Title

Enhancing Agroecosystem Nitrogen Management: Microbial Insights for Improved Nitrification Inhibition

Authors

Fabian Beeckman[1,2,a], Laure Annetta[1,2,a], Mario Corrochano-Monsalve[3,4,a], Tom Beeckman[1,2], Hans Motte[1,2,*]

Affiliations

[1]Department of Plant Biotechnology and Bioinformatics, Ghent University, Technologiepark 71, 9052 Ghent, Belgium.

[2]VIB Center for Plant Systems Biology, Technologiepark 71, 9052 Ghent, Belgium.

[3] Departamento de Genética, Antropología Física y Fisiología Animal, Facultad de Ciencia y Tecnología, Universidad del País Vasco (UPV/EHU), Leioa, Spain.

[4]Instituto Multidisciplinar Para el Estudio del Medio ‘‘Ramon Margalef’’, Universidad de Alicante, Carretera de San Vicente del Raspeig s/n, 03690 San Vicente del Raspeig, Spain.

[a]These authors equally contributed to this work.

*Corresponding author: hans.motte@psb.ugent.be

Abstract

Nitrification is a key microbial process in the nitrogen cycle that converts ammonia to nitrate. Excessive nitrification, typically occurring in agroecosystems, has negative environmental impacts, including eutrophication and greenhouse gas emissions. Nitrification inhibitors (NIs) are widely used to manage nitrogen in agricultural systems by reducing nitrification rates and improving nitrogen use efficiency. However, the effectiveness of NIs can vary depending on the soil conditions, which in turn affect the microbial community and the balance between

different functional groups of nitrifying microorganisms. Understanding the mechanisms underlying the effectiveness of NIs and how this is affected by the soil microbial communities or abiotic factors is crucial for promoting sustainable fertilizer practices. Therefore, this review examines the different types of NIs and how abiotic parameters can influence the nitrifying community, and as such the efficacy of NIs. By discussing the latest research in this field, we provide insights that could facilitate the development of more targeted, efficient, or complementary NIs that improve the application of NIs for sustainable management practices in agroecosystems.

1. Introduction

Agricultural systems rely heavily on the application of ammonium (NH_4^+)-containing fertilizers to achieve high productivity [1]. However, substantial amounts of the applied nitrogen (N) are lost, leading to environmental concerns and inefficiencies in N utilization [2]. This loss is strongly affected by ammonia (NH_3) oxidation, the first and rate-limiting step in nitrification and a crucial part of the global N cycle [3,4]. The oxidation of NH_3 in agroecosystems is mainly performed by chemolithoautotrophic microorganisms that require NH_3 as an energy source, but also heterotrophic microorganisms that are primarily dependent on organic compounds, may oxidize NH_3 as a secondary metabolism [5,6]. Whereas the biochemistry of heterotrophic NH_3 oxidation is diverse and poorly understood [6], chemolithoautotrophic NH_3 oxidation is much better characterized. Here, NH_3 is oxidized into nitrite (NO_2^-) via hydroxylamine (NH_2OH) and nitric oxide (NO), through the consecutive actions of the ammonia monooxygenase (AMO) enzyme and hydroxylamine dehydrogenase (HAO) or through yet to be identified enzymes (Figure 1) [7,8]. In the second step of nitrification, NO_2^- is converted into NO_3^-, which can leach from the soil [9]. NO_3^- can also be converted back to NO_2^- and ammonium (NH_4^+) through DNRA (Dissimilatory Nitrate Reduction to Ammonium) [10], but more likely, NO_3^- is further processed to NO and nitrous oxide (N_2O) through anaerobic denitrification (Figure 1) [4,10]. N_2O can also be produced through nitrifier denitrification or via biotic/abiotic conversion of metabolic intermediates [7]. Soil oxygen (O_2) levels determine whether nitrifier denitrification or anaerobic denitrification (so-called coupled nitrification-denitrification) occurs [8,11,12]. The formed N_2O is a precursor of ozone-depleting nitrogen oxides (NOx) and a strong greenhouse gas [8]. Nonetheless, N_2O can be reduced to innocuous dinitrogen gas (N_2) by bacteria possessing the nitrous oxide reductase enzyme (N_2OR), encoded by *nosZ* [4,13]. Finally, atmospheric N_2 might be fixed by endosymbiotic or free-living microorganisms that express *nifH*, encoding nitrogenase, to reduce N_2 to NH_3 [4].

