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Influence of nitrate and nitrite concentration on N_2O production via dissimilatory nitrate/nitrite reduction to ammonium in *Bacillus paralicheniformis* LMG 6934

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Abstract

Until now, the exact mechanisms for N_2O production in dissimilatory nitrate/nitrite reduction to ammonium (DNRA) remain underexplored. Previously, we investigated this mechanism in *Bacillus licheniformis* and *Bacillus paralicheniformis*, ubiquitous gram-positive bacteria with many industrial applications, and observed significant strain dependency and media dependency in N_2O production which was thought to correlate with high residual NO_2^-. Here, we further studied the influence of several physicochemical factors on NO_3^- (or NO_2^-) partitioning and N_2O production in DNRA to shed light on the possible mechanisms of N_2O production. The effects of NO_3^- concentrations under variable or fixed C/N-NO_3^- ratios, NO_2^- concentrations under variable or fixed C/N-NO_2^- ratios, and NH_4^+ concentrations under fixed C/N-NO_3^- ratios were tested during anaerobic incubation of soil bacterium *B. paralicheniformis* LMG 6934 (previously known as *B. licheniformis*), a strain with a high nitrite reduction capacity. Monitoring of growth, NO_3^-, NO_2^-, NH_4^+ concentration, and N_2O production in physiological tests revealed that NO_3^- as well as NO_2^- concentration showed a linear correlation with N_2O production. Increased NO_3^- concentration under fixed C/N-NO_3^- ratios, NO_2^- concentration, and NH_4^+ concentration had a significant positive effect on NO_3^- (or NO_2^-) partitioning ($[N–NH_4^+]/[N–N_2O]$) toward N_2O, which may be a consequence of the (transient) accumulation and subsequent detoxification of NO_2^-. These findings extend the information on several physiological parameters affecting DNRA and provide a basis for further study on N_2O production during this process.

KEYWORDS
ammonification, dissimilatory nitrate/nitrite reduction to ammonium, nitrate respiration, nitrogen assimilation

1 | INTRODUCTION

Nowadays, there is an increasing concern about the year-by-year rising emissions of N_2O from soil, as it is a potent greenhouse gas that damages the ozone layer (Daniel et al., 2007; Solomon et al., 2007; Wuebbles, 2009). Denitrification has been considered as the dominant NO_3^- reducing process in soil, in which NO_3^- is sequentially converted to NO_2^-, NO, N_2O, and N_2. However, recently, field

surveys (Bu et al., 2017; Silver, Herman, & Firestone, 2001; Silver, Thompson, Reich, Ewel, & Firestone, 2005; Song, Lisa, & Tobias, 2014; Yin et al., 2017) and research with pure cultures (Bleakley & Tiedje, 1982; Mania, Heylen, Spanning, & Frostegård, 2014; Smith & Zimmerman, 1981; Stremińska, Felgate, Rowley, Richardson, & Baggs, 2012; Sun, De Vos, & Heylen, 2016) have suggested that NO_3^--ammonifying bacteria could be a significant source of N_2O. Ammonification or dissimilatory NO_3^- reduction to NH_4^+ (DNRA) is the reduction in NO_3^- to NH_4^+, via NO_2^- (Cole, 1996; Simon, 2002), with the concomitant production of nonstoichiometric amounts of N_2O amounting to around 3%–36% of consumed NO_3^- (Bleakley & Tiedje, 1982). DNRA can follow different scenarios, with respiratory membrane-bound NarG, cytoplasmic NasBC, or periplasmic NO_3^- reductase NapA for NO_3^- reduction to NO_2^-, followed by NO_2^- reduction to NH_4^+ via cytoplasmic nitrite reductase NirB or a periplasmic nitrite reductase NrfA (Bothe, Ferguson, & Newton, 2006), with NirB induced under high NO_3^- concentration and NrfA induced by low NO_3^- concentration (Wang & Gunsalus, 2000). The exact mechanisms for N_2O production remain underexplored. They may differ between ammonifiers and most likely depend on the enzymes involved in the DNRA process. In *Escherichia coli* K-12, NO was shown to be produced by NrfA under the regulation of Fnr and mutants lacking Hmp, NarG or Fnr did not produce NO (Corker & Poole, 2003). In *Salmonella enterica* serovar *Typhimurium*, NarGHI was responsible for NO generation from NO_2^- (Gilberthorpe & Poole, 2008). The produced NO in these two bacteria will be reduced to N_2O by flavohemoglobin Hmp and the di-iron-centered flavorubredoxin NorV with its NADH-dependent oxidoreductase NorW. Hmp is phylogenetically widespread in both denitrifying bacteria and nondenitrifiers. It can oxidize NO to NO_3^- in the presence of oxygen and reduce NO to N_2O under anoxic conditions (Kim, Orii, Lloyd, Hughes, & Poole, 1999). However, not Hmp but NorVW (Gomes et al., 2002) may be the significant source of N_2O, which can detoxify NO under micro-oxic or anaerobic conditions (Torres et al., 2016). Besides, canonical NO reductase—Nor, which mostly exists in denitrifiers, was also found in certain DNRA bacteria. For instance, *Bacillus vireti* LMG 21834^T performs DNRA by NarG, NrfA, and Nor (CbaA), with additional NosZ partially reducing N_2O to N_2 (Mania, Heylen, Spanning, & Frostegård, 2016; Mania et al., 2014). Similarly, *Bacillus paralicheniform*is LMG 6934, LMG7559 (renamed since 2015 (Dunlap, Kwon, Rooney, & Kim, 2015)), and *Bacillus licheniform*is LMG17339 possess NarG, NirBD, and Nor, but not NosZ (Sun et al., 2016). While, the mutants of *Salmonella typhimurium Typhimurium* lacking Hmp, NorV, and NrfA and of *E.coli* lacking NirB, NrfA, NorV, and Hmp still can reduce NO, suggesting that there are other mechanisms of NO reduction uncharacterized (Mills, Rowley, Spiro, Hinton, & Richardson, 2008).

