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ORIGINAL RESEARCH

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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-$NO_x^-$ | $\Delta OD_{600}$ | 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 ($\Delta OD_{600}$), 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).

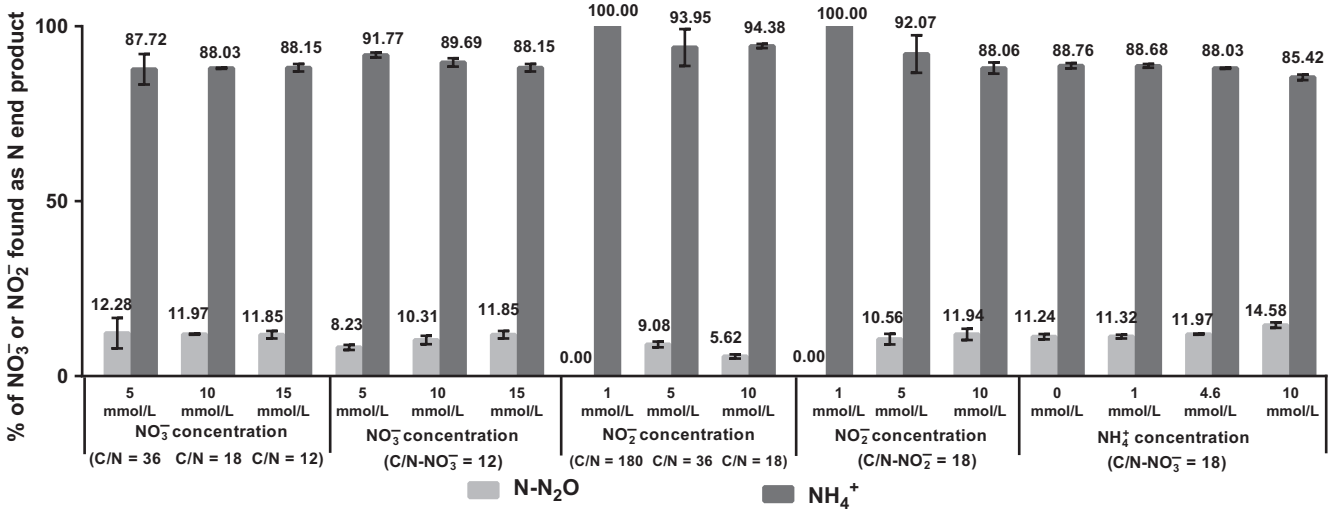


**FIGURE 1** Production of nitrous compounds by *Bacillus paralicheniformis* LMG 6934 in different mineral media after 72-hr anaerobic incubation. Percentages of end products of anaerobic $NO_3^-$/$NO_2^-$ reduction in mineral medium with increasing $NO_3^-$ concentration under variable C/N-$NO_3^-$ 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 ($\Delta OD_{600}$) (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 ($\Delta OD_{600}$) 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).

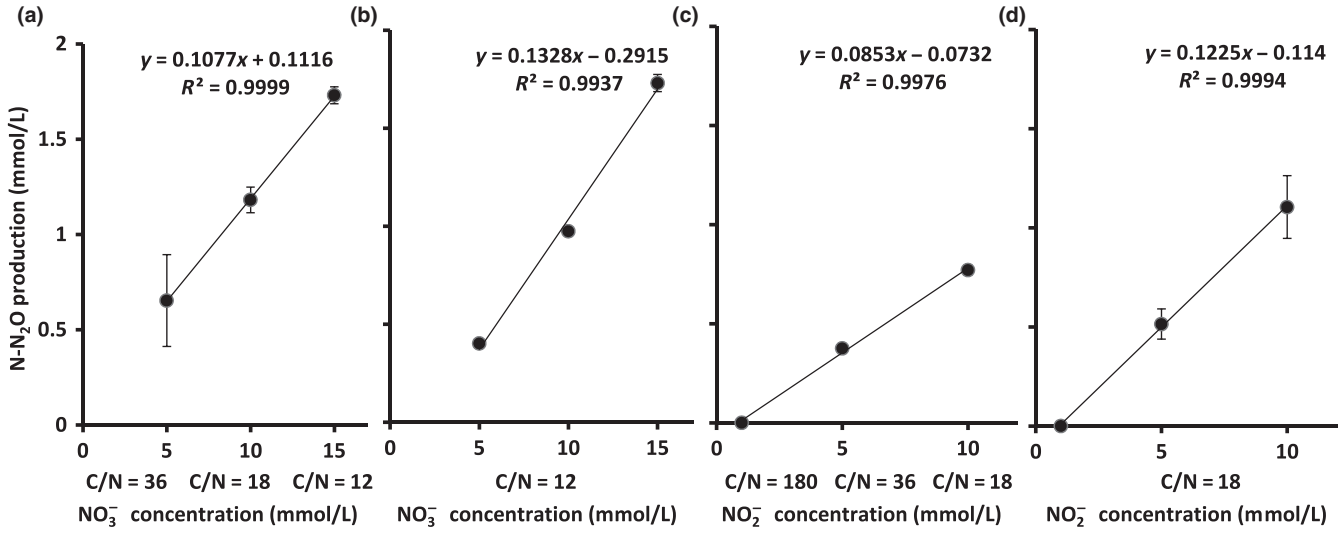


**FIGURE 2** N–$N_2O$ production by *B. paralicheniformis* LMG 6934 in different mineral media after 72-hr anaerobic incubation. Media tested are supplemented with the following: (a) increased $NO_3^-$ concentration under variable C/N-$NO_3^-$ ratio of 36 ($n$ = 2), 18, and 12; (b) increased $NO_3^-$ 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

