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Relative efficacy and stability of biological and synthetic nitrification inhibitors in a highly nitrifying soil: Evidence of apparent nitrification inhibition by linoleic acid and linolenic acid

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Abstract

Biological nitrification inhibition is a plant-mediated rhizosphere process where natural nitrification inhibitors can be produced and released by roots to suppress nitrifier activity in soil. Nitrification is one of the critical soil processes in the nitrogen (N) cycle, but unrestricted and rapid nitrification in agricultural systems can result in major losses of N from the plant–soil system (i.e., by NO_3^- leaching and gaseous N emissions). In this study, we explored the potential efficacy of biological nitrification inhibitors (linoleic acid [LA] and linolenic acid [LN]) and a proven efficient synthetic (dicyandiamide [DCD]) nitrification inhibitor on N dynamics, nitrous oxide (N_2O) and carbon dioxide (CO_2) emissions in a highly nitrifying soil. ^{14}C-labelled LA, LN and DCD mineralization was determined in a parallel experiment to explore the fate of inhibitors after application. We found that LA and LN had no effect on soil NH_4^+ concentrations, but significantly decreased NO_3^- concentrations. Soil that received DCD had lower NO_3^- and higher NH_4^+ concentrations than the control (soil without nitrification inhibitors). LA and LN increased the cumulative N_2O and CO_2 emissions when they were applied at high concentrations (635 or 1,270 mg kg^{-1} dry soil). LA and LN had a much greater mineralization rate than that of DCD: 47–56%, 37–61% and 2.7–5.5%, respectively, after 38 days incubation. We conclude that in contrast to the direct inhibition of nitrification caused by DCD, addition of LA and LN may cause apparent nitrification inhibition by promoting microbial immobilization of soil NH_4^+ and/or NO_3^-. Future studies on nitrification inhibitors need to clearly differentiate between the direct and indirect effects that result from addition of these compounds to soil.

Highlights

- The efficacy and stability of nitrification inhibitors in a highly nitrifying soil were explored.

- This study supports efforts to mitigate N losses and improve nitrogen use efficiency of inputs.
- Addition of LA, LN and DCD can decrease NO_3^- concentration, but their modes of action may be different.
- The apparent effect of LA and LN on soil NO_3^- concentration could be indirect.

KEYWORDS

^{14}C labelling, carbon dioxide, immobilization, mineralization, nitrification inhibitor, nitrous oxide

1 | INTRODUCTION

In the past decades, the global supply of nitrogen (N) fertilisers has increased dramatically, and is estimated to reach 171 million tons in 2020 (FAO, 2017). Chemical fertilisers represent the main input of N to agriculture soils (61% of the total), with additional N supplied via livestock manures (16%), symbiotic and associative N fixation (18%) and atmospheric N deposition (5%) (Lassaletta, Billen, Grizzetti, Anglade, & Garnier, 2014). Although the use of synthetic N fertilisers is central to maintaining food security, their use is also strongly associated with many of the world's most serious environmental problems (e.g., marine eutrophication, global warming, ozone depletion and air pollution) (Erisman et al., 2013). These issues are directly associated with the inefficient use of fertiliser N and large losses of N from agricultural systems either in gaseous, for example ammonia (NH_3), nitrous oxide (N_2O) and dinitrogen (N_2), or aqueous forms (dissolved organic N, nitrate (NO_3^-)) (Gardiner et al., 2016). The global average N use efficiency (NUE) (the percentage of applied fertiliser N recovered from the crop) is very low (*ca*. 47%) with little improvement seen in the last 30 years (Lassaletta et al., 2014). There is therefore an urgent need to devise practical and cost-effective solutions to promote greater capture of fertiliser N by crop plants and to minimize N loss pathways (e.g., leaching, surface run-off, denitrification and volatilization). One of the proposed strategies is the targeted use of chemicals to control the rate of key N transformations in the soil that result in the losses of N to the environment, for example urea → ammonium (NH_4^+) and $NH_4^+ \rightarrow NO_3^-$.

Nitrification is a key soil process, responsible for the conversion of NH_4^+ to NO_3^- (Firestone & Davidson, 1989). It is a two-step microbially mediated process carried out by chemoautotrophic nitrifying bacteria, first oxidizing NH_4^+ to nitrite (NO_2^-) and then oxidizing NO_2^- to NO_3^- (Firestone & Davidson, 1989). In recent years, fungi-driven heterotrophic nitrification was observed and is also important for NO_3^- production (Chen et al., 2015). Two groups of soil microorganisms, ammonia-oxidizing bacteria (AOB) (mainly *Nitrosomonas* spp. and *Nitrosospira* spp.) and ammonia-oxidizing archaea (AOA), are largely responsible for the biological oxidation of NH_4^+ to NO_3^- (Beeckman, Motte, & Beeckman, 2018; Leininger et al., 2006; Taylor, Zeglin, Dooley, Myrold, & Bottomley, 2010). Nitrification, nitrifier-denitrification and denitrification are primarily biologically mediated processes in soil that are responsible for N_2O generation (Gardiner et al., 2016; Hofstra & Bouwman, 2005; Smith, McTaggart, & Tsuruta, 1997; Tubiello et al., 2013). However, denitrification cannot take place without the substrate NO_3^-. Thus, controlling nitrification represents a good potential way to simultaneously improve NUE, reduce greenhouse gas emissions and attenuate NO_3^- leaching.

Synthetic nitrification inhibitors (NIs), such as dicyandiamide (DCD), 3,4-dimethylpyrazol-phosphate (DMPP) and 2-chloro-6-(trichloromethyl)-pyridine (Nitrapyrin), have been developed for use in agriculture to help slow nitrification and reduce soil N losses (Li et al., 2008; Menéndez, Barrena, Setien, González-Murua, & Estavillo, 2012; Weiske, Benckiser, Herbert, & Ottow, 2001; Wu et al., 2007). The synthetic NIs specifically suppress the ammonia monooxygenase (AMO) pathway within nitrification (Subbarao et al., 2008). In addition to improving NUE (Monaghan, Smith, & Klein, 2013; Wu et al., 2007), the application of NIs may also improve the economic and environmental footprint of food production, and in some cases improve agronomic yield benefit (Li et al., 2018). In the case of DCD, the application of low doses of N-sources applied to or deposited on grassland soils (10 to 50 mg kg^{-1} soil) has been shown to reduce N_2O emissions by 26–82%, and carbon dioxide (CO_2) emissions by 7% (Chadwick et al., 2018; Di & Cameron, 2016; Weiske et al., 2001). Despite their proven benefits, however, synthetic NIs suffer from a number of challenges that may limit their adoption. These include: (a) lack of chemical stability and variable responses in different soil types and moisture/temperature regimes (Marsden et al., 2016; McGeough, Watson, Müller, Laughlin, & Chadwick, 2016; Menéndez et al., 2012), (b) lack of cost-effective and practical delivery strategies to spatially target NI application in

the field (e.g., urine patches) (Ledgard et al., 2008; Luo et al., 2015; Minet et al., 2016, 2018; Welten, Ledgard, & Luo, 2014), and (c) recent evidence that synthetic NIs (e.g., DCD) can contaminate grazed grass (Kim et al., 2012) and be taken up by plants (Marsden, Scowen, Hill, Jones, & Chadwick, 2015), finding their way into the human food chain (Lucas, 2013), resulting in negative public perceptions.