As denitrification and DNRA are the two well-known NO_3^--consuming pathways in soil, with the former contributing to nitrogen loss to the atmosphere and the latter mainly leading to nitrogen retention in soil, studies with respect to different factors influencing these two pathways have been widely performed. It is well known that DNRA is favored over denitrification at higher C/N-NO_3^- ratios or NO_3^- limitation (Van den Berg, Van Dongen, Abbas, & Van Loosdrecht, 2015; Yoon, Cruz-Garcia, Sanford, Ritalahti, & Löffler, 2015), higher pH (Schmidt, Richardson, & Baggs, 2011; Yoon, Cruz-Garcia, et al., 2015), higher temperature (Ogilvie, Rutter, & Nedwell, 1997; Yoon, Sanford, & Loeffler, 2015), and certain NO_2^- to NO_3^- ratios (Schmidt et al., 2011; Yoon, Sanford, et al., 2015). However, the influence of these environmental drivers on NO_3^- partitioning to NH_4^+ and N_2O in DNRA remains underexplored, although increased understanding might help unravel the underlying mechanisms and regulation of N_2O production. Early work by Smith showed that higher C/NO_3^- ratios under constant or decreasing NO_3^- concentration (Smith, 1981) favored NO_3^- partitioning to N_2O in *Citrobacter* sp. with glucose as energy source and suggested that N_2O production was induced by (transient) accumulation of NO_2^-. However, recently, it was found, both in batch and continuous cultures of *Citrobacter* sp. and *Bacillus* sp., that low C/N-NO_3^- (C limitation, N sufficiency) ratios resulted in higher NO_2^- accumulation accompanied by higher N_2O production compared to high C/N-NO_3^- with constant initial glycerol concentration as carbon source and variable NO_3^- concentration (Stremińska et al., 2012).

It has been generally known that NH_4^+ inhibits assimilatory NO_3^- reduction (general N control) (Schreier, Brown, Hirschi, Nomellini, & Sonenshein, 1989; Stouthamer, 1976), increases growth rate of cells (Sun, De Vos, & Willems, 2017), and does not repress dissimilatory NO_3^- reduction (Konohana, Murakami, Nanmori, Aoki, & Shinke, 1993). In *B. licheniformis*, NO_3^- reductase activity increased with rising initial concentrations of NH_4^+, but with an upper limit of 46 mmol/L, suggesting that the activity is not for NO_3^- assimilation but for other physiological functions containing a dissimilatory NO_3^- reduction (Konohana et al., 1993). However, no previous work has been performed on the influence of NH_4^+ on N_2O production in DNRA. As NH_4^+ can react with multiple nitrogen regulation sensors (TnrA, CodY, and GlnR) and the mechanism of N_2O production and regulation of nitrogen metabolism are underexplored in DNRA strains, it is possible that NH_4^+ can influence NO_3^- partitioning to N_2O.

B. (para)licheniformis is a spore-forming gram-positive bacterium that can be isolated from soils and plant material all over the world but was never reported to be pathogenic for either animals or plants (Sneath, Mair, Sharpe, & Holt, 1986). In our previous study, we investigated three strains of *B. (para)licheniform*is (as mentioned above) which were disguised as denitrifiers and proved that they are N_2O emitters performing DNRA probably by expression of *narG*, *nirB*, *qNor*, and *hmp*, with up to one-third of all NO_3^- converted to N_2O (Sun et al., 2016). They are therefore suitable model organisms to study the mechanism of N_2O production during DNRA and to supplement the insights of environmental drivers influencing DNRA. Following our observation of N_2O production being correlated to high residual NO_2^-, here we used the soil bacterium *B. paralicheniformis* LMG 6934, selected for its high nitrite tolerance and efficient nitrite reduction ability, to study in detail the influence of NO_3^-, NO_2^-, and NH_4^+ concentrations on N_2O production via DNRA in batch cultures.

2 | MATERIALS AND METHODS

2.1 | Strains

Bacillus paralicheniformis LMG 6934 was obtained from the BCCM/LMG bacteria collection. It was grown aerobically at 37°C on TSA for 2 days, followed by two subcultivations on TSA before use in growth experiments in mineral media.

2.2 | Growth experiments

Anaerobic growth experiments were performed in mineral medium (containing 4.6 mmol/L NH_4^+) supplemented with 10 mmol/L potassium NO_3^- as electron acceptor and 30 mmol/L glucose as electron donor unless stated otherwise. Mineral medium was as described by Stanier, Palleroni, and Doudoroff (1966), including 10 mmol/L phosphate buffer (pH 6.92 ± 0.05), 2.3 mmol/L $(NH_4)_2SO_4$, 0.4 mmol/L $MgSO_4{\cdot}7H_2O$, 0.04 mmol/L $CaCl_2{\cdot}2H_2O$, 27 µmol/L EDTA, 25 µmol/L $FeSO_4{\cdot}7H_2O$, 10 µmol/L $ZnSO_4{\cdot}7H_2O$, 25 µmol/L $MnSO_4{\cdot}H_2O$, 3.8 µmol/L $CuSO_4{\cdot}5H_2O$, 2 µmol/L $Co(NO_3)_2{\cdot}6H_2O$, and 0.196 µmol/L $(NH_4)_6Mo_7O_{24}{\cdot}24H_2O$. Serum vials (120 ml) were soaked in 1 mol/L HCl overnight to remove growth inhibiting substances and subsequently washed five times with distilled water before use. Serum vials with 50 ml medium were sealed with black butyl rubber stoppers. After autoclaving, the headspace of the serum vials was replaced via five cycles of evacuating and refilling with helium. Serum vials were inoculated (1% v/v) with a bacterial suspension of OD_{600} of 1.0 ± 0.05. Each growth experiment was performed in triplicate, and noninoculated media in duplicate were included to check for potential nitrosation reactions in sterile medium, which were proved negligible after measurement. After inoculation, serum vials were incubated at 37°C, 150 rpm, for 72 hr for endpoint analysis or for 192 hr for detailed growth experiments. Gas samples and culture samples were taken at the start and the end of the experiment, or at various time points over the incubation for detailed analysis (see below).

