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de Klein, Cecile A. M.; Bowatte, Saman; Simon, Priscila; Arango, Jacobo; Cárdenas, Laura M.; Chadwick, D; Pijlman, Jeroen ; Rees, RM; Richards, Karl G; Subbarao, Guntur; Whitehead, David

Published in:
Biology and Fertility of Soils

DOI:
[10.1007/s00374-022-01631-2](https://doi.org/10.1007/s00374-022-01631-2)

Print publication: 01/04/2022

Document Version
Peer reviewed version

[Link to publication](#)

Citation for published version (APA):

de Klein, C. A. M., Bowatte, S., Simon, P., Arango, J., Cárdenas, L. M., Chadwick, D., Pijlman, J., Rees, RM., Richards, K. G., Subbarao, G., & Whitehead, D. (2022). Accelerating the development of biological nitrification inhibition as a viable nitrous oxide mitigation strategy in grazed livestock systems. *Biology and Fertility of Soils*, 58, 235-240. <https://doi.org/10.1007/s00374-022-01631-2>

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1 **Accelerating the development of biological nitrification inhibition as a viable nitrous**
2 **oxide mitigation strategy in grazed livestock systems**

3

4 Cecile AM de Klein^{1*}, Saman Bowatte², Priscila Simon¹, Jacobo Arango³, Laura M
5 Cardenas⁴, David R Chadwick⁵, Jeroen Pijlman⁶, Robert M Rees⁷, Karl Richards⁸, Guntur V
6 Subbarao⁹, David Whitehead¹⁰

7 ¹AgResearch Invermay, Mosgiel, New Zealand, ORCID number: 0000-0001-7840-0934

8 ²AgResearch Grasslands, Palmerston North, New Zealand

9 ³International Center for Tropical Agriculture, Cali, Colombia

10 ⁴Rothamsted Research, Sustainable Agriculture Sciences, North Wyke, Okehampton, UK

11 ⁵Bangor University, Bangor, UK

12 ⁶Louis Bolk Institute, Bunnik, The Netherlands

13 ⁷Scotland's Rural College, Edinburgh, UK

14 ⁸Teagasc, Wexford, Ireland

15 ⁹Japan International Research Center for Agricultural Sciences, Ibaraki, Japan

16 ¹⁰Manaaki Whenua – Landcare Research, Lincoln, New Zealand

17 * Corresponding author: cecile.deklein@agresearch.co.nz

18

19 **Keywords:** BNI; animal urine; livestock systems; nitrous oxide; research priorities

20

21 **Abstract**

22 This position paper summarizes the current understanding of biological nitrification
23 inhibition (BNI) to identify research needs for accelerating the development of BNI as a N₂O
24 mitigation strategy for grazed livestock systems. We propose that the initial research focus
25 should be on the systematic screening of agronomically desirable plants for their BNI

26 potency and N₂O reduction potential. This requires the development of *in-situ* screening
27 methods that can be combined with reliable N₂O emission measurements and microbial and
28 metabolomic analyses to confirm the selective inhibition of nitrification. As BNI-induced
29 reductions in N₂O emissions can occur by directly inhibiting nitrification, or via indirect
30 effects on other N transformations, it is also important to measure gross N transformation
31 rates to disentangle these direct and indirect effects. However, an equally important challenge
32 will be to discern the apparent influence of soil N fertility status on the release of BNIs,
33 particularly for more intensively managed grazing systems.