In a 'closed' N-cycle, < 10% of NH_3 is oxidized to NO_3^- [8]. This contrasts with agroecosystems, where large anthropogenic N inputs have changed the balance of different N-cycle microorganisms, resulting into a shift towards nitrification and denitrification. As such, N fertilization leads to significant N_2O emissions and NO_3^- leaching from the soil, resulting in climate change, and severe environmental pollution [2,14]. This is obviously problematic and calls for measures to reduce greenhouse gas emissions from agriculture, preferably without lowering crop productivity and hence human nutrition.

One highly cost-effective approach to reduce N_2O emissions involves employing nitrification inhibitors (NIs) to block NH_3 oxidation [15]. These inhibitors have moreover been demonstrated to decrease NO_3^- leaching, while having in general positive effects on crop yield [16,17]. The current portfolio of approved NIs is however limited, and their efficiency varies between fertilizer strategies, soils, crops and climate regions [16]. Multiple recent studies advanced our current understanding of nitrification and the actions of (novel) inhibitors or addressed how abiotic factors shape the soil NH_3 oxidizing microbial community. These findings offer new insights into the factors affecting NI efficacy. Therefore, we synopsize the current literature, and discuss (1) the main actors of nitrification in agroecosystems and their respective NH_3 oxidation pathways, (2) how distinct molecular and chemical aspects of NIs affect their target-specificity, and finally (3) integrate how abiotic factors shape the NH_3 oxidizing microbial community and as such determine the efficiency of different NIs. Ultimately, this synthesis provides guidance for future research, aiming to develop new, enhanced, or complementary NIs. It will furthermore support a more rationalized selection of the appropriate (combination of) NIs for the right conditions.

2. Nitrification in agroecosystems: actors and pathways

NH_3 oxidation, the initial and rate-limiting step in nitrification (see above), is mainly performed by three co-occurring chemolithoautotrophic microorganisms [5]: ammonia-oxidizing bacteria (AOB), ammonia-oxidizing archaea (AOA), and complete ammonia-oxidizing (comammox) bacteria (Figure 1) [18]. The latter, together with nitrite-oxidizing bacteria (NOB), can complete nitrification and convert NO_2^- into NO_3^-.

AOB belong to the class of *Betaproteobacteria*, represented by the genera *Nitrosomonas* and *Nitrosospira*, or the class of *Gammaproteobacteria*, with the less soil-relevant genus

Nitrosococcus. AOA comprise only one class, *Nitrososphaera*, belonging to the phylum of *Nitrososphaerota* [8,19]. Finally, comammox bacteria all belong to the *Nitrospira* genus lineage II, including an A- and B-clade, which are all present in soil, but only clade A members were isolated so far [20,21].

The three groups of chemolithoautotrophic ammonia-oxidizers all have an AMO enzyme that catalyzes the oxidation of NH_3 to NH_2OH, which is subsequently oxidized to NO and finally to NO_2^- through an unidentified component (Figure 1) [4,8,22]. AMO enzymes in AOB and AOA harbor copper (Cu)-containing active sites (Figure 1), which suggests an important role of Cu in NH_3 oxidation [18,23,24]. This is further supported by the fact that NIs are often Cu-chelators, though particularly those targeting AOB. Indeed, although AOA growth is inhibited by reduced Cu availability [25], they are not sensitive to inhibition by Cu-chelators [26]. Another difference concerns NH_3 oxidation kinetics: while cellular NH_3 affinities are mostly higher in AOA compared to AOB, AOA are more quickly saturated than AOB, implying an advantage for AOB or AOA at higher or lower NH_3 levels, respectively (Figure 2) [8]. Still, there is a lot of variation in NH_3 oxidation kinetics within AOA, and certain AOA, for instance *Ca.* Nitrosocosmicus franklandus, show similar cellular NH_3 affinities as their bacterial counterparts [27]. In AOA, a NirK enzyme is suggested to function as NH_2OH-oxidizing or NO-oxidizing enzyme (Figure 1). Here NO would act as a co-substrate of NH_2OH and together form two NO_2^- molecules [28]. Alternatively, NO might act as an intermediate metabolite to form one NO_2^- molecule [29]. NO-scavengers like PTIO (2-phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl 3-oxide) are strong AOA inhibitors [26], confirming the importance of NO in the archaeal nitrification (Figure 1; Table 1).