Mineral media with different supplements were designed and tested to study the effect of several factors on NO_3^- partitioning to NH_4^+ and N_2O: (1) different NO_3^- concentrations (5 mmol/L, 10 mmol/L, and 15 mmol/L) and 30 mmol/L glucose resulting in variable C/N-NO_3^- ratios of 36, 18, and 12; (2) different NO_3^- concentrations (5 mmol/L, 10 mmol/L, and 15 mmol/L) under identical C/N-NO_3^- ratio of 12 (glucose 10 mmol/L, 20 mmol/L, and 30 mmol/L, respectively); (3) different NO_2^- concentrations without NO_3^- (1 mmol/L, 5 mmol/L, and 10 mmol/L) and 30 mmol/L glucose resulting in variable C/N-NO_2^- ratios of 180, 36, and 18; (4) different NO_2^- concentrations (1 mmol/L, 5 mmol/L, and 10 mmol/L) under identical C/N-NO_2^- ratio of 18 (glucose 3 mmol/L, 15 mmol/L, and 30 mmol/L, respectively); (5) different NH_4^+ concentrations (0 mmol/L, 1 mmol/L, 4.6 mmol/L, and 10 mmol/L) and 10 mmol/L NO_3^-, 30 mmol/L glucose, resulting a C/N-NO_3^- ratio of 18. Under all conditions, incubation was limited to 72 hr for endpoint analysis. However, in addition, in setup (4), serum vials were also incubated for a longer period of 192 hr and the complete NO_2^- reduction process was followed over time, and growth and nitrogen compound concentrations were monitored at several time points to study the mechanism of N_2O production.

2.3 | Analytical procedures

Samples of 1 ml were taken from cultures through the rubber septum of serum vials with sterile syringes for growth determination and colorimetric determination of NH_4^+, NO_3^-, and NO_2^-. Growth was determined by measuring the optical density OD_{600} of 100 µl sample in duplicate in microtiter plates and standardized to 1 cm path length using PathCheck Sensor of the spectrophotometer (Molecular Devices, Spectramax plus 384, USA). Samples left were centrifuged at 17,949*g* for 2 min to remove the cells, and supernatants were kept frozen at -20°C until colorimetric determination. NH_4^+ concentration was determined with the salicylate-nitroprusside method (absorption at a wavelength of 650 nm) (Baethgen & Alley, 1989), and NO_2^- and NO_3^- concentrations were determined with Griess reaction (Griess, 1879) and Griess reaction with cadmium (Cataldo, Haroon, Schrader, & Youngs, 1975; Navarro-Gonzalvez, Garcıa-Benayas, & Arenas, 1998), respectively. For endpoint measurements, NH_4^+ production was corrected per strain for the amount of NH_4^+ assimilated based on OD_{600} values obtained. Standard curves covered ranges suitable for the tested media and were strictly linear with an R_2 of 0.99. For determination of N_2O, 1 ml sample of the headspace of serum vials was taken with sterile syringes and was injected into a gas chromatograph (Compact GC with EZChrom Elite Software, Interscience, Netherlands, 2012, column molsieve 5A 7*0.32 mm and Rt-Q Bond 3*0.32 mm). N_2O concentrations were corrected for pressure and solubility based on Henry's law. Henry's constant for N_2O is 0.025 mol/L/atm at 25°C.

Statistical differences in end product concentration (OD_{600}, NO_3^-/NO_2^-/NH_4^+ concentration, N_2O production) and ratios of N-NH_4^+ production to N–N_2O production (indicating NO_3^- partitioning to NH_4^+ and N_2O) in the tests of different environmental drivers were processed using factorial ANOVA and least significant difference post hoc testing in IBM SPSS 23 or the nonparametric Kruskal–Wallis *H* test.

3 | RESULTS AND DISCUSSION

3.1 | NO_2^- reduction ability

Already three decades ago, it was suggested that N_2O production during DNRA originates from detoxification of accumulated NO_2^- (Bleakley & Tiedje, 1982; Smith, 1983). Our previous study demonstrated that *B. paralicheniformis* LMG 6934 had a high NO_2^- tolerance of 10 mmol/L and could efficiently perform DNRA by reducing all intermediary NO_2^- to NH_4^+ and N_2O (Sun et al., 2016), while *B. paralicheniformis* LMG 7559 showed a NO_2^- tolerance of 6.29 ± 0.39 mmol/L, and both LMG 7559 and *B. licheniformis* LMG 17339 had residual NO_2^- (2.76 mmol/L ± 0.57 mmol/L, 4.88 mmol/L ± 0.60 mmol/L) after 72-hr incubation probably due to their lower tolerance to the toxic effect of NO_2^-. Less N_2O was produced by LMG 6934 than by LMG 7559