34

35 **Introduction**

36 Nitrous oxide (N₂O) is a powerful greenhouse gas (GHG) with a global warming potential
37 close to 300 times that of carbon dioxide. Globally, agriculture contributes around 52% of
38 anthropogenic N₂O emissions, with animal urine patches the largest N₂O source in grazed
39 livestock systems (Tian et al. 2020). The inhibition of soil nitrification, which is the
40 conversion of ammonium to nitrate, has been shown to reduce N₂O emissions, with much of
41 the existing understanding of the abatement potential based on studies using synthetic
42 nitrification inhibitors (SNIs; de Klein et al. 2011; Di and Cameron 2016; Minet et al. 2016a;
43 Chadwick et al. 2018). However, there is increasing evidence that plant-induced biological
44 nitrification inhibition (BNI), defined here as attenuation of the nitrification process resulting
45 from the introduction of plant secondary metabolites into the soil through root exudation or
46 turnover of plant tissue, can also reduce N₂O emissions (Subbarao et al. 2013; Byrnes et al.
47 2017; Villegas et al. 2020). Although SNIs provide the flexibility to target applications at
48 specific times, locations and doses to maximize N₂O reduction, BNIs offer other advantages
49 such as i) a root delivery network reaching into nitrifying sites in the soil, ii) affecting both
50 ammonia mono-oxygenase (AMO) and hydroxylamine oxidoreductase (HAO), two enzymes

51 involved in nitrification (compared to SNI which only acts on the AMO pathway), iii) not
52 requiring synthetic production and mechanical application and therefore potentially lowering
53 costs, iv) potential for continuous formation in growing plants, and v) being more natural,
54 thus offering the potential for greater public acceptance. On the other hand, the effectiveness
55 of BNI relies on soil incorporation of plant tissue containing BNI compounds, or the
56 rhizodeposition of BNI active compounds into the soil. The latter is modulated by many
57 plant-soil interactions and as yet poorly understood (Nardi et al. 2020). We acknowledge that
58 SNI and BNI could be complementary as N₂O mitigation options for grazed livestock
59 systems, but we focus here specifically on the potential of BNIs and their development as a
60 recognized N₂O mitigation strategy for livestock systems. The following sections summarize
61 our current understanding and identify key research needs for accelerating this development
62 along key stages of the innovation pipeline (Figure 1):

- 63 (1) Identifying candidate forage species with the genetic capacity to synthesize BNI
64 compounds (discovery)
- 65 (2) Maximizing the BNI capacity of these compounds in soils with agronomically viable
66 species (proof of concept)
- 67 (3) Managing species within systems to maintain BNI effect and productivity (proof of
68 function)
- 69 (4) Implementing systems to incentivize farmers to adopt BNI as a N₂O mitigation
70 strategy (recognized mitigation option)

71

72 **Discovery: Which source-plants have the genetic capacity to regulate BNI?**

73 Much of the work to date has focused on (sub)tropical systems and common agricultural
74 plants that have been shown to exhibit the BNI trait naturally including *Brachiaria*
75 *humidicola* (syn. *Urochloa*), wheat, sorghum, maize, rice, (Subbarao and Searchinger 2021),

76 and *Elymus* grass (Li et al. 2022). There is some evidence that the temperate forb species
77 plantain (*Plantago lanceolata*) may also exhibit BNI effects (Judson et al. 2019). For all these
78 species BNI-active root exudates have been identified and many of these plants have genetic
79 variation in BNI capacity among wild populations and modern cultivars (Navarrete et al.
80 2016; Nardi et al. 2020; Subbarao et al. 2021). Recent research has also demonstrated that the
81 BNI trait from the wild grass *Leymus racemosus* can be successfully transferred via inter-
82 specific hybridization into elite wheat cultivars without disrupting agronomic features or
83 using regulated gene technologies (Subbarao et al. 2021). Therefore, key elements of success
84 at the ‘discovery’ stage are that high potency source-plants containing the BNI trait are
85 identified and that interventions to transfer the trait from potentially raw germplasm sources
86 into elite forage cultivars can occur within agronomic constraints. These efforts will benefit
87 from deciphering the fundamental genetic control of BNI traits in source plants, knowledge
88 of the candidate genes influencing BNI trait expression, and highly efficient means of
89 screening for BNI expression in candidate source populations and large-scale breeding
90 populations.

