and Lincoln University demonstration farm (LD). Values are means of four replicates (± standard deviation), $n = 4$. Solid lines represent the exponential curve fitted using Equation 1.
et al. (2013). This was reflected in the absence of (−7 kPa), or relatively lower (−5 kPa), N₂ emissions from the sandy textured AD soil, again likely to be the result of the diffusivity in the AD soil being > −0.006 (Balaine et al., 2016). Thus, in support of our second hypothesis, declining diffusivity invoked greater rates of denitrification regardless of C substrate type.

We also hypothesized that the ratio of N₂O:(N₂O + N₂) would decrease as the denitrification rate increased but this did not occur. This suggests there were factors other than simply the rate of denitrification influencing this ratio. The rate of denitrification can potentially differ due to microbial community composition, microbial biomass, or the way in which a specific soil's microbial community utilizes an applied C substrate. For example, Giles, Morley, Baggs, and Daniell (2017) found that 120 h after a single input of glucose, glutamine or citric acid, differences in the N₂O and N₂ emissions resulted from differences in C substrate use efficiency. In a study of 13 grassland soils, Samad et al. (2016b) found that the rate of soil denitrification was also closely linked to anoxic C mineralization (r² = 0.89), measured for 40 h after removal of oxic conditions. Wakelin et al. (2017) found that increasing soil P status increased both microbial biomass and mineralization of added C substrates. Both soil C availability and P status have also been shown to influence soil N cycling (e.g., O’Neill et al., 2021). Hence, the relatively low rate of denitrification observed in the AD soil at −3 kPa may have resulted from both the lower organic matter content and P status of the AD soil generating differences in microbial biomass and microbial community structure that in turn affected how, and at what rate, the applied C substrates were used.

The NO₃⁻ concentration can also affect the N₂O: (N₂O + N₂) ratio (Conthe et al., 2020). The decline in N₂O emissions by day 14, under glucose and acetate addition, most likely occurred because soil NO₃⁻ concentrations had also decreased over time. Soil NO₃⁻ is a preferred electron acceptor to N₂O (Giles, Morley,
Baggs, & Daniell, 2012) and decreasing soil NO$_3^-$ concentrations enables increasing N$_2$O reductase activity. For example, after applying organic substrates, Sen-bayram et al. (2012) found that the transformation of N$_2$O to N$_2$ occurred more rapidly once soil NO$_3^-$ concentrations decreased below 20 mg kg$^{-1}$ soil. At day 14, this was the case for the LU and LD soils treated with glucose at all matric potentials, and for the LU and LD soils treated with acetate at $-3$ kPa. Similarly, the increase in soil pH over time will have favoured N$_2$O reductase activity (Firestone & Davidson, 1989; Samad, Bakken, et al., 2016a). This is because low soil pH (≤6.1) diminishes or prevents reduction of N$_2$O, primarily by precluding a successful assembly of functional N$_2$O reductase (Liu, Frostegård, & Bakken, 2014). Consequently, it is also possible that the higher N$_2$ fluxes observed under acetate could be due partially to the higher soil pH observed under the acetate treatment.

In support of the third hypothesis, on day 3 acetate enhanced N$_2$O reduction to N$_2$, relative to glucose, in all three soils (Figure 3), with a lower N$_2$O:(N$_2$O + N$_2$) ratio observed under acetate (0.81) than glucose (0.91). This effect was not present at day 14 due to the diminished production of N$_2$O and the dominance of N$_2$ as a denitrification product as noted above. Previously, Paul et al. (1989) and Morley et al. (2014) showed that the efficiency of N$_2$O reduction to N$_2$ was substrate dependent. It has been suggested that acetate is more efficient than glucose in promoting N$_2$O reduction, possibly due to the differential metabolism of glucose and acetate, with acetate directly entering the tricarboxylic acid (TCA) cycle (Gunina, Dippold, Glaser, & Kuzyakov, 2014), and producing compounds directly linked to the electron transport chain (Conthe et al., 2020; Gottschalk, 1986). However, although the dominance of N$_2$ production precluded observing the possible effect of acetate on the N$_2$O:(N$_2$O + N$_2$) ratio at day 14, the more than twofold higher emissions of N$_2$ under the acetate-treated LU and LD soils, compared with the glucose-treated LU and LD soils, show that, in addition to enhancing N$_2$O reduction, acetate also increased the overall rate of denitrification at day 14. Gunina et al. (2014) showed that, under nonsaturated soil water conditions, similar initial uptakes of glucose and acetate by soil microorganisms occurred after 10 days, but more glucose $^{13}$C than acetate $^{13}$C was recovered from the extractable microbial biomass, which was interpreted as the result of a higher use efficiency for glucose than acetate. Sugars are metabolized by microbes via glycolysis prior to glucose-C being incorporated into cell components or entering the TCA cycle (Bore, Kuzyakov, & Dippold, 2019) and glucose is recognized as providing the main source of C for a wide range of microbial communities (Paterson, Gebbing, Abel, Sim, & Telfer, 2007), providing more energy than acetate for microbial processes (Paul et al., 1989). However, glucose efficiency as a denitrification C substrate may decline if fermentative bacteria compete with denitrifiers for C (Paul et al., 1989). Given that acetate is generally considered to be a non-fermentable substrate (van den Berg, Elíasáiro, Kuenen, Kleerebezem, & van Loosdrecht, 2017), the lower N$_2$ emissions observed on day 14 in the LU and LD soils under glucose may have also resulted from greater microbial competition for glucose between fermentative organisms and denitrifiers. However, the fact that the glucose-treated AD soil had similar N$_2$ emissions to the acetate-treated soil at day 14, suggests that the microbial community in the AD soil was also responding differently to substrate addition with respect to the LU and LD soils due to potential effects of the lower P and C status on the microbial biomass and community structure as noted above. Recent studies have reported increases in N$_2$O production 2 to 3 weeks after an initial denitrification-induced flux of N$_2$O is observed, potentially as the result of ensuing mineralization and nitrification (Wu et al., 2017). This may occur depending on soil organic matter content and aeration status. However, observation of such effects was beyond the scope of this experiment.

