EDITORIAL

Recommendations about soil Biological Nitrification Inhibition (BNI) studies

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In 2019-2021, Biology and Fertility of Soils published 14 research articles focusing on BNI and added an additional publication in the form of a Special Issue on "Biological Nitrification Inhibition", which included an editorial, two reviews, a position/opinion paper and nine research articles (see *Biology and Fertility of Soils* 58 issue 3, 2022), reflecting the broad research interest in this topic. Moreover, a literature search on Scopus using the term "BNI" returned 103 documents, 97 of which were research articles and 6 were reviews. Published BNI studies fall broadly into two categories: 1) those that focus on screening and 2) those focusing more on testing BNI in soil systems, with some studies including both aspects of BNI research. The main goal of screening studies is to identify plants with BNI potential by collecting and subsequently chemical characterization of

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compounds capable of inhibiting ammonia oxidation in pure culture of nitrifiers. An unquestionable merit of these studies is that they have led to the discovery of several compounds with BNI potential, clarification of the release mechanisms as well as their inhibitory capacity of pure culture of nitrifiers. Returning to the testing of BNI in soils, a common aspect shared by these studies is that the effects of either plants with BNI capacity or BNI compounds are assessed using DNA-based approaches coupled with measurements of potential nitrification activity and/or net nitrification rate (i.e. the balance between production and consumption of NO_3^-). Here, decrease in abundances of ammonia oxidisers, i.e. ammonia oxidizing archaea (AOA) and ammonia oxidising bacteria (AOB), estimated by quantitative PCR (qPCR), along with reduced soil NO_3^- concentrations, net nitrification rates or potential nitrification activity, is generally interpreted as support or evidence for BNI (Kaur-Bhambra et al. 2022). However, both DNA-based approaches and net rate measurements have important limitations that BNI researchers need to recognize before inferring causation (Stark and Hart 1997; Nardi et al. 2020). Drawbacks of these approaches are reported below.

1) DNA-based approaches, i.e. assessment of the pool of *amoA* genes, do not discriminate between DNA released from living cells and DNA released from dead cells. In addition, even if sourced from living cells, the detection of the gene does not necessarily imply its expression. Therefore, changes in gene abundances only mirror changes in the functional potential and not activity (Nannipieri et al. 2020). Limitations of DNA-based approaches may partially be circumvented if combined with measurements of gene transcripts, i.e., *amoA* mRNA, with the assumption of shorter half-life of transcripts compared to that of genes. Undoubtedly, BNI studies would benefit from the concurrent analysis of gene abundance, transcripts and the transcripts/gene abundance ratio, the latter being a proxy of gene

expression. However, as the detection of gene transcripts mirrors the potential to produce protein, changes in transcripts abundances may not necessarily reflect *in situ* activity changes (Nicol et al. 2008). Using the transcript/gene abundance ratios instead of transcript abundance alone may better describe transcriptional activity and is therefore more related to the metabolic activity of the cells (Freitag and Prosser 2009; Nicol and Prosser 2011). However, factors influencing the extent to which the above mentioned RNA-based analyses reflect enzyme activity should be recognized and results interpreted with caution (Prosser and Nicol 2008).

2) As mentioned above, DNA-based approaches are coupled with measurements of potential nitrification activity (PNA) or net nitrification, also known as potential nitrification rate (PNR), and net nitrification rate. However, neither PNA nor net nitrification rates provide information about the *in situ* nitrifying activity, which can be reflected by the gross nitrification rate (i.e. production of NO_3^-) using ^{15}N isotope techniques (see below). In addition, as the PNA is biased toward favoring AOB (Hazard et al. 2021), its use is not recommended neither to infer BNI nor to study the effects of BNI on ammonia oxidizers because of the high risk for drawing misleading conclusions. Net nitrification quantifies the balance between productive and consumptive NO_3^- processes, with values ranging from negative to positive, while it does not provide information on gross production of NO_3^-, for which negative values are impossible. Gross NO_3^- production may occur but does not necessarily lead to NO_3^- accumulation if NO_3^- consuming processes such as immobilization, plant uptake, denitrification, dissimilatory nitrate reduction to ammonium (DNRA) and NO_3^- leaching are higher (Davidson et al. 1992).

These aspects cannot be ignored by BNI researchers, as BNI implies the reduction of gross nitrification rate. It is therefore surprising that despite these limitations, and with few exceptions (Subbarao et al. 2009; Vázquez et al. 2020; Egenolf et al. 2022; Lan et al. 2022; Teutscherová et al. 2022), measurements of net nitrification rates still dominate BNI literature. BNI can be defined as the property of certain plants that determine a reduction of gross nitrification through direct effects on nitrifying microorganisms. Therefore, it is crucial for BNI to understand 1) whether plants inhibit *in-situ* nitrification activity, i.e. gross nitrification, and 2) what the driving mechanisms are, which are not yet clear. Answering these questions will provide mechanistic insights into BNI and at the same time deepen our knowledge of how the soil N cycling is influenced by the interactions between plants and microorganisms. To address these questions, hypotheses about gross nitrification inhibition and inhibition mechanisms i.e. direct or indirect (Nardi et al.

2020), should be clearly stated and tested using appropriate measurements of *in-situ* activity, e.g. via ^{15}N tracing techniques. A first hypothesis in BNI research should relate to whether NO_3^- production, i.e. gross nitrification, is inhibited. If experimental results support this hypothesis, the driving mechanism of inhibition, i.e. direct or indirect inhibition, must be clarified. Here, specific hypotheses can be made about the competition between nitrifiers and other microorganisms or plants. Since all individual gross N fluxes in soil-plant systems occur simultaneously, they should also be determined simultaneously. This requires advanced ^{15}N tracing tools (Müller et al. 2004, 2007; Kelly and Wood 2006; Inselsbacher et al. 2013; He et al. 2020; Jansen-Willems et al. 2022), that can unambiguously quantify concurrent gross N transformations and determine whether direct (BNI) or indirect mechanisms cause nitrification inhibition in the rhizosphere.

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