91

92 **Proof of concept: What is the N₂O reduction potential and how can the BNI effect be**
93 **maximized?**

94 The reduction potential of N₂O emissions through BNI depends on the microbial community
95 composition, abundance and activity of nitrifiers. The common understanding is that N₂O
96 originating from nitrification is largely produced by ammonia oxidizing bacteria (AOB) and
97 much less so by ammonia oxidizing archaea (AOA) (Prosser et al. 2020). However, a recent
98 study with pure cultures of the AOA *Nitrosopumilus maritimus* showed that this AOA can
99 also produce N₂O from nitrification (Kraft et al. 2022). These authors showed that under the
100 anaerobic conditions of the study, the AOA was capable of generating and re-using oxygen

101 (O₂) to support their metabolic activity. This suggests that AOA can perform nitrification and
102 produce N₂O under anaerobic conditions. However, the implications of this finding for
103 managed livestock systems requires further investigation, as increased N availability in these
104 systems is likely to favor AOB over AOA (Egenolf et al. 2022). In addition, the relative
105 contribution of AOA vs AOB to nitrification in different ecosystems is not fully understood
106 yet.

107 N₂O reductions due to BNI have been measured for some key tropical and subtropical
108 grass species, including *Brachiaria humidicola* and Guinea grass (*Megathyrsus maximus*)
109 (Subbarao et al. 2013; Byrnes et al. 2017; Villegas et al. 2020). Byrnes et al. (2017) showed
110 that soils containing a *Brachiaria* cultivar with high BNI capacity emitted 60% less N₂O from
111 urine patches than soils with low BNI capacity cultivars, and Villegas et al. (2020) identified
112 varieties of Guinea grass with high N₂O reduction potentials. Both studies found a direct link
113 between N₂O reduction and BNI, i.e., reduced nitrifier bacteria abundance and nitrification
114 rates. In temperate climate systems, plantain (*Plantago lanceolata*) has been suggested as a
115 species with BNI activity (de Klein et al. 2020), but comprehensive investigation into a direct
116 link between N₂O reduction and BNI activity is lacking. Furthermore, experimental results on
117 the effect of plantain on N₂O emissions from livestock urine are inconsistent, with both
118 reductions and increases in N₂O observed (Luo et al. 2018; Simon et al. 2019; Pijlman et al.
119 2020; Bracken et al. 2021). It is commonly accepted that BNI is an adaptive mechanism that
120 plants use to conserve mineral nitrogen (N) in soils where the competition between plants and
121 microbes for limited N is high. So, one hypothesis is that the inconsistency in the results from
122 intensively managed systems could be attributable to variation in soil N fertility status, with
123 high soil N fertility possibly downregulating the expression of the BNI trait, and thus the N₂O
124 reduction potential. A recent study indeed suggested that, while BNI seems to determine net
125 nitrification rates in extensive pasture systems with *B. humidicola*, inter- and intra-

126 competition for N between microbes and plants appeared to be the main determinant in
127 intensive systems (Egenolf et al. 2022). However, the impact of soil N fertility status on BNI-
128 trait expression is yet to be systematically investigated; more studies into this effect are
129 needed. This should include experiments under controlled conditions in greenhouses that
130 enable a focus on specific controlling factors, as well as field trials to investigate the impact
131 under grazing conditions. There are also other potential factors that regulate the release of
132 BNI compounds, including soil pH, soil moisture content, soil aeration and nematode activity
133 (Wurst et al. 2010; Zhang et al. 2022), that warrant systematic testing in field studies. As it is
134 difficult to separate BNI effects from other plant effects on soil N transformations and
135 microbial community, such studies should measure gross N transformation rates to
136 disentangle the direct and indirect effects of root exudates on soil nitrification (Nardi et al.
137 2020; Ma et al. 2021). In addition, studies should combine N₂O measurements with
138 metabolomics and microbial analysis to confirm both the release of BNI compounds as well
139 as nitrifier inhibition of nitrification, thus directly linking any reduction in N₂O emissions
140 with BNI under field conditions.