The fact that the AD soil amended with acetate did not reach steady-state CO$_2$ emissions at $-3$ kPa, despite comparable diffusivity with the LU and LD soils at this matric potential, indicates the microbial pool utilizing acetate was still growing, and this is also reflected in relatively low denitrification emissions at $-3$ kPa in the AD soil at day 3. The lower P status and lower soil C concentration in the AD soil, reflected in the lower DOC concentrations in the control (water only) treatment, may also have resulted in a lower microbial biomass initially being present. The fact that the DOC values were an order of magnitude lower in the glucose-treated AD soil, at $-5$ and $-7$ kPa, aligns with the concurrent enhanced diffusivity of these treatments, with an increased oxygen supply driving the CO$_2$ emissions response in the AD soil in these treatments.

Besides substrate decomposition, CO$_2$ emissions may also result from substrate-induced priming, stimulating the decomposition of native soil C (Schimel & Weintraub, 2003; Shahbaz, Kumar, Kuzyakove, Börjesson, & Blagodatskaya, 2018). Thus, it is also possible that the observed CO$_2$ emissions were partly due to priming effects. However, the aim of this study was not to determine priming effects. Future studies are required to examine potential interactions between priming effects and N$_2$O:(N$_2$O + N$_2$) emissions ratio and gross denitrification rates, as mediated by soil types and C substrate quantity and quality.
The positive response of soil CO₂ emissions to decreasing matric potential that was observed (Figure 5) is in agreement with Groffman and Tiedje (1991), who determined the response of soil CO₂ emissions across the full range of soil water content to be parabolic. For both substrates this positive response was driven strongly by the highest cumulative CO₂ emissions that occurred in the AD soil at the highest diffusivity levels (−5 and −7 kPa), where N₂O and N₂ emissions were relatively low or non-existent.

5 | CONCLUSIONS

By varying soil matric potential to manipulate relative gas diffusivity, emissions of CO₂ and N₂O were measured from three soils amended with NO₃ and C substrates over 14 days. The results highlight that soil microbial responses to C substrate depend on soil relative gas diffusivity and substrate type. Soil relative gas diffusivity influenced both denitrification and C substrate utilization, with the latter also able to generate anaerobic conditions for denitrification by enhancing O₂ demand. Carbon substrate also regulated denitrification products: acetate initially (day 3) produced lower peak N₂O emissions and lower N₂O/N₂ ratios than glucose. After 14 days, the denitrification emissions were dominated by N₂, with soils higher in organic matter content and with finer texture (lower diffusivity) having twofold greater N₂ emissions under acetate compared with glucose. The time taken to reach steady-state CO₂ emissions, and the maximum rate of CO₂ emissions, varied with C substrate and soil relative gas diffusivity, the latter being a function of soil type.

ACKNOWLEDGEMENTS

This work was funded by the New Zealand Agricultural Greenhouse Gas Research Centre (NZAGRC) and the China Scholarship Council (File no. 201606180104) to support Yuan Li. The authors especially thank Roger Cresswell, Kethsiri Alwis, Emily Huang and Qian Liang, members of the analytical service at Lincoln University, for assisting with laboratory analytical work. We acknowledge also Leanne Hassall, Neil Smith, John Hunt, Graeme Rogers, Carmen Medina Carmona, Marion Delacoux des Roseaux, Adriana Medina Cipagauta, Sephra Rayner, David Rex and Zach Simpson for their practical assistance.

AUTHOR CONTRIBUTIONS

Yuan Li: Formal analysis; methodology; writing-original draft. Timothy Clough: Conceptualization; methodology; resources; supervision; writing-review & editing. David Whitehead: Conceptualization; resources; supervision; writing-review & editing.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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How to cite this article: Li, Y., Clough, T. J., Moinet, G. Y. K., & Whitehead, D. (2021). Emissions of nitrous oxide, dinitrogen and carbon dioxide from three soils amended with carbon substrates under varying soil matric potentials. European Journal of Soil Science, 1–15. https://doi.org/10.1111/ejss.13124