Finally, for comammox, the importance of Cu is not clear. In contrast to AOA, several Cu-chelators are able to inhibit comammox nitrification (Figure 1) [26]. Comammox bacteria show high sequence similarity of *amo* and *hao* genes with AOB [30]. In general, comammox AMO shows a higher cellular NH_3 affinity, even higher than AOA. This corresponds to higher yield, but slower growth rates [21].

Next to the chemolithoautotrophic ammonia-oxidizers, also heterotrophic nitrifiers may contribute to NH_3 oxidation. Heterotrophic nitrifiers, encompassing both bacteria and fungi, use diverse but poorly characterized nitrification pathways [6,31]. Heterotrophic nitrifiers commonly oxidize organic N, but certain heterotrophic bacteria and fungi also oxidize NH_3

[6,31]. Some heterotrophic bacteria have an AMO enzyme, but their *amo* genes are not targeted by PCR primers designed for autotrophic nitrifiers, and general primers for heterotrophic AMO are lacking [6]. Additionally, no specific inhibitors of heterotrophic nitrifiers are known. This, together with their diversity in biochemistry makes it complicated to estimate the contribution of heterotrophic nitrifiers to the overall nitrification or NH_3 oxidation [6]. Often, the residual nitrification occurring when chemolithoautotrophic NIs are applied is considered to be heterotrophic nitrification, but this assumes that those NIs block all chemolithoautotrophic nitrification in soil, which is highly unlikely. ^{15}N labelling could be used to distinguish heterotrophic nitrification from organic N versus NH_3 oxidation, but not to distinguish chemolithoautotrophic NH_3 oxidation versus heterotrophic NH_3 oxidation [6]. In other words, it is currently unknown to what extent heterotrophic nitrifiers contribute to NH_3 oxidation in soil.

3. Nitrification inhibitors: different types and targets

NIs are valuable tools to reduce N-losses and the environmental impact in modern agricultural practices (see above). Currently, however, only a handful NIs are registered to be used in Europe (https://echa.europa.eu/nl/fertilizer-list-ann-1-f) or US (https://www.npirs.org/ppis/), including the commonly applied DCD (dicyandiamide), DMPP (3,4-dimethylpyrazole phosphate), DMPSA (3,4-dimethyl pyrazole succinic acid), and nitrapyrin (2-chloro-6-(trichloromethyl)-pyridine). More NIs are described in literature, often for laboratory use only, and novel NIs are still developed or identified (e.g. [26,32]), including new synthetic nitrification inhibitors (SNIs) and biological nitrification inhibitors (BNIs). BNIs, naturally occurring molecules, are often plant metabolites (see Table 1 for examples) [33]. As these different types of NIs show distinct chemical and molecular properties, we here discuss how this potentially defines their microbial targets, and as such their efficiency.

Multiple NIs, including DCD, DMPP and DMPSA, exhibit Cu-chelation properties and, owing their specific action on the AMO enzyme, are presumed to impede the function of Cu as a co-factor (Figure 1; Table 1) [26,34,35]. The chelating capacity as such is however not sufficient. DMPSA for example, despite being a Cu-chelator, only inhibits nitrification after degradation to DMP [36]. Still, Cu-DMP complexes easily dissociate, indicating a weak binding [34], and analysis in pure bacterial cultures reveals that DMP efficacy remains unaffected even in a Cu-saturated medium [36]. This could indicate that either (1) the mode of action is not reliant on the chelation capacity of DMP or (2) that the presence of Cu in the medium does not influence

the process, for instance, because DMP might directly target the enzyme's active site or because the intracellular DMP:Cu ratio might be different than the ratio in the environment.

The SNI nitrapyrin does not chelate Cu but is thought to directly block the Cu-binding site of the AMO enzyme [37]. Although experiments showed that nitrapyrin targets both AOA and AOB *in vitro* (Figure 1) [38], this might not be the case in soil. Indeed, nitrapyrin affects AOB but not AOA abundance in soil (Figure 1) [39], while co-application of nitrapyrin with an AOA-targeting NI improves the nitrification inhibition [26], suggesting that nitrapyrin targets mainly AOB in soil.