TABLE 1 Overview of growth tests of *Bacillus paralicheniformis* LMG 6934

Media supplements	C/N-NO_x^-	ΔOD_{600}	Concentration (mmol/L)		
			NO_3^- or NO_2^- consumed	NH_4^+ produced	N_2O produced
5 mmol/L NO_3^-	36	0.60^{aA} (0.10)	5.23^{aA} (0.15)	4.80^{aA} (0.27)	0.33^{aA} (0.12) *
10 mmol/L NO_3^- #	18	0.71^{aAB} (0.20)	9.87^{bA} (0.43)	$8.69\ A^{b}$ (0.36)	0.59^{bA} (0.03)
15 mmol/L NO_3^- ##	12	0.76^{a} (0.09)	14.67^{c} (1.13)	12.94^{c} (1.15)	0.87^{c} (0.02)
5 mmol/L NO_3^-	12	0.22^{aB} (0.03)	4.91^{aA} (0.21)	4.50^{aA} (0.23)	0.20^{aA} (0.01)
10 mmol/L NO_3^-	12	0.50^{bA} (0.05)	9.55^{bA} (1.13)	8.57^{bA} (1.11)	0.49^{bB} (0.01)
15 mmol/L NO_3^- ##	12	0.76^{c} (0.09)	14.67^{c} (1.13)	12.94^{c} (1.15)	0.87^{c} (0.02)
1 mmol/L NO_2^-	180	0.35^{a} (0.02)	1.17^{a} (0.01)	1.17^{a} (0.01)	0^{a} (0.00)
5 mmol/L NO_2^-	36	0.51^{bA}(0.02)	6.19^{bB} (0.17)	5.71^{bB} (0.15)	0.19^{abA} (0.16)
10 mmol/L NO_2^-	18	0.66^{cA} (0.03)	13.76^{cB} (0.97)	12.99^{cB} (0.99)	0.39^{bC} (0.01)
1 mmol/L NO_2^-	18	0.22^{a} (0.01)	0.99^{a} (0.01)	0.99^{a} (0.01)	0^{a} (0.00)
5 mmol/L NO_2^-	18	0.52^{bA} (0.06)	4.87^{bA} (0.06)	4.35^{bA} (0.07)	0.26^{bA} (0.04)
10 mmol/L NO_2^-	18	0.95^{cBC} (0.10)	9.57^{cA} (0.17)	8.53^{cA} (0.16)	0.55^{cABC} (0.08)
0 mmol/L NH_4^+	18	0.67^{aAB} (0.08)	10.32^{aAB} (1.34)	9.16^{aA} (1.26)	0.58^{aA} (0.04)
1 mmol/L NH_4^+	18	0.82^{aB} (0.02)	10.95^{aAB} (0.18)	9.71^{aA} (0.20)	0.62^{aA} (0.02)
4.6 mmol/L NH_4^+ #	18	0.71^{aAB} (0.20)	9.87^{aA} (0.43)	8.69^{aA} (0.36)	0.59^{aA} (0.03)
10 mmol/L NH_4^+	18	0.87^{aB} (0.03)	8.99^{aA} (0.99)	7.68^{aA} (0.91)	0.65^{aA} (0.04)

Growth (ΔOD_{600}), electron acceptors (NO_3^- or NO_2^-) consumption, NH_4^+ production (measured concentrations of NH_4^+ corrected for loss through assimilation), and N_2O production after 72-hr incubation under different media composition are shown. All NO_3^- added was consumed by the end of the experiment. Standard deviations are given between brackets (n = 3 if not stated otherwise). Statistics were determined via one-way ANOVA or nonparametric tests accordingly. Significant differences (p < .05) of each parameter (OD_{600}, NO_3^- or NO_2^- consumption, NH_4^+, and N_2O production) within the same experiment (five experiments: (i) NO_3^- concentration test under variable C/N$^-$ NO_3^- ratio, (ii) NO_3^- concentration test under fixed C/N– NO_3^- ratio, (iii) NO_2^- concentration test under variable C/N$^-$ NO_3^- ratio, (iv) NO_2^- concentration test under fixed C/N$^-$ NO_3^- ratio, and (v) NH_4^+ concentration test (with initial 10 mmol/L NO_3^-)) are displayed as different lowercase letters (combined lower letters are used to indicate nonsignificance for multiple variables). Significant differences in each parameter between four different experiments when 5 mmol/L NO_3^-/NO_2^- or 10 mmol/L NO_3^-/NO_2^- supplied is displayed as capital letters.

*n = 2.

#or ##indicates data from the same test analyzed twice in different experiment interpretation.

and LMG 17339, and less NO_3^- partitioning to N_2O was observed as well ([N-NH_4^+]/[N–N_2O] of 4.24 ± 0.29 vs 1.49 ± 0.82, 0.71 ± 0.09, respectively) (Sun et al., 2016 and unpublished data therein). To uncover factors affecting N_2O production during DNRA, here, NO_2^- reduction was anaerobically tested in LMG 6934 at concentrations of 1 mmol/L, 5 mmol/L, and 10 mmol/L under variable C/N-NO_2^- ratios of 180, 36, and 18 and fixed C/N-NO_2^- ratios of 18. After 72-hr incubation, growth was observed under all NO_2^- concentrations tested, with all NO_2^- converted to NH_4^+ or N_2O, thus confirming its high tolerance to NO_2^- (Table 1; Figure 1). Indeed, compared with other DNRA strains (Sun et al., 2016) belonging to *Bacillus* sp. and *Citrobacter* sp. (Stremińska et al., 2012), *B. licheniformis* (Konohana et al., 1993), and *Pseudomonas stutzeri* D6 (Yang, Wang, & Zhou, 2012), LMG 6934 showed a high NO_2^- reduction ability, with up to 10 mmol/L of initial NO_2^- consumed. Furthermore, up to 15 mmol/L NO_3^- was converted to NH_4^+ (>85%) and N_2O (<15%) with no residual NO_2^- at the end of the experiment. The high NO_2^- reduction ability observed in our tests with high NO_3^- or NO_2^- concentration might partly be due to increased NirB activity (Wang & Gunsalus, 2000).