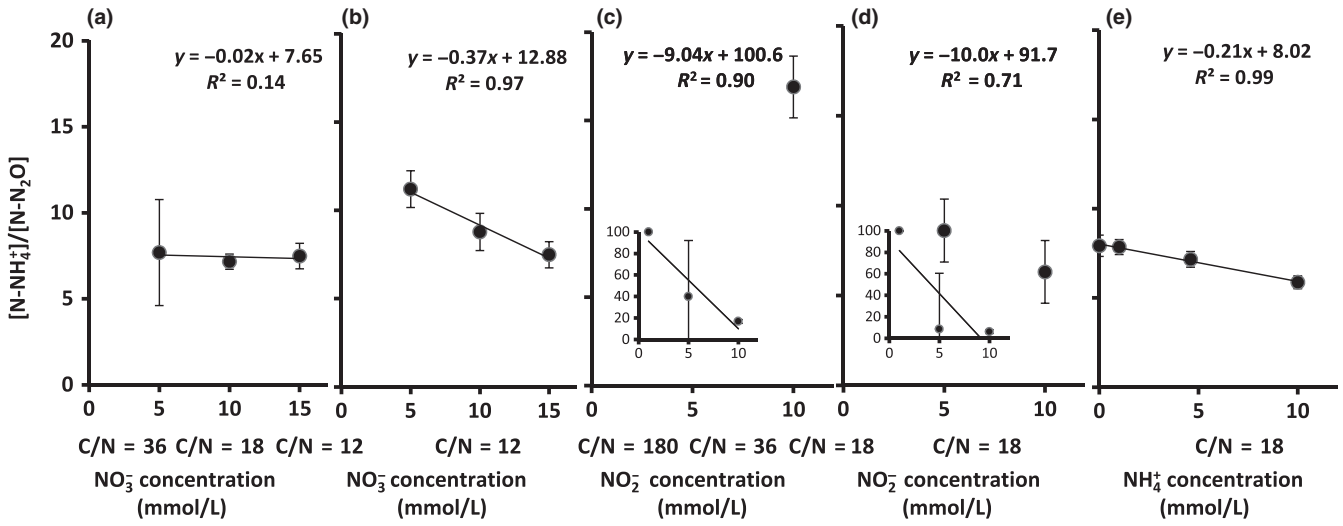


**FIGURE 3** Ratio of N-$NH_4^+$ production to N–$N_2O$ production by *B. paralicheniformis* LMG 6934 after 72-hr anaerobic incubation in mineral media. Mineral medium supplemented with the following: (a) increasing $NO_3^-$ concentration under variable C/N- $NO_3^-$ ratio of 36 ($n$ = 2), 18, and 12; (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

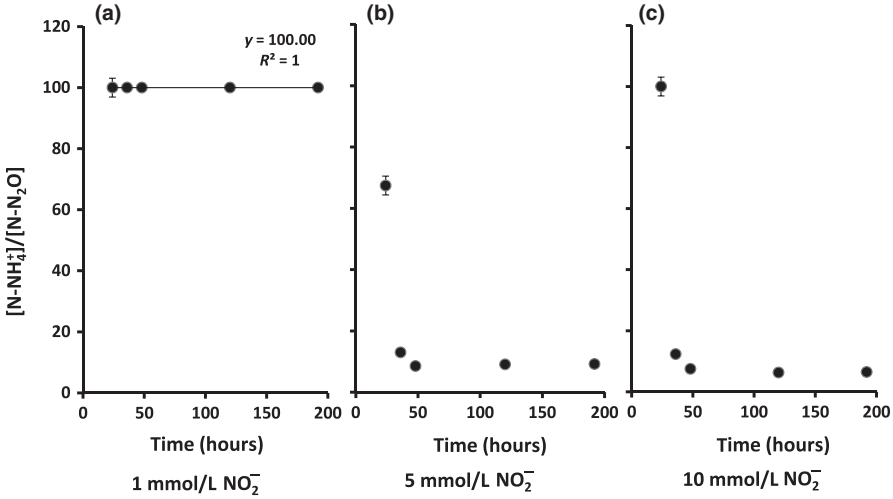


**FIGURE 4** Ratio of N–$NH_4^+$ production to N–$N_2O$ production during 192 hr of anaerobic incubation of *B. paralicheniformis* LMG 6934 in mineral medium supplemented with $NO_2^-$ under fixed C/N- $NO_2^-$ ratio of 18: (a) 1 mmol/L $NO_2^-$ added; (b) 5 mmol/L $NO_2^-$ added; and (c) 10 mmol/L $NO_2^-$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