Biological nitrification inhibition is a plant-mediated rhizosphere process where NIs are produced and released from roots that can suppress nitrifier activity in soil (Subbarao et al., 2006). Harnessing this potential to promote greater NUE is highly desirable and has several benefits over synthetic NIs, including: low cost, delivery through the entire root zone, continuous production, greater public acceptability and lower carbon (C) footprint. Most biological nitrification inhibitors (BNIs) released by plants inhibit nitrification by suppressing both AMO and hydroxylamine oxidoreductase (HAO) enzymatic pathways in *Nitrosomonas* (Subbarao et al., 2008, 2015). *Brachiaria humidicola* is a common tropical pasture grass that contains substantial amounts of BNIs within its root and shoot tissues (Subbarao et al., 2006, 2007). Of these BNIs, brachialactone has been found to contribute 60–90% of the inhibitory activity released from the root (Subbarao et al., 2009). In addition, two other BNIs (i.e., linoleic acid [LA] and linolenic acid [LN]) have been identified from the shoot tissue of *Brachiaria humidicola* (Subbarao et al., 2008). When applied to soil as pure compounds, LA and LN have been shown to promote NH_4^+ retention and reduce NO_3^- levels (Subbarao et al., 2008). Most research has focused on the effects of BNIs on soil receiving ammonium-based fertiliser (Subbarao et al., 2008, 2013; Subbarao, Rondon, et al., 2007; Sun, Lu, Yu, Kronzucker, & Shi, 2016) or urine (Byrnes et al., 2017). However, little is known about the effects of BNIs on "residual" soil NH_4^+-N, especially that produced in strongly nitrifying soils.

The aims of our study were therefore to: (a) determine the relative effect of LA, LN and DCD on "residual" NH_4^+ and NO_3^- concentrations, (b) evaluate the effect of LA, LN and DCD on N_2O and CO_2 emissions from soil, and (c) explore the stability (mineralization rate) of LA, LN and DCD in soil. In addition, we use our results to explore if reported nitrification inhibition by biological NIs could actually be the result of an indirect effect due to microbial immobilization of N, stimulated by the addition of available C in LA and LN.

2 | MATERIALS AND METHODS

2.1 | Soil properties

A sandy loam textured Eutric Cambisol collected from a sheep-grazed fertilized grassland in north Wales was used for this study (53°24′N, 4°02′W) (Table 1). This soil was chosen as it is known to possess very high nitrification rates (Jones, Shannon, Murphy, & Farrar, 2004). The soil had not been previously exposed to LA, LN or DCD, and had not been grazed for >3 months prior to collection. Four independent replicate soil samples (0–10 cm depth) were collected, and sieved to pass 2 mm, then stored at 4 °C in loosely sealed bags for 5 days to wait for the incubation experiment to be prepared. Each replicate soil sample collected was used as an experimental replicate ($n = 4$).

Soil moisture content was determined after oven drying (105°C, 24 h), and soil organic matter content determined by loss-on-ignition in a muffle furnace (450°C, 16 h) (Ball, 1964). Soil pH and electrical conductivity (EC) were measured on fresh soil using standard electrodes (1:2.5 (w/v) soil to distilled water). Total soil C and N concentrations were determined on oven-dried soil using a CHN2000 analyser (Leco Corp., St. Joseph, MI, USA). Extractable NH_4^+ and NO_3^- concentrations were measured colorimetrically on 1:5 (w/v) fresh soil to 1 M KCl extracts, using the methods of Mulvaney (1996) and Miranda, Espey, and Wink (2001), respectively.

2.2 | Effect of LA, LN and DCD on soil nitrification

To characterize the effect of LA, LN and DCD on soil nitrification, a soil incubation experiment was conducted. Pure compounds of LA, LN and DCD were added to 450 g of the sandy loam soil in containers (volume: 850 mL; Length × Width × Height: 137 × 104 × 120 mm) at a range of concentrations. LA and LN were applied at 12.7, 127, 635 and 1,270 mg kg^{-1} dry soil (equivalent to 10, 100, 500 and 1,000 mg kg^{-1} wet soil), which are similar dose rates to the pure compounds of LA and LN used

TABLE 1 Properties of soils (0–10 cm) used in the incubation experiments

Soil property	Eutric Cambisol
Moisture content (%)	25.14 ± 0.06
Organic matter (%)	5.26 ± 0.29
pH	5.47 ± 0.01
Electrical conductivity (μS cm^{-1})	103.4 ± 0.49
Total carbon (g kg^{-1} dry soil)	22.13 ± 1.19
Total nitrogen (g kg^{-1} dry soil)	2.33 ± 0.13
NH_4^+-N (mg kg^{-1} dry soil)	4.17 ± 0.05
NO_3^--N (mg kg^{-1} dry soil)	21.29 ± 1.20

Note: Values represent means ± standard error of the mean ($n = 4$).

by Subbarao et al. (2008). DCD was added at the concentration of 12.7, 63.5 and 127 mg kg^{-1} dry soil (equivalent to10, 50 and 100 mg kg^{-1} wet soil). The inclusion of DCD was to act as reference treatments of a known synthetic NI with a proven effect on nitrification. NI applied at the concentration of 0 mg kg^{-1} dry soil was set as the control treatment. To ensure uniform mixing of the small quantities of NIs in the soil, the NIs were first mixed with sterile fine-grained quartz sand. Firstly, LA and LN were dissolved in a small amount of ethanol, which was then mixed with fine quartz sand (50 μL ethanol g^{-1} sand) and evaporated to dryness under a stream of air. The NI-labelled sand was then mixed into the soil (0.065 g sand g^{-1} wet soil). For the DCD treatments, DCD was dissolved in distilled water and mixed with the same quartz sand and added to soil as described above. In the control treatment, the same amount of sterile fine quartz sand was applied to the soil.

The experiment consisted of two sets of containers. One set of containers was used for regular soil sampling, and another set of containers was used for greenhouse gas sampling. Containers (850 mL) containing the NI-labelled soil (450 g soil $container^{-1}$) were covered with Parafilm® (Bemis Inc, Neenah, WI, USA) to allow gas exchange but retain moisture. Every 3 days, the containers were weighed and deionised water was added if it was necessary to maintain soil moisture. The containers were incubated in the dark in a temperature-controlled room at 10 °C, the mean annual air temperature in northwest Wales (Hill et al., 2015). The soil water status during the experiment was maintained at 60% water filled pore space (WFPS) to optimize conditions for nitrification (Mosier, Duxbury, Freney, Heinemeyer, & Minami, 1996). The incubation experiment lasted 38 days. During that time, soil and gas samples were collected every 2 or 3 days during the first 2 weeks after NI application. Afterwards, sampling continued at a frequency of once or twice per week. Soils in the containers were not disturbed when soil samples were collected.

At each sampling time, soil (5 g) was extracted with 25 mL of 1 M KCl in an orbital shaker at 200 rev min^{-1} (1 h, 20°C), the extracts were centrifuged (10 min, 3,800 *g*), filtered through a Whatman No.1 filter paper, and stored at −20 °C to await analysis for NH_4^+ and NO_3^- as described above. For greenhouse gas sampling, air-tight lids fitted with a septum were attached to the incubation vessels, and syringes (20 mL) fitted with hypodermic needles were used to collect two gas samples from the headspace (0 and 60 min after the lids were closed). The increase in gas concentration in the headspace was assumed to be linear over 1 h, based on headspace gas analysis of replicated vessels filled with the same quantity of soil at the same %WFPS and temperature (see Figure S1 for details; N_2O, $R^2 = 0.936$; CO_2, $R^2 = 0.993$). Gas samples were transferred to pre-evacuated 20-mL headspace glass vials fitted with rubber butyl septa crimp caps. Gas samples were analysed by gas chromatography (GC) (Clarus 580 GC; PerkinElmer Corp., Waltham, MA, USA) equipped with an electron capture detector (ECD) for N_2O detection and a flame ionization detector (FID) for CO_2. Standards of N_2O and CO_2 were placed in vials, stored and analysed at the same time as the samples.