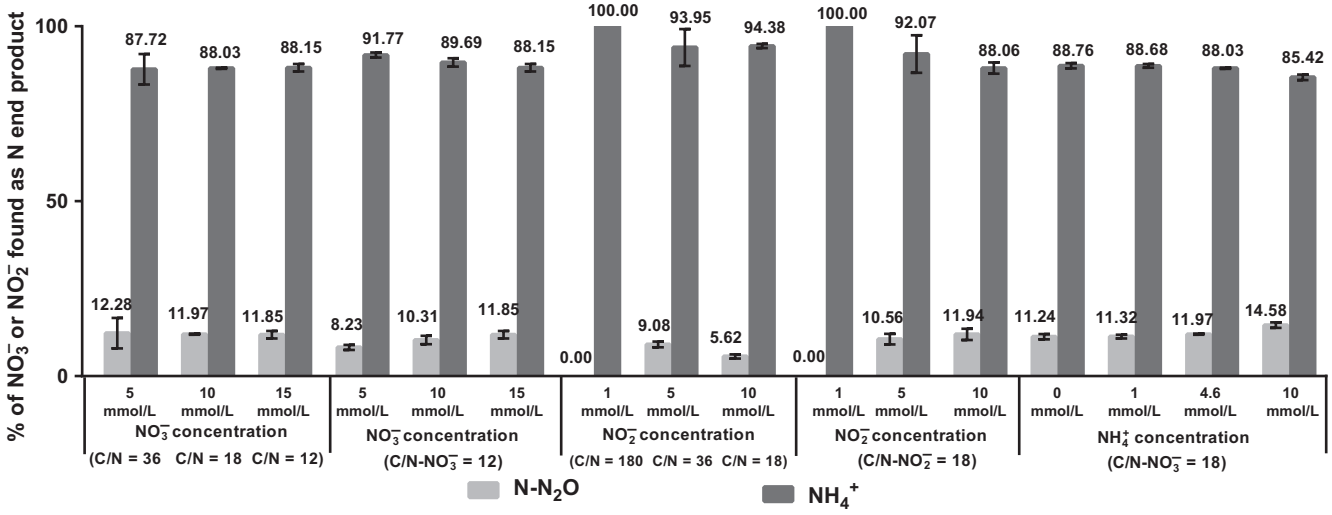

FIGURE 1 Production of nitrous compounds by *Bacillus paralicheniformis* LMG 6934 in different mineral media after 72-hr anaerobic incubation. Percentages of end products of anaerobic NO_3^-/NO_2^- reduction in mineral medium with increasing NO_3^- concentration under variable C/N-NO_3^- ratio (n = 2 for C/N ratio of 36); with increasing NO_3^- concentration under fixed C/N-NO_3^- ratio of 12 (for 15 mmol/L NO_3^-, it is the same experiment as above, the same data used twice for analysis); with increasing NO_2^- concentration under variable C/N-NO_2^- ratios; with increasing NO_2^- concentration under fixed C/N-NO_2^- ratio of 18; with increasing NH_4^+ concentration under fixed C/N-NO_3^- ratio of 18. Error bars represent standard deviation (n = 3 if not stated otherwise). Measured concentrations of NH_4^+ were corrected for loss through assimilation

3.2 | Influence of NO_3^- and NO_2^- concentration on N_2O production

Anaerobic growth experiments with 5, 10, and 15 mmol/L NO_3^- under variable C/N-NO_3^- ratios of 36, 18, and 12 after 72-hr incubation revealed that NO_3^- or intermediate NO_2^- was completely converted to N_2O or NH_4^+ for all conditions tested and growth ceased and sporulation started due to either NO_3^- limitation for respiration or carbon source (glucose) limitation for fermentation. Growth (ΔOD_{600}) (including sporulation), consumption of NO_3^-, production of NO_v and NH_4^+ are summarized in Table 1. Percentages of NO_3^- or NO_2^- converted to N_2O or NH_4^+ under different conditions are shown in Figure 1. Percentage of NO_3^- recovery as N_2O and growth (ΔOD_{600}) under 10 mmol/L NO_3^- condition agreed with previous observations (Sun et al., 2016).

With a constant 30 mmol/L glucose and variable C/N-NO_3^- ratios of 36, 18, and 12, the rising NO_3^- concentration had an influence on N_2O production (p = .0018) and NH_4^+ production (p = .000027), with higher NO_3^- concentrations leading to production of more NH_4^+ and more N_2O (Table 1; Figure 2a). Different NO_3^- concentrations had no significant influence on NO_3^- partitioning ([N–NH_4^+]/[N–N_2O]) (p = .417) (Figure 3a). Growth did not significantly increase with NO_3^- concentration (p = .287) (Figure 3a), and this may because excess glucose (initial 30 mmol/L) supports fermentation and sporulation. Smith (1981) showed that, in *Citrobacter*, higher C/N-NO_3^- ratios with constant NO_3^- concentration favor NO_3^- partitioning to N_2O. In our study, the opposite was apparently found: A higher C/N-NO_v ratio led to less N_2O produced. However, the higher C/N-NO_3^- ratios here were created by lowering NO_3^- concentration with glucose at 30 mmol/L. We hypothesize that lower NO_3^- concentration would lead to lower NO_2^- concentration resulting in a lower toxic effect and less need for its reduction to nontoxic N_2O. To confirm that a rising NO_3^- concentration and exclude the influence of C/N-NO_3^- ratio, which might be strain-dependent (Stremińska et al., 2012), the same experiment was repeated under fixed C/N-NO_3^- ratio of 12. Again, after 72-hr anaerobic incubation, all NO_3^- or NO_2^- was completely converted to N_2O or NH_4^+ without any residual NO_2^- left for all conditions tested. As expected, growth increased with a rising NO_3^- concentration under fixed C/N-NO_3^- ratio (p = .000128) and was supported by fermentation of glucose and NO_3^- respiration. NH_4^+ production (p = .000101) and N_2O production ($p = 4.95 \times 10^{-9}$) showed a positive correlation with the rising NO_3^- concentration (Table 1; Figure 2b). In addition, increased NO_v concentration from 5 to 10 mmol/L promoted NO_3^- partitioning to N_2O and negatively impacted its partitioning to NH_4^+ (p = .008) (Figure 3b), but this effect was statistically not significant when increasing from 10 to 15 mmol/L NO_3^- (p = .155).