For most other NIs, the mechanism of action or target specificity is less clear (Table 1). Few novel NIs also chelate Cu and act specifically on the AMO enzyme [26]. Certain BNIs specifically target this AMO enzyme as well, but others seem to have a more general action, and target at least both the AMO and HAO enzyme (Table 1) [33]. Even though some target both AOB and AOA (Table 1), they might also show more unspecific responses. MHPP for example, is even shown to interact with the plant auxin signaling (Figure 1; Table 1) [40] and could cause undesired side-effects when applied to the field. This is not surprising, as BNIs often are metabolites with other functions in their source organism [33]. This advocates for careful evaluation of such molecules before possible use in the field.

Also some simple, synthetic molecules such as alkynes are able to perform nitrification inhibition (Table 1) [41,42]. 1-alkynes are thought to compete with NH_3 at the NH_3-binding site of the AMO enzyme [41,42]. Interestingly, short-chain alkynes (e.g. acetylene) inhibit both AOB and AOA, whereas the long-chain variants (e.g. 1-octyne) target only AOB (Figure 1; Table 1) [41,43]. This indicates that archaeal AMO has a narrower substrate range and might partially explain why AOA-specific NIs are difficult to discover and almost not available.

Indeed, only few AOA-targeting NIs are described. For nitrapyrin, which is not registered for use in Europe, there are doubts about its action on AOA (see above). Different short-chain alkynes target both AOA and AOB but are impractical to use in agriculture because of their gaseous state while they are also highly unspecific. They even inhibit, amongst others, N_2OR thereby preventing reduction of N_2O to N_2 (Figure 1) [44]. As such, they are only used in experimental settings. PTIO, an NO-scavenger often used in lab-scale experiments, inhibits AOA and comammox bacteria (Figure 1) [26,45]. Its weak or absent responses in soil [18,46],

and its possible interference with other pathways [47,48] makes it unsuitable to be used in agricultural settings. Recently, new archaeal NIs were discovered, including ethoxyquin (EQ) and its derivate 2,6-dihydro-2,2,4-trimethyl- 6-quinone imine (QI), possibly acting as an NO-scavenger as well [32,49,50], simvastatin, which interferes with the archaeal membrane biosynthesis and hence is not specific for AOA [51], or 4-[1,6-bis(propan-2-yl)-1H-pyrazolo[3,4-b]pyridine-4-carbonyl]thiomorpholine (SIAS) that was identified in a high-throughput screen using the AOA *Nitrososphaera viennensis* (Table 1) [26].

Finally, no NIs that target heterotrophic nitrifiers were described. Although heterotrophic nitrification seems to be limited in agroecosystems, this type might be important in specific conditions [52]. Hence, whereas heterotrophic nitrifier-targeting NIs would in any case be useful to better understand the contribution of heterotrophic nitrifiers (see also above), their discovery and application might further optimize nitrification inhibition and reduce environmental N-pollution.

Table 1: Origin, mode of action, targets, and effects on microbiota of nitrification inhibitors.