141

142 **Proof of function: How can the BNI trait for N₂O reduction be optimized in grazed**
143 **systems?**

144 Once our understanding of the links between BNI-induced N₂O reduction and soil N status or
145 other regulators is improved, the question is how this can be optimized in grazed livestock
146 systems? This may be especially relevant in legume-containing pasture systems, where there
147 is a strong interaction between soil N fertility status and legume content of the sward, or in
148 grazed systems, where urine deposition results in localized rapid increases in soil N and soil
149 pH. Furthermore, to meet improved productivity as well as environmental outcomes,
150 enhancement of the BNI trait should not compromise the viability of the system through

151 unintended consequences on agronomic characteristics of the species such as, productivity,
152 palatability, nutritional value, persistence, winter hardiness, and drought resilience. To date,
153 there is no evidence to suggest that the BNI-trait has a yield penalty either in pastures or in
154 grain crops when comparing BNI-capable varieties with non-BNI capable varieties of the
155 same species (Subbarao and Searchinger 2021). In addition, a BNI-induced increase in farm
156 N use efficiency (NUE) provides the opportunity to reduce farm N inputs and any associated
157 N₂O emissions. A recent LCA modelling study suggested that the impacts from BNI-wheat
158 with 40% nitrification inhibition by 2050 could reduce both N fertilizer requirements and
159 GHG emissions by about 15%, and improve NUE at the farm scale by almost 17% (Leon et
160 al. 2021). However, there is limited research on the effects of BNI species on soil, rumen and
161 farm level N cycling in grazed systems, which severely limits our ability to assess the full
162 impact of BNI species on farm scale GHG emissions. More specifically, due to the apparent
163 inverse relationship between soil N fertility and BNI, a key question is whether there is a
164 ‘sweet spot’ of N fertility in managed grazed livestock systems: one that supplies N sufficient
165 to promote exudation of BNI compounds and thus conserve N, yet not too low that plant
166 production is significantly compromised? Another key question is if, and how, the release of
167 BNI compounds is affected by transient changes in soil N and pH in urine patches in grazed
168 systems. In addition, the effect of grazing intensity on soil aeration and root exudation (Sun et
169 al. 2017), and their subsequent impacts on microbial community composition and function
170 also warrant further investigation. Finally, for optimizing BNIs within grazed systems there
171 may be advantages in synthesizing ‘BNI active’ plant compounds that are delivered to the
172 soil via surface application or in animal feeds (Minet et al. 2016b). Although this would
173 eliminate some advantages of BNIs over SNIs, as discussed above, it could provide a solution
174 in the shorter-term, whilst longer-term plant screening and breeding programmes are
175 developed and root-delivery of BNI compounds is maximized.

176

177 **Recognized mitigation option: How can farmers be recognized for BNI-induced N₂O**
178 **reduction in grazing systems?**

179 For farmers to be recognized for achieving N₂O reductions, the effect of the intervention on
180 total N₂O emissions needs to be accounted for in GHG inventory methodologies and on-farm
181 accounting tools. To the best of our knowledge, BNI is not (yet) recognized as a N₂O
182 mitigation technology in national GHG inventories nor in on-farm accounting tools. This not
183 only requires robust evidence of the efficacy of BNI and the ability to predict N₂O reductions
184 under a range of temporal and spatially variable conditions, but it also requires the ability to
185 accurately estimate and record the BNI ‘activity’ of plants. For BNI-active plants in grazed
186 systems this means being able to demonstrate and verify the effects of their presence in the
187 swards and the conditions that influence their efficacy in N₂O reduction.