In contrast to a rising NO_3^- concentration under variable C/N-NO_3^- ratios, a rising NO_2^- concentration under variable C/N-NO_2^- ratio did show a positive effect on NH_4^+ production (p = .027) and N_2O production (p = .034) and resulted in an increasing growth (p = .000017) supported by fermentation and/or respiration as stated above. However, why this excess glucose did not result in similar growth by fermentation as it did in NO_3^- concentration tests is unclear. As expected, with more NO_2^- consumed in the media, more NH_4^+ and N_2O were produced, resulting in more cell growth (Table 1; Figure 2c). In addition, increase in NO_2^- concentration had a significantly positive influence on NO_2^- partitioning to N_2O but the significance was only shown between 1 mmol/L and 10 mmol/L NO_2^- (p = .00028) (Figure 3c), which is also the case for the amount of N_2O produced (Table 1).

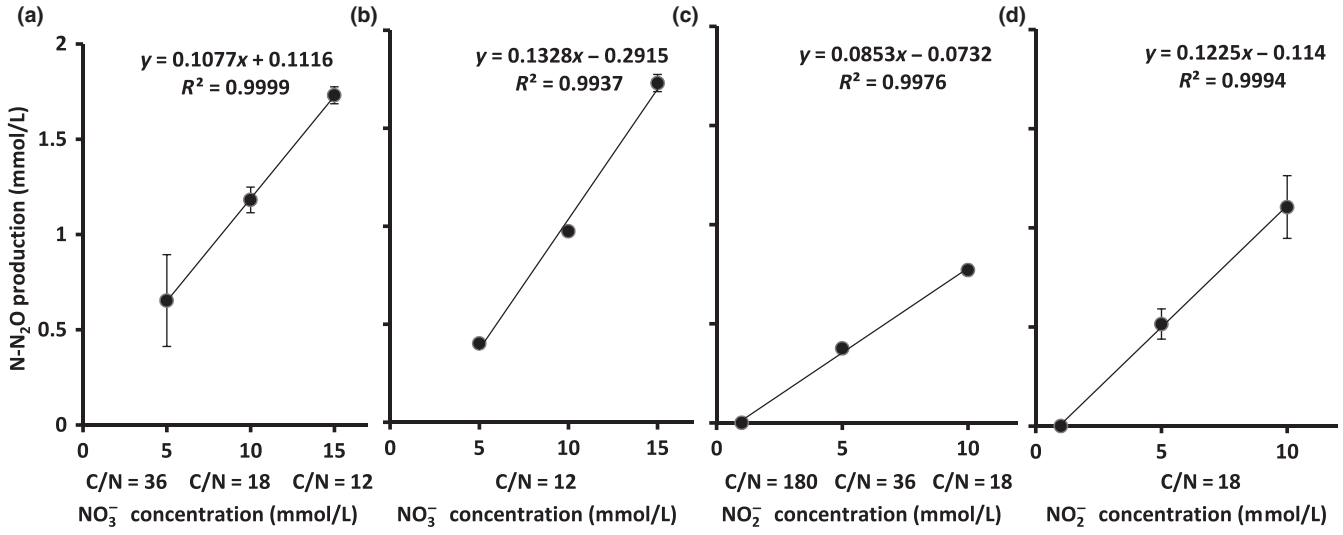

FIGURE 2 N–N_2O production by *B. paralicheniformis* LMG 6934 in different mineral media after 72-hr anaerobic incubation. Media tested are supplemented with the following: (a) increased NO_3^- concentration under variable C/N-NO_3^- ratio of 36 (n = 2), 18, and 12; (b) increased NO_3^- concentration under fixed C/N-NO_3^- ratio of 12; (c) increased NO_2^- concentration under variable C/N-NO_2^- ratio of 180, 36, and 18; (d) increased NO_2^- concentration under fixed C/N-NO_2^- ratio of 18. Error bars represent standard deviation (n = 3 if not stated otherwise). Trend line equations and R-squared value are given