2.3 | Mineralization of ^{14}C-labelled LA, LN and DCD within soil

In a parallel experiment, a ^{14}C-labelling approach (Marsden et al., 2016) was used in the incubation experiment to assess the stability of LA, LN and DCD in soil; that is, their mineralization rate. ^{14}C-labelled LA, LN and DCD (American Radiolabelled Chemical Inc., St Louis, MO, USA) were added to 5 g of soil (collected as in section 2.1) contained in sealed polypropylene tubes (50 mL) using the same method described above (section 2.2), and at the same range of concentrations (LA and LN applied at 12.7, 127, 635 and 1,270 mg kg^{-1} dry soil; DCD at 12.7, 63.5 and 127 mg kg^{-1} dry soil). Soils were incubated at 10 °C in the dark for 38 days.

At the beginning of the incubation, the ^{14}C activity of substrates solution (^{14}C-labelled LA, LN and DCD) added to the soil was determined by liquid scintillation counting after mixing with HiSafe 3 scintillant (4 mL) (PerkinElmer Corp.). After adding the ^{14}C-labelled NIs to the soil, a vial containing 1 M NaOH (1 mL) was placed above the soil surface to absorb any $^{14}CO_2$ evolved (capture efficiency >95%; Boddy, Hill, Farrar, & Jones, 2007) and the tubes were sealed. The $^{14}CO_2$ traps were changed two or three times in the first 2 weeks, after which they were changed weekly. The ^{14}C activity of the NaOH solution was then determined by liquid scintillation counting after mixing with 4 mL HiSafe 3 scintillant. After 38 days, the soil (5 g) was extracted by shaking with either 25 mL ethanol or distilled water (1 h, 200 rev min^{-1}), the extracts were centrifuged (10 min, 3,850 *g*) and the ^{14}C of the supernatant was determined by liquid scintillation counting as described above.

2.4 | Data calculations

The effect of LA, LN and DCD on soil nitrification was characterized after the 38-day incubation study.

Treatment effect on soil NO_3^- concentration was estimated as Equation (1) (Subbarao et al., 2007):

$$\text{Treatment effect on } NO_3^- \text{ concentration} = \left(1 - \frac{NO_3^- - \text{N concentration in treatment}}{NO_3^- - \text{N concentration in control}}\right) \times 100\%. \quad (1)$$

Fluxes of N_2O and CO_2 were estimated from the slope of the linear regression between headspace concentrations at the two time-points, as in Equations (2) and (3) (MacKenzie, Fan, & Cadrin, 1998):

$$F_{N-N_2O} = \frac{28}{22.4} \times \frac{dc}{dt} \times \frac{V \times 60}{W}, \quad (2)$$

$$F_{C-CO_2} = \frac{12}{22.4} \times \frac{dc}{dt} \times \frac{V \times 60}{W}, \quad (3)$$

where F_{N-N_2O} is the flux of N-N_2O in μg kg^{-1} dry soil h^{-1}, F_{C-CO_2} is the flux of C-CO_2 in μg kg^{-1} dry soil h^{-1}, 28 is the molar mass of N in N_2O, 12 is the molar mass of C in CO_2, 22.4 is the molar volume of an ideal gas at standard temperature and pressure, $\frac{dc}{dt}$ is the initial rate of change in concentration with time in ppb min^{-1}, V is the volume of the headspace in m^3, W is the dry weight of soil added to the container in kg, and 60 converts minutes to hours.

Cumulative N_2O and CO_2 emissions, were calculated from estimated mean daily fluxes as Equation (4) (Li, Sørensen, Olesen, & Petersen, 2016):

$$F_{k+1} = \frac{1}{2}\sum_{1}^{k}(\triangle_i \times (f_i + f_{i+1})), \quad (4)$$

where F_{k+1} is the cumulative flux from d 1 to d (k + 1) in μg N kg^{-1} dry soil or μg C kg^{-1} dry soil, $\triangle_i$ is the time interval between the d i and d (i + 1) in h, and f_i is the mean flux on the d i in in μg kg^{-1} dry soil h^{-1}.

The mineralization rate of ^{14}C-labelled LA, LN and DCD was determined as Equation (5) (Marsden et al., 2015):

$$\text{Mineralization rate}\,(\%) = \frac{^{14}\text{C activity of NaOH solution}}{^{14}\text{C activity of substrate}} \times 100\%. \quad (5)$$

Potential soil microbial N immobilization (predicted value) was calculated indirectly. We used the % C mineralized (from the $^{14}CO_2$ measurements) of the NIs (Figure 4) to estimate the total C available to the soil microbial biomass, using the individual C contents (i.e., based on their molecular structures; LA: $C_{18}H_{32}O_2$; LN: $C_{18}H_{30}O_2$; DCD: $C_2H_4N_4$). The microbial N demand needed to assimilate the C-rich substrates was calculated, in mg N kg^{-1} dry soil (predicted value), using the standard C:N ratio of the soil microbial biomass of 8:1 (Chen, Zhu, & Zhang, 2003). Although we recognize there may be some variation in the C:N of the microbial biomass, we based the choice of this ratio (value) on the average from Xu, Thornton, and Post's (2013) global analysis of >3,000 data points from the world's major biomass. For every C molecule assimilated, two are consumed for energy through respiration; thus, 24 C molecules would be needed for every N molecule assimilated (Manzoni, Taylor, Richter, Porporato, & Ågren, 2012).

The observed amount of N immobilization was calculated indirectly from the extractable soil mineral N measurements minus cumulative N_2O loss as in Equation (6), in mg N kg^{-1} dry soil (observed value). These calculations were made on all concentrations for the LA, LN and DCD treatments at d 6, d 11, d 14 and d 35.

$$\begin{aligned}\text{N immobilized} = &\left[\left(NH_4^+ - \text{N} + NO_3^- - \text{N in control}\right)\right. \\ &\left. - \left(NH_4^+ - \text{N} + NO_3^- - \text{N in treatment}\right)\right] \\ &- \left(\left(\text{cumulative } N_2O \text{ from treatment}\right)\right. \\ &\left. - \left(\text{cumulative } N_2O \text{ from control}\right)\right). \quad (6)\end{aligned}$$

2.5 | Statistical analysis

A repeated measurement analysis of variance (RMANOVA) was used to test the effect of concentrations of NI (LA, LN or DCD) on soil NH_4^+ and NO_3^-, daily CO_2 flux and effect of treatment on soil NO_3^- concentration during the incubation period. A one-way ANOVA was applied to determine the effect of LA, LN or DCD concentrations on cumulative N_2O, CO_2 and mineralization rate after the incubation (d 38). In addition, a linear regression analysis was undertaken to relate the predicted microbial N immobilization (predicted value, section 2.4) and observed N immobilization (observed value, section 2.4) as a result of added available C in the LA and LN treatments. A linear regression analysis was conducted to relate the cumulative N_2O and CO_2 in the LA and LN treatments, respectively. All statistical analyses were performed in SPSS Statistics 25.0 (IBM Inc., Armonk, NY, USA).