188

189 **Conclusions**

190 For BNI to be successfully exploited as a N₂O mitigation option in grazed livestock systems,
191 we identified key questions along key stages of the innovation pipeline and possible
192 approaches to address these (Table 1). We propose that the initial research focus should be
193 prioritized on the ‘discovery’ and ‘proof of concept’ stages. Firstly, the systematic screening
194 of agronomically desirable plants and cultivars to identify their ability to synthesize and
195 exude BNI compounds (i.e., do these plants have the genetic blueprint for BNI?) requires the
196 development of *in-situ* screening methods that can be combined with reliable N₂O emission
197 measurements as well as measurements of gross N transformation rates. To ensure that any
198 N₂O reduction can be assigned to BNI, these measurements should also be accompanied by
199 microbial and metabolomic analyses to confirm the selective inhibition of nitrification.
200 Secondly, whilst understanding the genetic regulation of BNI is a key first step, an equally

201 important challenge will be to discern the apparent influence of soil N fertility status or other
202 soil and climatic factors on the release of the BNIs, particularly for more intensively managed
203 grazing systems. The expansion of an existing BNI consortium (Subbarao and Searchinger
204 2021) to develop a coordinated global programme to address the research gaps we identified
205 here may be a key step towards accelerating the development of BNI as a N₂O mitigation
206 option in both (sub)tropical and temperate livestock systems.

207

208 **Acknowledgements**

209 This project was part-funded by the New Zealand Government, in support of the objectives of
210 the Livestock Research Group of the Global Research Alliance on Agricultural Greenhouse
211 Gases.

212

213 **Statements and Declarations**

214 Conflict of Interest: The authors declare that they have no conflict of interest

215

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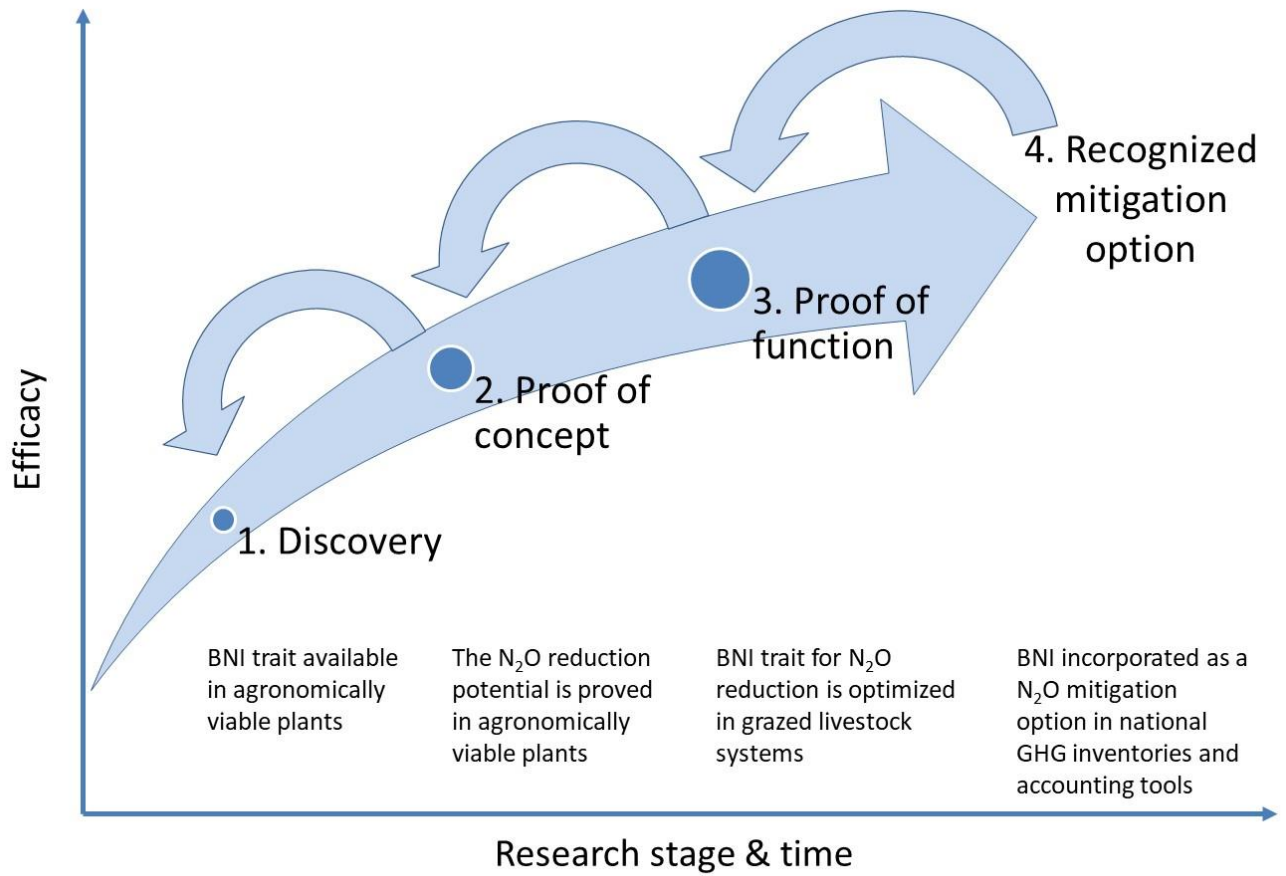
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324