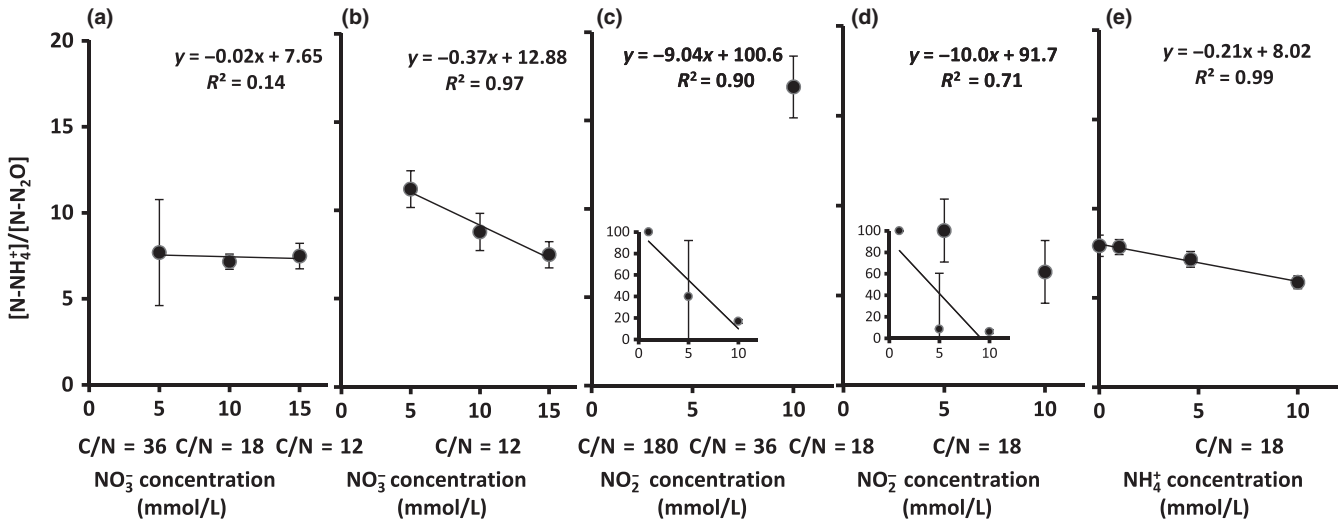

FIGURE 3 Ratio of N-NH_4^+ production to N–N_2O production by *B. paralicheniformis* LMG 6934 after 72-hr anaerobic incubation in mineral media. Mineral medium supplemented with the following: (a) increasing NO_3^- concentration under variable C/N- NO_3^- ratio of 36 (n = 2), 18, and 12; (b) increasing NO_3^- concentration under fixed C/N-NO_3^- ratio of 12; (c) increasing NO_2^- concentration under variable C/N-NO_2^- ratio of 180, 36, and 18; (d) increasing NO_2^- concentration under fixed C/N- NO_2^- ratio of 18; (e) increasing NH_4^+ concentration under fixed C/N- NO_3^- ratio of 18. Error bars represent standard deviation (n = 3 if not stated otherwise). The inserted figure in panel C and panel D is the complete figure of the test with a [N–NH_4^+]/[N–N_2O] range from 0 to 100. Trend line equations and R-squared value are given

Similarly, increasing NO_2^- concentration under fixed C/N-NO_2^- ratio of 18 also showed a positive effect on growth (p = .000049), NH_4^+ production (p = 1.9996E−8), and N_2O production (p = .000033) (Table 1; Figure 2d). Likewise, rising NO_2^- concentration had a significantly positive influence on NO_2^- partitioning to N_2O, but the significance was only shown between 1 mmol/L and 5 mmol/L or 10 mmol/L NO_2^- (p = 7.5916E−11) (Figure 3d). To further study the conditions affecting N_2O production during DNRA, growth was monitored over a 192-hr incubation period. As expected, NH_4^+ was produced during incubation, accompanied by N_2O production and NO_2^- partitioning to N_2O at first increased, becoming stable after 48 hr (Figure 4).

In summary, a linear but nonstoichiometric correlation was observed for the first time between NO_3^- or NO_2^- concentration and N_2O production (Figure 2), which may be useful for further studies of N_2O production calculation or interpretation of its regulation. In addition, increasing NO_3^- concentration under fixed C/N-NO_3^- ratio but not under variable C/N-NO_3^- ratios and increasing NO_2^- concentration under variable as well as fixed C/N-NO_2^- ratios significantly

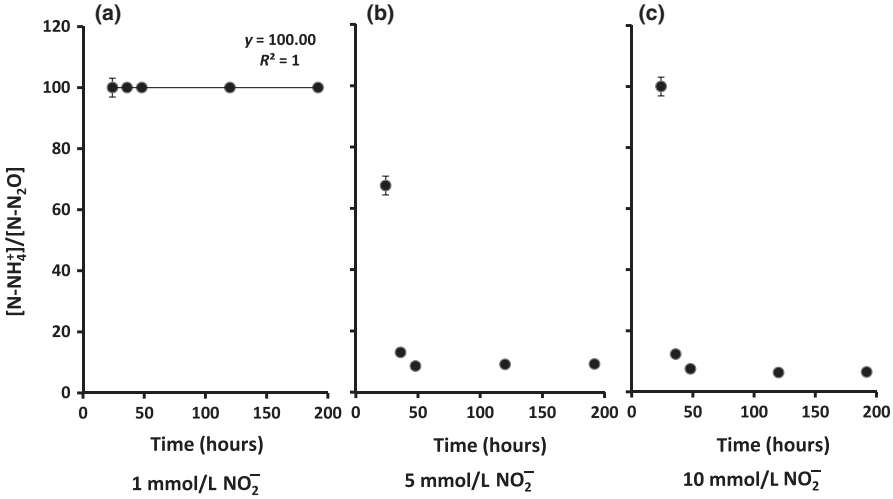

FIGURE 4 Ratio of N–NH_4^+ production to N–N_2O production during 192 hr of anaerobic incubation of *B. paralicheniformis* LMG 6934 in mineral medium supplemented with NO_2^- under fixed C/N- NO_2^- ratio of 18: (a) 1 mmol/L NO_2^- added; (b) 5 mmol/L NO_2^- added; and (c) 10 mmol/L NO_2^- added