3 | RESULTS

3.1 | Ammonium

During the monitoring period, NH_4^+ concentration varied significantly ($p_{time} < 0.001$, Table 2) with incubation

TABLE 2 Repeated measurement analysis of variance (RMANOVA) on soil NH_4^+ and NO_3^- concentrations, treatment effect on soil NO_3^- concentration and daily CO_2 fluxes in the linoleic acid (LA), linolenic acid (LN) and dicyandiamide (DCD) treatments

Source	NI		Time		NI × Time	
	df	*F*	*df*	*F*	*df*	*F*
	LA					
NH_4^+	4	0.4	7	113.9***	28	1.8*
NO_3^-	4	423.1***	7	25.5***	28	4.3***
Treatment effect on NO_3^-	3	2,772.1***	7	3.8**	21	1.7
Daily CO_2 flux	4	166.3***	8	50.8***	32	10.5***
	LN					
NH_4^+	4	1.1	7	115.1***	28	3.2**
NO_3^-	4	52.0***	7	36.6***	28	2.6**
Treatment effect on NO_3^-	3	67.1**	7	6.7***	21	2.2*
Daily CO_2 flux	4	148.4***	8	62.2***	32	11.9***
	DCD					
NH_4^+	3	87.3***	7	33.7***	21	4.2***
NO_3^-	3	49.0**	7	26.5***	21	4.4***
Treatment effect on NO_3^-	2	82.0**	7	9.1***	14	4.7**
Daily CO_2 flux	3	9.2**	8	23.6***	24	4.5***

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.
Abbreviations: *df*, degree of freedom.

time and showed a similar trend in the LA, LN and DCD treatments (Figure 1a–c). The soil NH_4^+ concentration increased during the first 8 days, then decreased over the following 27 days, with a small additional increase at d 27 in the LA, LN and DCD treatments. During the incubation period, there were no significant effects of LA ($p = 0.804$) or LN ($p = 0.431$) on soil NH_4^+ concentration. The NH_4^+ concentrations in the DCD 10, DCD 50 and DCD 100 treatments remained significantly higher than that in the control (without NI), reaching 4.7 mg N kg^{-1} dry soil, 12.4 mg N kg^{-1} dry soil and 15.8 mg N kg^{-1} dry soil after incubation (in the control, 0.8 mg N kg^{-1} dry soil). Throughout the monitoring period, DCD significantly affected soil NH_4^+ concentrations ($p < 0.001$), with soil NH_4^+ concentrations increased as the concentration of DCD increased at almost all sampling days (with the exception of d 6 and d 11).

3.2 | Nitrate

Soil NO_3^- concentrations increased slowly during the experimental period, and varied significantly ($p_{time} < 0.001$, Table 2) with the incubation time in the LA, LN and DCD treatments (Figure 1d–f). Compared with the control, the addition of LA ($p < 0.001$), LN ($p < 0.001$) and DCD ($p < 0.01$) significantly decreased soil NO_3^- concentrations. There was almost no effect of the LA 10 treatment on soil NO_3^- concentration (averaging a reduction of 0.6%; Figure 1g). During the monitoring period, the LA 100, LA 500 and LA 1,000 treatments resulted in average reductions in soil NO_3^- concentrations of 16.5%, 63.2% and 93.5%, respectively. The concentration of LN required to reduce soil NO_3^- concentration was substantially higher than that for LA (Figure 1h), with the LN 100, LN 500 and LN 1,000 treatments resulting in average reductions in soil NO_3^- concentrations of 11.5%, 36.8% and 50.8%. For DCD, the effect on soil NO_3^- concentration significantly increased as DCD concentration increased ($p < 0.05$–0.01, Figure 1i), with soil NO_3^- concentration reductions of 15.0%, 31.1% and 39.6% for the DCD 10, DCD 50 and DCD 100 treatments, respectively.

3.3 | N_2O emissions

Generally, cumulative N_2O emissions in the LA and LN treatments increased as the concentrations increased (Figure 2a,b). In the LA 500 and LA 1,000 treatments, the cumulative N_2O emissions were significantly higher than those in the control, LA 10 and LA

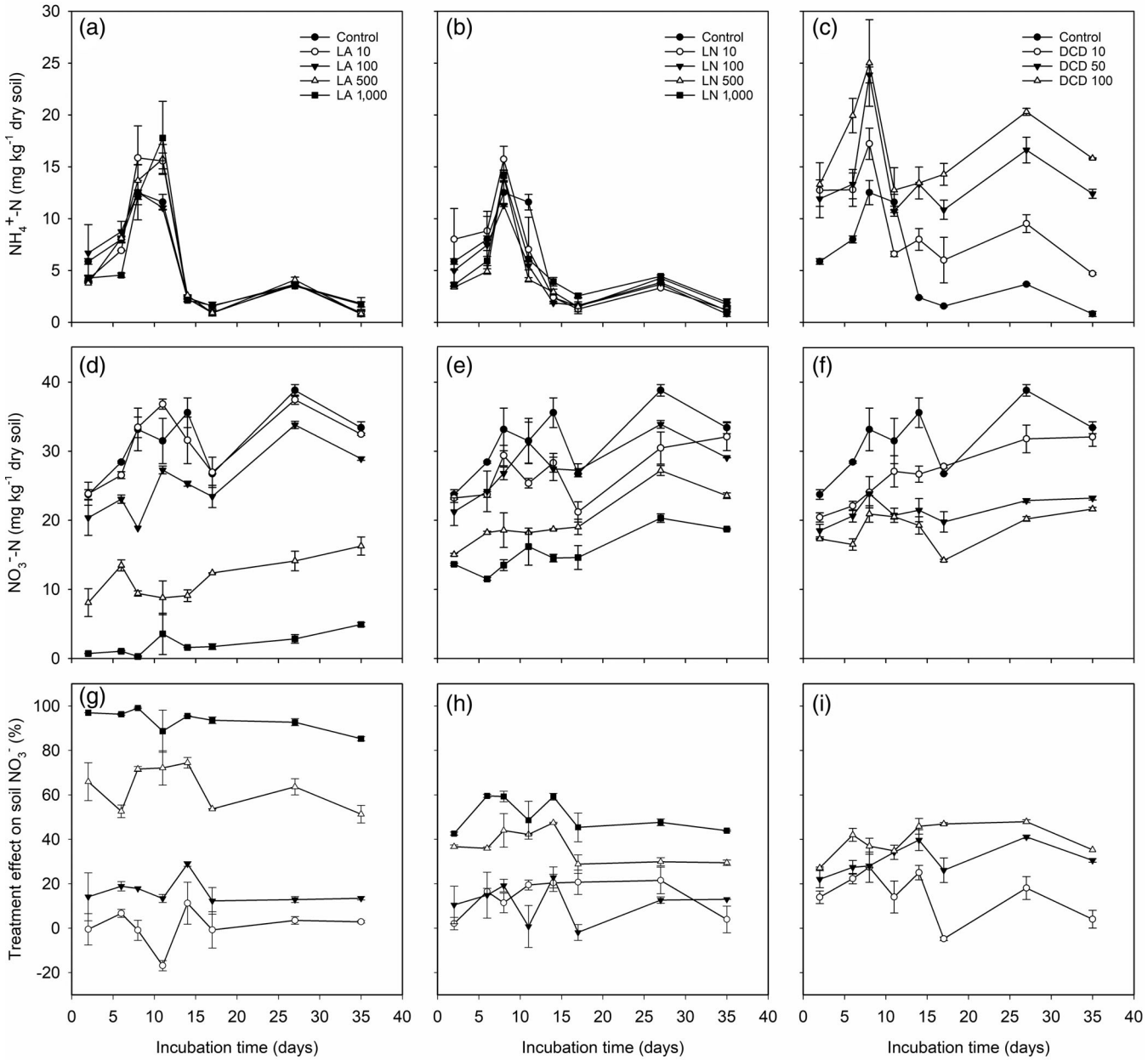

FIGURE 1 Effect of different concentrations of linoleic acid (LA, panels (a), (d), (g)), linolenic acid (LN, panels (b), (e), (h)) and dicyandiamide (DCD) (panels (c), (f), (i)) on soil NH_4^+, NO_3^- concentrations and treatment effect on soil NO_3^- concentration during a 38-day incubation at 10°C. Error bars represent standard error of the mean ($n = 4$). Note: the same control treatment is common to all panels