325

326 *Figure 1: Stages of development pipeline for BNI as a N_2O mitigation option and desired*

327 *outcome of each stage.*

328

Table 1: Biological nitrification inhibition – key research questions and recommendations for future work for developing BNI into a viable strategy for reduction N₂O emissions from grazed livestock systems.

	1. Stage of development Discovery	2. Proof of concept	3. Proof of function	4. Recognized mitigation option
Desired outcome at each stage	Existence of BNI trait Trait is available in agronomically viable plants	Expression of BNI trait for N₂O reduction Factors that maximize the expression of the BNI trait in plants are understood. The N ₂ O reduction potential is proved in BNI capable plants that are agronomically viable.	BNI trait optimised in systems BNI-induced N ₂ O reduction potential optimized in agronomically viable systems	BNI recognised in calculators BNI-induced N ₂ O reduction potential incorporated into national GHG inventories and on-farm GHG accounting tools
Summary of knowledge or capability gaps	Lack of knowledge of high potency sources. Lack of predictive tools to identify candidate sources. Lack of genetic knowledge and rapid screening methods.	Lack of understanding of the drivers of the expression of the BNI trait in grazed systems.	Lack of understanding of the impact of BNI-capable species or mixed swards on other important outcomes. Lack of understanding of impacts of treading-induced changes in aeration and exudation on BNI.	Lack of consistent and robust evidence on BNI technology for mitigating N ₂ O emissions.
Selected key research questions	What high potency BNI sources are available for pasture species? Are those sources amenable to plant breeding?	What conditions maximize the expression of the BNI trait in agronomically viable temperate species? How does the BNI trait expression change in urine patches, and in legume-based pastures?	How can the use of BNI-capable plants be optimized in livestock systems to optimize agro-economic and environmental benefits? How to balance N fertility to promote BNI expression without compromising yield.	What is the effect of incorporating BNI-capable plants in livestock systems on the annual N ₂ O emission factors for urine and other N sources?
Approach to fill knowledge gaps	Develop and apply consistent rapid screening methods to survey within and among agronomically viable species for BNI potency and N ₂ O reduction potential.	Screen BNI-capable plants under different conditions to elucidate relationships between key variables and BNI trait expression to maximize efficacy. Measure N ₂ O and gross N transformation rates and combine with microbial and metabolomic analysis.	Field studies measuring the effect of the management of BNI-capable plants on a range of agro-economic and environmental metrics. Grazing studies to measure the effect of grazing pressure on soil aeration and root exudation, and subsequently N ₂ O emissions.	Comprehensive validation under range of conditions quantifying the effect of BNI on N ₂ O emission factors for urine and other N sources.