increased NO_3^- partitioning to N_2O in *B. paralicheniformis* LMG 6934 (Figure 3). The latter may be a direct effect of NO_2^-, probably by action of NirB, while NO_3^- may work through a combined effect of C/N-NO_3^- ratio and NO_3^- concentration. Higher NO_3^- concentration under fixed C/N-NO_3^- ratio promotes NO_3^- partitioning to N_2O, and this agrees with physiological data of a previous study (Smith, 1981). It indeed makes sense that, under higher NO_3^- concentration, more NO_2^- transiently accumulates and therefore needs to be detoxified, leading to a higher proportion of NO_3^- to N_2O. This agrees with the observation in NO_2^- batch tests. Non-negligibly, the C/N-NO_3^- referred to was the initial ratio. The C/N-NO_3^- ratio varied during the batch incubation tests. Constant C/N-NO_3^- in a chemostat setup is suggested for further study.

3.3 | Influence of NH_4^+ concentration on N_2O production

It is known that NH_4^+ can repress NO_3^- assimilation causing NO_2^- to accumulate (Schreier et al., 1989; Stouthamer, 1976); however, it does not inhibit nitrate reduction for dissimilation toward NH_4^+ (Konohana et al., 1993). Here, we tested its effect on N_2O production and used NH_4^+ concentrations of 0 mmol/L, 1 mmol/L, 4.6 mmol/L (standard), and 10 mmol/L in the presence of 10 mmol/L NO_3^- under a fixed C/N-NO_3^- ratio of 18. After 72-hr incubation, growth was obtained under all NH_4^+ concentrations, even without NH_4^+ added (Table 1; Figure 1). All NO_3^- was converted to NH_4^+ or N_2O, with some samples reaching up to approx. 10 mmol/L NH_4^+ produced (Table 1). There was no statistically significant effect of NH_4^+ concentration on growth ($p = .12$) as expected, and similar results were observed for NH_4^+ production ($p = .12$) or N_2O production ($p = .11$), again confirming that LMG 6934 is a vigorous ammonifier able to produce and take up sufficient NH_4^+ for growth. However, there was a significant effect of NH_4^+ on NO_3^- partitioning to N_2O but only in medium with the highest NH_4^+ concentration (10 mmol/L) compared with media with lower NH_4^+ concentration ($p = .000932$) (Figure 3e). This observation requires further confirmation with higher NH_4^+ concentrations, and this mechanism behind this effect requires in-depth study.

Thus, anaerobic growth was not repressed by NH_4^+ (starting from 10 mmol/L initial NH_4^+, an NH_4^+ concentration as high as 18.47 ± 0.10 mmol/L was measured after incubation), which is in agreement with previous studies on *Bacillus* sp. and *Citrobacter* sp. (Smith & Zimmerman, 1981). Almost no difference in growth was obtained under different NH_4^+ concentrations. Similar observations were described with *B. licheniformis* No. 40-2, a strain isolated from a hot spring but under aerobic conditions (Konohana et al., 1993).

3.4 | Ecological relevance and future perspectives

Here, we demonstrated that indeed NO_3^- as well as NO_2^- concentration shows a linear correlation with N_2O production and increasing concentrations lead to more partitioning to N_2O which may be a direct result of NO_2^- detoxification. This linear correlation is media-dependent and may be strain-dependent, as was found in our previous study when comparing three *Bacillus* strains in different media conditions (Sun et al., 2016). The underlying mechanisms, however, remain elusive. Further studies are required to assess whether these effects apply for other DNRA strains and under field conditions. Such information may in future contribute to the estimation of environmental N_2O emissions based on in situ measurements of environmental parameters. Furthermore, we also observed that higher NH_4^+ concentration could lead to more NO_3^- partitioning to N_2O. Canonical NO reductase (Nor) is widespread among denitrifiers and nondenitrifiers and efficient for NO reduction to N_2O. The genome of strain LMG 6934 encodes for quinol-dependent NO reductase (qNor) as well as Hmp (Sun et al., 2016). Hmp, however, has not been fully proved to be physiologically relevant as protection from nitrosative stress (Torres et al., 2016). Therefore, as there was no growth defect caused by NO toxicity under the conditions tested, it can be hypothesized that qNor rather than Hmp may be a significant source of N_2O in LMG 6934. However, it still remains unclear whether NO generation is by NarG, NirBD, or both of them.

This study contributed to characterization of DNRA performance under different environmental drivers, including increasing NO_3^-, NO_2^-, and NH_4^+. Although we used relatively high concentrations of

NO_3^- or NO_2^-, they are still relevant as comparable concentrations can exist in the environment (Reisenauer, 1966; Wolt, 1994), for example during fertilization events of agricultural land (Dechorgnat et al., 2011). We realize that the N_2O production during ammonification might be considered negligible compared to that during canonical denitrification, especially when considering LMG 6934 is highly tolerant to NO_2^-. Nevertheless, ammonifiers are widely distributed in the environment and DNRA is considered the preferred NO_3^- reduction process in agricultural soils as it retains N in the system (Mania et al., 2014). Therefore, future N_2O mitigation strategies promoting DNRA need to consider the potential concomitant N_2O production. In this respect, *B. paralicheniformis* LMG 6934, which under laboratory conditions produces less N_2O than some other DNRA bacteria (Sun et al., 2016), is an interesting strain. It was originally isolated from garden soil, showing nonfastidious growth and is nonpathogenic and may thus be a good candidate for application in agricultural fields, to promote DNRA over denitrification. This would favor nitrogen retention, increasing efficiency of nitrogen fertilizer applied and, to a certain degree, reducing N_2O emission from the soil.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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