100 treatments ($p < 0.01$–0.001), and no significant differences ($p > 0.05$) were observed between the control, LA 10 and LA 100 treatments. Similar effects were also observed in the LN treatments. After the 38-day incubation, the cumulative N_2O emissions in the LA 500 treatment and LA 1,000 treatment were 201 μg N kg^{-1} dry soil and 271 μg N kg^{-1} dry soil, respectively, whereas the cumulative N_2O emissions in the LN 500 and LN 1,000 treatments were 138 μg N kg^{-1} dry soil and 156 μg N kg^{-1} dry soil. During the monitoring period, there was no significant effect ($p > 0.05$) of the concentration of DCD on soil cumulative N_2O emission (Figure 2c). After 38 days of incubation, the cumulative N_2O emissions were 58.1 μg N kg^{-1} dry soil, 87.9 μg N kg^{-1} dry soil, 95.0 μg N kg^{-1} dry soil and 64.7 μg N kg^{-1} dry soil in the control, DCD 10, DCD 50 and DCD 100 treatments, respectively.

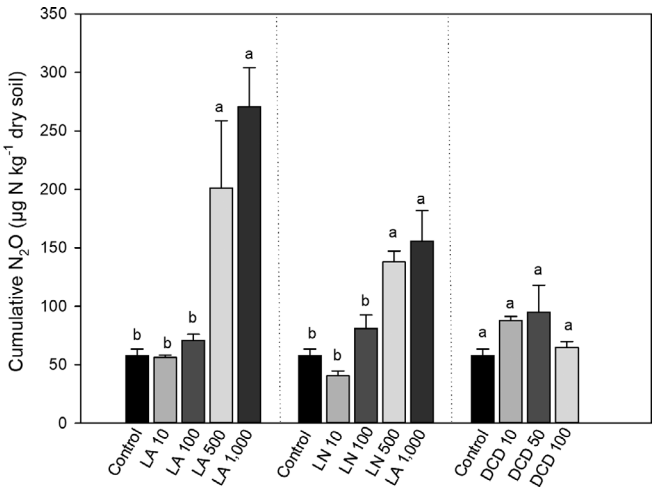

FIGURE 2 Effect of different concentrations of linoleic acid (LA), linolenic acid (LN) and dicyandiamide (DCD) on cumulative N_2O emissions during a 38-day incubation at 10 °C. Error bars represent standard error of the mean ($n = 4$). Different letters indicate significant differences between treatments at $p < 0.05$ by Least Significant Difference (LSD) test

3.4 | CO_2 emissions

As shown in Figure 3a–c, the daily CO_2 emissions varied significantly ($p_{time} < 0.001$, Table 2) with incubation time. In the LA, LN and DCD treatments, daily CO_2 emissions increased rapidly from d 1 to d 4, and then decreased gradually. At d 4, the peak CO_2 emissions in the LA 500 and LA 1,000 treatments were 1.1 mg C kg^{-1} dry soil h^{-1} and 1.6 mg C kg^{-1} dry soil h^{-1}, and were 1.4 mg C kg^{-1} dry soil h^{-1} and 2.1 mg C kg^{-1} dry soil h^{-1} in the LN 500 and LN 1,000 treatments, respectively. However, in the control, the CO_2 emissions declined rapidly from d 1 to d 6, and then decreased gradually during the remainder of the 38-day incubation period. During the incubation period, daily CO_2 emissions were significantly affected by the application of LA, LN and DCD ($p < 0.01$–0.001).

In the LA 10 treatment, the cumulative CO_2 emissions were significantly ($p < 0.01$) lower, with a reduction rate of 27.7% compared to the control. No significant ($p > 0.05$) effects of LN addition at lower concentrations (control, LN 10 and LN 100) on cumulative CO_2 emissions were observed. LA and LN applied at 635 and 1,270 mg kg^{-1} dry soil significantly ($p < 0.001$) increased the cumulative CO_2 emissions, with an increase of 86.5% and 176% in the LA treatments, and 68.5% and 189% in the LN treatments, respectively. There were no significant differences between the control and DCD 10 treatment ($p = 0.185$), and between the control and DCD 100 treatment ($p = 0.283$). In the DCD 50 treatment, the cumulative CO_2 emission was significantly lower ($p < 0.01$), with a reduction of 26.8%.

3.5 | Microbial mineralization of ^{14}C-labelled LA, LN and DCD

During the incubation period, the overall patterns of LA (Figure 4a) and LN (Figure 4b) mineralization were similar. The mineralization of LA and that of LN were initially rapid (d 1 to d 6) and became progressively slower over the 38-day incubation period. After the 38-day incubation period, the total mineralization rate averaged 52.6%, ranging from 46.9% to 55.7% in the LA treatments, and averaged 50.7%, ranging from 36.6 to 60.7%, in the LN treatments. In comparison with LA and LN, the mineralization rate of DCD was much lower (Figure 4c), with a total mineralization rate of 5.5, 2.9 and 2.7% in the DCD 10, DCD 50 and DCD 100 treatments after the 38 days of incubation.

During the monitoring period, cumulative CO_2 emissions above those of the control treatment (cumulative CO_2 emissions in the LA/LN treatments minus those in the control, y in mg C kg^{-1} dry soil) were significantly related with the amount of $^{14}CO_2$ (x in mg C kg^{-1} dry soil) ($p < 0.001$), as measured using the ^{14}C-labelled LA and LN. The relationship for LA was $y = 0.62x\text{-}27.85$ ($R^2 = 0.982$) and for LN was $y = 0.58x\text{-}14.44$ ($R^2 = 0.982$). The apparent linear relationship suggests that the additional CO_2 emissions in the LA/LN 500 and LA/LN 1,000 treatments were mainly associated with the mineralization of added LA and LN.

At the end of the 38 days of incubation, the amount of ^{14}C-labelled BNIs and DCD remaining in the soil were quantified by extraction in water or ethanol (Table 3). In the water-based extraction, only 2.1–2.6% of ^{14}C-labelled LA and 2.7–2.8% of the ^{14}C-labelled LN remained, compared with 20.6–25.3% of the ^{14}C-labelled DCD. In the LA and LN treatments, the quantities detected from the ethanol extraction were greater than those from water extractions, namely, 3.9–5.2% ^{14}C-labelled LA and 4.2–5.5% ^{14}C-labelled LN, with only 3.3–6.8% of the ^{14}C-labelled DCD being detected in the ethanol extractions. In the LA, LN and DCD treatments, 37.2–45.4%, 30.9–55.9% and 64.5–73.2% of the ^{14}C-labelled substrates were not recovered in the water and ethanol extractions, indicating immobilization of the remaining ^{14}C by the soil biomass or the formation of organo-mineral complexes. As there is no satisfactory technique (e.g., chloroform-fumigation extraction) for assessing the quantity of isotope contained in the microbial biomass (Glanville, Hill, Schnepf, Oburger, & Jones, 2016), this could not be verified.