	Compound	Origin	(Putative) mode of action	Microbial target	(Presumed) enzyme target	Other functions / Non-target effects	Ref.
BNIs	*1,9-decanediol*	*Oryza sativa*	Unknown	AOB + AOA	AMO	Promotes root growth in *Arabidopsis* through ABA and PIN2-mediated auxin signaling	[53–55]
	Brachialactone	*Brachiaria humidicola*	Unknown	AOB	AMO + HAO		[56]
	MHPP	*Sorghum bicolor*	Unknown	AOB + AOA	AMO	Interacts with auxin signaling in *Arabidopsis*	[40,57]
	Sakuranetin	*Sorghum bicolor*	Unknown	AOB + AOA	AMO + HAO	Phytoalexin of rice	[55,58]
	Sorgoleone	*Sorghum bicolor*	Unknown	AOB	AMO + HAO	Allelopathic compound; phytotoxic; and herbicide / Wide range effects	[58–60]
SNIs used in agriculture	DCD	Synthetic	Cu-chelator	AOB	AMO	Phytotoxic	[32,35,38,61–64]
	Nitrapyrin	Synthetic	Binds to Cu-binding site	AOB + AOA(?)	AMO		[32,37,38,62,65–67]
	DMP(P)	Synthetic	Cu-chelator	AOB	AMO		[34,63,68–71]
	DMPSA	Synthetic	Cu-chelator, precursor of DMP	AOB	AMO		[34,71,72]
SNIs used in laboratories	Acetylene	Synthetic	Competitive inhibitor	AOB + AOA	AMO	Inhibitor of other monooxygenases and nitrous oxide reductase	[41,42,44]
	1-octyne	Synthetic	Competitive inhibitor	AOB	AMO		[41,68,73]
	PTIO	Synthetic	NO-scavenger	AOA			[38,74–76]
	SIAS	Synthetic	Unknown	AOA			[26]
	Simvastatin	Synthetic	Blocks archaeal cell wall synthesis	Archaea (incl. AOA)		Other archaea	[51]
	EQ/QI	Synthetic	Antioxidant, NO-scavenger	AOB + AOA			[32,49,50]

Abbreviations: BNI: biological nitrification inhibitor; SNI: synthetic nitrification inhibitor; MHPP: methyl 3-(4-hydroxyphenyl) propionate; DCD: dicyandiamide; DMP(P): 3,4-dimethyl-1H-pyrazole (phosphate); DMPSA: 2-(3,4-dimethyl-1h-pyrazol-1-yl) succinic acid; SIAS: 4-[1,6-bis(propan-2-yl)-1H-pyrazolo[3,4-b]pyridine-4-carbonyl]thiomorpholine; PTIO: 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide; AMO: ammonia monooxygenase; HAO: hydroxylamine oxidoreductase.

4. Soil microbial community-dependent variability in nitrification inhibition efficiency

The efficiency of NIs varies between soils, and hence seems to depend on the microbial community present, possibly controlled by abiotic factors. A better knowledge on these affecting factors could help in predicting the nitrifying community, and as such which type(s) of NI should be used on certain fields to maximize their efficiency.

Several recent correlation studies between environmental factors, soil characteristics, microbiome and/or NI efficiency aimed to assess these effects (e.g. [3,63,68,69,77–84]), but the results are not always clear and sometimes contradictory between different studies. A main limitation in such studies is the throughput in which nitrification inhibition efficiency is tested in soil: as soil nitrification assays are relative labor intensive, such studies often evaluate only one or two nitrification inhibitors in a limited set of soils, often only two to four, which makes it hard to draw strong conclusions. Furthermore, for now, such studies mostly make use of the not applicable acetylene and octyne, or one of the commonly applied NIs DMPP or DCD, while it would be of interest to compare more NIs and assess their efficiency in relation to different parameters. Nevertheless, some studies show interesting correlations, though to be taken with caution.

The organic matter or clay content, for example, seems to both be negatively correlated with DCD efficiency (Figure 1) [82,83], possibly due to sorption of this NI by clay particles and organic matter, resulting in less mobility and availability. Nitrapyrin also shows high absorption to organic matter [85], whereas DMPP shows less sorption to the soil matrix than DCD [86,87]. Hence, DMPP or possibly other NIs that show low adsorption might be preferred in clay soils or soils with high organic matter. Such parameters could also affect the NI efficiency through their effects on the microbial community, more particular on the abundance and the composition of the ammonia-oxidizing community. Organic matter, for example, can be used by heterotrophic nitrifiers, which may compete with the chemolithoautotrophic ammonia-oxidizers, but are not inhibited by any NI [6,88]. This possibly results in less efficient nitrification inhibition in soils with high organic matter. Likewise a low pH, a high C:N ratio, and even the presence of certain plants generally favor heterotrophic nitrification and might as well affect NI efficiency [5,31,88]. Low pH and high C:N ratios are often found in non-agricultural environments, for example in forests [52]. As a result, agricultural fields with a balance towards heterotrophic nitrification are typically fields that were converted from forests

[55 and references therein]. Hence, efficient nitrification inhibition after land use change might require novel types of NIs that are currently not available.