3.6 | Soil microbial N immobilization

There was a strong linear relationship between the predicted value (potential soil microbial N

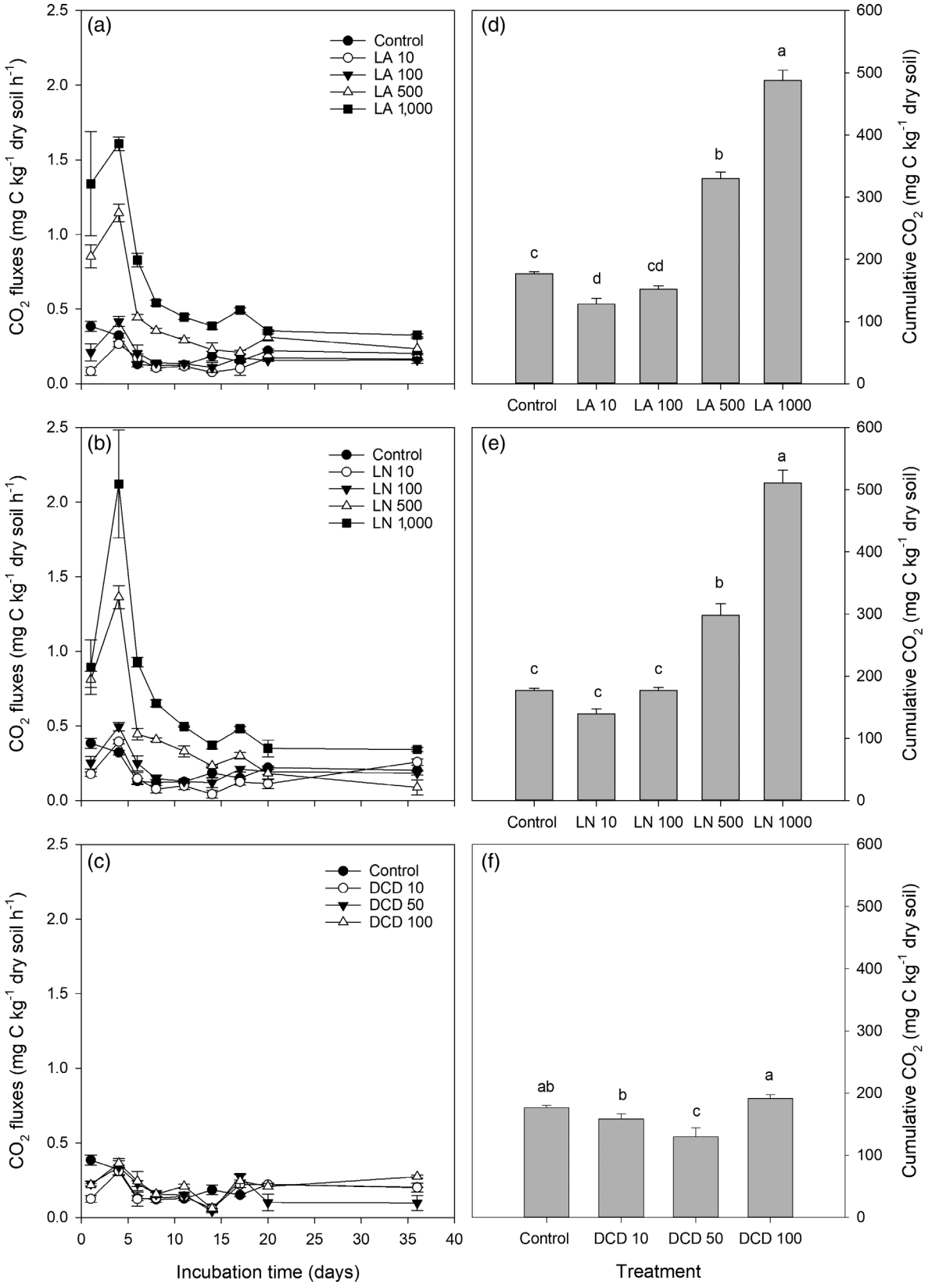

FIGURE 3 Effect of different concentrations of linoleic acid (LA, panels (a), (d)), linolenic acid (LN, panels (b), (e)) and dicyandiamide (DCD) (panels (c), (f)) on CO_2 fluxes and cumulative CO_2 emissions during a 38-day incubation at 10 °C. Error bars represent standard error of the mean ($n = 4$). Different letters indicate significant differences between treatments at $p < 0.05$ by LSD test

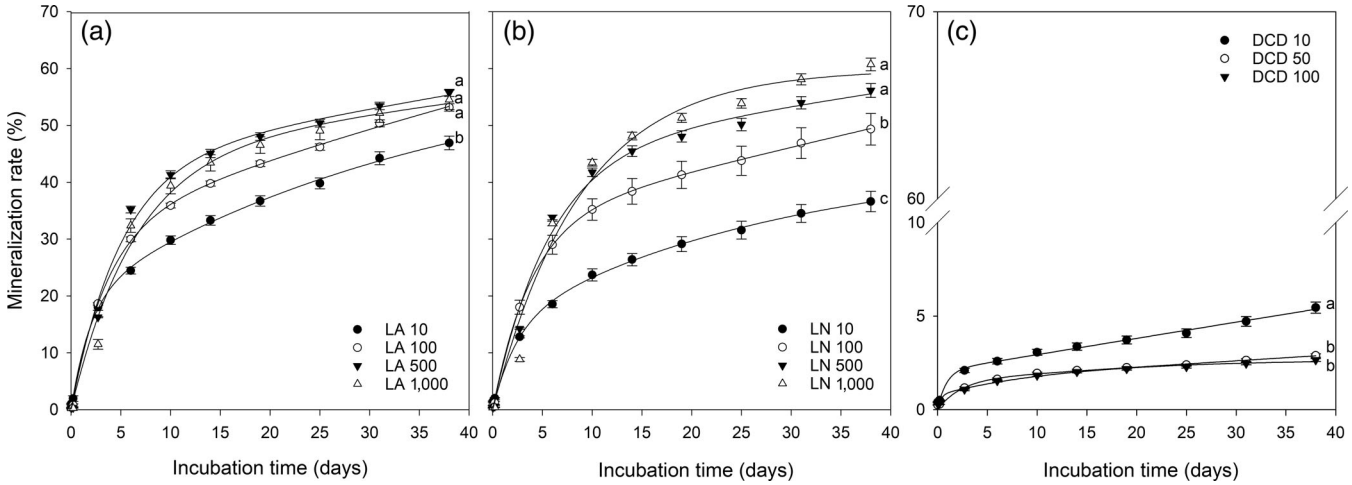

FIGURE 4 Effect of nitrification inhibitor concentrations on mineralization rate of ^{14}C-labelled linoleic acid (LA, panel (a)), linolenic acid (LN, panel (b)) and dicyandiamide (DCD) (panel (c)) in a sandy clay loam soil during a 38-day incubation at 10°C. Error bars represent standard error of the mean ($n = 4$). Different letters indicate significant differences between treatments at $p < 0.05$ by LSD test

TABLE 3 ^{14}C-labelled linoleic acid (LA), linolenic acid (LN) and dicyandiamide (DCD) extracted from soil at the end of the 38-day incubation period

NI	^{14}C-compound in water (%)	^{14}C-compound in ethanol (%)
LA		
LA 10	2.6 ± 0.4 c	5.1 ± 0.8 ab
LA 100	2.1 ± 0.3 c	4.4 ± 1.2 bc
LA 500	2.6 ± 0.7 c	3.9 ± 1.0 bc
LA 1,000	3.1 ± 0.2 c	5.2 ± 0.6 ab
LN		
LN 10	2.8 ± 0.2 c	4.7 ± 0.5 abc
LN 100	2.8 ± 0.3 c	5.5 ± 0.4 ab
LN 500	2.7 ± 0.1 c	4.2 ± 0.5 bc
LN 1,000	3.2 ± 0.4 c	5.2 ± 0.3 ab
DCD		
DCD 10	23.2 ± 2.9 ab	6.8 ± 0.4 a
DCD 50	20.6 ± 2.5 b	3.3 ± 0.6 bc
DCD 100	25.2 ± 2.4 a	5.0 ± 0.2 abc

Note: Different letters indicate significant differences between treatments for each extractant at $p < 0.05$ by Least Significant Difference (LSD). Values represent means ± standard error of mean ($n = 4$).

immobilization as a result of the added available C in the LA and LN) and observed value (the observed amount of N immobilization) for the LA (Figure 5a, $p < 0.001$) and LN treatments (Figure 5b, $p < 0.01$). This linear relationship between predicted and observed immobilization values indicates that LA and LN application results in microbial N immobilization of NH_4^+ and/or NO_3^-. This effect was not observed for DCD addition in this study (Figure 5c, $p > 0.05$).

4 | DISCUSSION

4.1 | Effects of nitrification inhibitors on soil NH_4^+ and NO_3^- concentrations

Nitrification inhibitors are capable of delaying the oxidization of NH_4^+ into NO_3^- effectively, to mitigate the negative impact of nitrate on the environment (Guo et al., 2013; Subbarao et al., 2008). Previous studies, where an additional source of NH_4^+ has been applied, have indicated that LA and LN show direct nitrification inhibition due to blocking the AMO and HAO enzymatic pathways, which play a critical role in the oxidation of NH_4^+ to NO_2^- in *Nitrosomonas* (Subbarao et al., 2008). In this study, with no added NH_4^+ source, and where soil NH_4^+ and NO_3^- concentrations were < 6 mg kg^{-1} and < 24 mg kg^{-1}, respectively, we observed that the addition of LA and LN decreased soil NO_3^- concentration significantly, but did not have an appreciable effect on the residual NH_4^+ concentration in soil (Figure 1). In contrast, the addition of DCD resulted in high soil NH_4^+ and low NO_3^- concentrations, corroborating the direct effect of this NI on NO_3^- formation, as seen in other studies (Chaves et al., 2006; McGeough et al., 2016).

If the inhibition of soil nitrification occurred in the LA and LN treatments during the incubation, the soil would retain relatively higher NH_4^+ and lower NO_3^- concentration compared to the control, as in the DCD treatments or the study in Subbarao et al. (2008). The NO_3^- concentration decreased significantly as expected,

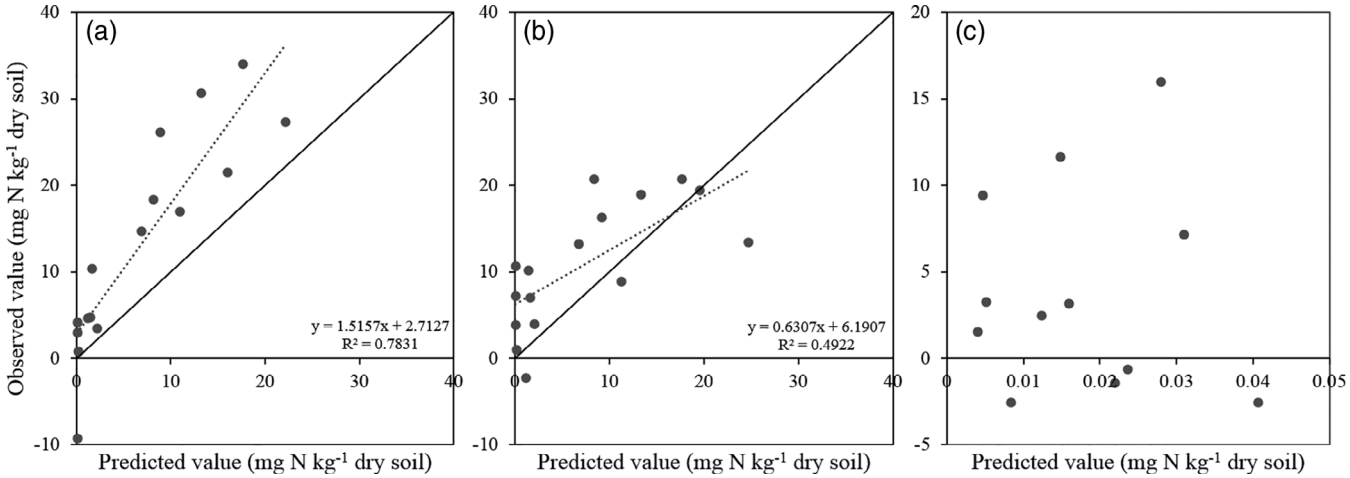

FIGURE 5 Relationship between predicted and observed N immobilization in the linoleic acid (LA, panel (a)), linolenic acid (LN, panel (b)) and dicyandiamide (DCD) (panel (c)) treatments

but the NH_4^+ concentration did not increase correspondingly in this study. A decline in NH_4^+ supply rather than toxicity of specific compounds to nitrifiers has at times explained low nitrification rates (Schimel, Van Cleve, Cates, Clausen, & Reichardt, 1996), and heterotrophic NO_3^- immobilization could occur when NH_4^+ concentrations are low (Rice & Tiedje, 1989). Thus, we hypothesize that the apparent inhibition of nitrification (i.e., reduction in soil NO_3^- concentration) observed when LA and LN are added to a highly nitrifying soil (with no NH_4^+ amendment) could be the result of microbial immobilization of NH_4^+ and/or NO_3^- (i.e., an indirect effect), in contrast to the direct inhibition proven for NIs such as DCD (Guo et al., 2013; Subbarao et al., 2008).

The linear relationship between the predicted microbial N immobilization (predicted value) using the ^{14}C-labelling method and observed N immobilization (observed value) (Figure 5) provided evidence for the immobilization effect of LA and LN. It is supported by the study by Li et al. (2020), in which fungal and bacterial NO_3^- immobilization activities were enhanced by *Paspalum notatum* residue input. Vázquez et al. (2020) also suggest that a combination of different mechanisms, particularly stimulation of N immobilization, may be responsible for the BNI capacity observed as low NO_3^- soil content and reduced N losses. Numerous studies have shown that the addition of labile C-rich substrates to soil can increase net N immobilization, and is an indicator of immediate microbial response to the C substrate (Chen et al., 2003; Magill & Aber, 2000; Vinten, Whitmore, Bloem, Howard, & Wright, 2002). The addition of organic C stimulates the growth of soil microorganisms until they become limited by N availability (Garten & Wullschleger, 2000; Martin & Johnson, 1995). Compared with DCD, the relatively rapid and high mineralization of LA and LN indicates that the addition of LA and LN represents a C source that is available to the soil microorganisms (Figure 4), and the linear relationship between the $^{14}CO_2$ and CO_2-C indicated that the mineralization of LA and LN was related to the CO_2 emissions from this source.

4.2 | Effects of nitrification inhibitors on soil N_2O emissions

In previous studies, researchers have focused on the effect of LA and LN on soil N transformations (Lu et al., 2019; Subbarao et al., 2008). In this study, we report for the first time the effect of LA and LN on N_2O emissions. Our results demonstrated that cumulative N_2O emissions were significantly greater in the higher-concentration BNI treatments. Both nitrification and denitrification processes are responsible for the N_2O emissions (Gardiner et al., 2016; Hofstra & Bouwman, 2005; Smith et al., 1997). These high N_2O emissions coupled with the lower soil NO_3^- concentrations in the 635 and 1,270 mg BNI kg^{-1} dry soil treatments suggest that denitrification, stimulated by the large amount of available C added in the LA and LN, may be another soil process responsible for the apparent inhibition of nitrification observed. The significant linear relationship in the LA ($p < 0.001$, $R^2 = 0.635$) and LN ($p < 0.001$, $R^2 = 0.793$) treatments between the cumulative N_2O and CO_2 may give support to the stimulated N_2O emissions via denitrification by the increased C availability. Dlamini et al. (2020) confirmed that slurry application resulted in the promotion of denitrification and this depends on the availability of the C compounds it contains.

In this study, DCD did not have a significant effect on the N_2O emissions, which is inconsistent with the fact that DCD can reduce direct soil N_2O emissions by 26%–91% (Cameron & Di, 2002; Cameron, Di, & Moir, 2014; Kelliher, Clough, Clark, Rys, & Sedcole, 2008; Smith, Klein, Monaghan, & Catto, 2008; Weiske et al., 2001; Zaman, Saggar, Blennerhassett, & Singh, 2009). This could be because total N_2O emissions were relatively low. In this study, the effects of BNIs and DCD on "residual" NH_4^+, on soil NH_4^+ and NO_3^- and N_2O and CO_2 emissions were explored, but we did not apply NH_4^+ fertiliser. A meta-analysis from Yang, Fang, Sun, and Shi (2016) supported that the efficiency of NIs positively varies with N fertiliser application rates, with higher N fertiliser rates often causing high N losses (Yang et al., 2016). This is also supported by the study by Li et al. (2018), in which the greater reduction in N_2O loss by NIs was observed with the higher baseline of N_2O emission (>20 kg N_2O-N ha^{-1}).

4.3 | Mineralization of nitrification inhibitors

To our knowledge, the factors that influence the efficacy of these specific BNIs have not been quantified. This is the first study to explore the degradation rates of LA and LN in soil directly using ^{14}C-labelled compounds. The mineralization rates of LA and LN observed in this study provide a reference for future research studies. The relatively low mineralization rates of DCD are consistent with other studies (e.g., Marsden et al., 2015; Singh, Saggar, Giltrap, & Bolan, 2008). DCD degrades to CO_2 and NH_4^+ via guanylic urea, guanidine and urea (Kelliher et al., 2008; Marsden et al., 2016). The half-life of DCD is strongly affected by soil temperature (Kelliher et al., 2008, 2014; McGeough et al., 2016; Singh et al., 2008). Researchers have quantified the relationship between temperature (T) and the time (t) taken for DCD concentration in soil to decline to half its application value ($t_{½}$) as $t_{½}$ (T) = 168 $e^{-0.084T}$ (Kelliher et al., 2008). In this study, the soil was incubated at a relatively low temperature (10 °C), which may explain the low mineralization rate of DCD.

4.4 | Direct and indirect inhibition of nitrification

The linear relationship between the predicted value and observed value based on the ^{14}C-labelling method provided direct evidence that LA and LN application to soil significantly increased soil microbial N immobilization and decreased NO_3^- concentration. Further research using ^{15}N-labelling techniques, and quantification of effects of BNI on the nitrifier population, for example using N cycling gene abundance (Lu et al., 2019), are needed to test this hypothesis directly and explore if reported nitrification inhibition by BNIs could actually be the result of an indirect effect due to microbial immobilization of N, stimulated by the addition of available C in LA and LN. However, low NO_3^- concentrations may also be the result of increased N_2O emissions, presumably via denitrification, following the supply of sufficient available C in the two highest additions of the BNIs, which was not verified in this study but could be explored in a future study using C_2H_2 inhibition (Mosier, Guenzi, & Schweizer, 1986), ^{15}N-labelling (Beline, Martinez, Marol, & Guiraud, 2001) or the direct measurements of N_2 and N_2O using a He/O_2 incubation system (Cárdenas, Hawkins, Chadwick, & Scholefield, 2003).

Because the apparent BNI effect (microbial immobilization and/or denitrification) was different between the 127 and 635 mg kg^{-1} BNI treatments, we suggest that further research is needed to explore the appropriate application rates of LA and LN needed to inhibit soil nitrification/increase N immobilization and decrease greenhouse gas emissions at the same time. In this study, LA and LN were added on an equivalent mass basis, and not an equivalent C loading basis, and DCD was included as a reference of a synthetic NI with a proven effect on nitrification inhibition (Monaghan et al., 2013; Wang et al., 2017), so was not applied on an equivalent C loading basis either. In future studies, we recommend that researchers investigating the effects of BNIs on nitrification rates include treatments that compare BNIs on an equivalent C loading basis, and perhaps include glucose and DCD reference treatments to help distinguish between real and apparent inhibition of nitrification. In addition, this study focused on soil N_2O and CO_2 emissions, but did not include NH_3 emissions. However, previous studies using NIs have retained higher soil NH_4^+ concentrations, thus increasing NH_3 emissions (Lam, Suter, Mosier, & Chen, 2017; Sánchez-Rodríguez et al., 2018; Soares, Cantarella, & de Campos Menegale, 2012). Attention should also be paid to NH_3 emissions when biological NIs are applied in future studies.

5 | CONCLUSIONS

Our results confirmed that the addition of LA, LN and DCD can decrease soil NO_3^- concentration, but their modes of action may be different. Our results suggest that

the apparent effect of LA and LN on soil NO_3^- concentration could be indirect under low-N conditions (no addition of fertiliser NH_4^+) due to the addition of sufficient labile C in the BNIs stimulating microbial immobilization of soil NH_4^+ and/or NO_3^-. We also demonstrated that LA and LN were much more rapidly mineralized than DCD in soil. Overall, we suggest that researchers exploring the effectiveness of BNIs consider whether any observed effects on NO_3^- concentration are the result of a direct or indirect effect, as this has implications for developing effective mitigation strategies for N_2O emission and NO_3^- leaching, and is something that has been overlooked.

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AUTHOR CONTRIBUTIONS

Study design: Yan Ma, Davey L. Jones and David R. Chadwick. Literature research: Yan Ma and David R. Chadwick. Experimental studies: Yan Ma.

Data acquisition: Yan Ma. Data analysis/interpretation: Yan Ma, Davey L. Jones, Jinyang Wang and David R. Chadwick. Statistical analysis: Yan Ma and Jinyang Wang.

Drafting the manuscript: Yan Ma. Revising the manuscript critically for important intellectual content: Yan Ma, Davey L. Jones, Laura M. Cardenas and David R. Chadwick.

CONFLICT OF INTERESTS

We declare that the authors have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

DATA AVAILABILITY STATEMENT

The data presented in this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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