Although it is difficult to measure, especially their contribution to NH_3 oxidation, heterotrophic nitrifiers have in general a limited contribution to nitrification in agricultural settings [5]. In particular when ammonium-containing fertilizers are applied, which do not provide a substrate for heterotrophs, heterotrophic nitrification will not, or hardly, contribute to the NH_3 oxidation. In contrast, high N-fertilization actually gives AOB a competitive advantage over other ammonia-oxidizing microorganisms because of their capacity to oxidize more NH_3 (Figure 2, Figure 3A, B) [8,89]. In any case, the N level in the soil significantly stimulates the overall ammonia-oxidizing community and is the most important determinant of nitrification rates [3]. As such, agricultural fields with a significant fertilization history generally exhibit high nitrification rates, in contrast to forests or recently converted fields [52]. Hence, although the latter hosts a dominant heterotrophic nitrifying community contributing to the gross nitrification, the total nitrification will be limited.

Other conditions may still shift the balance within the ammonia-oxidizing community. A major determinant for this balance is pH. Whereas a more alkaline pH positively affects nitrification [5], AOA usually outnumber AOB in acidic soils (Figure 2) [8]. Thus, pH seems to shift the AO community, which could affect NI efficiency. Indeed, at least for the AOB-targeting NIs DMPP, DCD or 1-octyne, different studies show that pH is a major factor that affects the NI efficiency [63,68,69,79–81]: in particular in acidic soils the nitrification inhibitory effect is generally weak. Because acetylene, targeting both AOA and AOB, does show a stronger inhibitory effect in acidic soils [68,80,81], a shift towards AOA-dependent nitrification seems to explain the weak effect of DMPP, DCD and 1-octyne, and advocates for co-application of AOA- and AOB-targeting nitrification inhibitors on acidic arable lands. Importantly, also in acidic soils, AOB may significantly contribute to nitrification [90]. This might partially be explained by the presence of AOB adapted to low pH: a recently isolated AOB was even able to grow at pH 2.5 [91]. Conversely, certain AOA strains are able to grow at high NH_3 concentrations [27,92] and AOA might dominate nitrification in alkaline soils [93], showing that predicting the actual contribution of each nitrifier group remains a challenging task. This is even more difficult for comammox bacteria. At least the currently isolated comammox bacteria show an NH_3 affinity that is higher than those of AOB and AOA, despite their lower oxidation rate [21]. This argues for a greater relevance only at more extreme conditions, and

therefore they could be considered less competitive than other ammonia-oxidizers in agricultural systems. Still, although several studies showed only marginal contributions from comammox bacteria [94,95], other studies show a possible importance [96,97], and a strong response of newly identified comammox strains to N fertilization [92]. This indicates a potentially more copiothropic lifestyle of at least some previously unknown comammox bacteria. Nevertheless, as comammox bacteria are inhibited by both AOA and AOB inhibitors [26,45], it is unlikely that their presence would affect NI efficiency.

Besides fertilization and pH, also the application of NIs themselves is an important driver of shifts in the AO community. Indeed, several studies report an induction of AOA by an AOB-targeting nitrification inhibitor, also in alkaline soils [63,68,79,81,89], probably due to the reduced competition for the NH_3 substrate (Figure 2). The opposite is true as well: inhibition of AOA results in an increase of AOB [26,51] (Figure 2). As such, nitrification inhibition efficiency is actually reduced by the use of one specific NI. In other words, it seems that co-application of AOA- and AOB-targeting NIs could optimize nitrification inhibition efficiency in any condition [26] (Figure 3C, D).

Cu-content in soil could also play a vital role in nitrification (inhibition). Indeed, higher amounts of Cu positively affect AOB abundance and nitrification rate (Figure 3A-B) [34]. It could be hypothesized that high Cu-content affect the efficacy of a copper chelating NI. At least a negative correlation between NI efficiency of the Cu-chelating DCD and Cu-content of soil was found [83], though this might also be attributed to a higher nitrification rate in such soils. Indeed, the negative correlation was also observed with other parameters that affect the nitrification rate [83], while slightly higher soil Cu contents do not per se change the efficiency of Cu-chelating NIs [34]. Remarkably, and commonly observed, is an unexpected increase in the abundance of *nosZI* after the application of Cu-chelating NIs, which is expected to result in higher complete denitrification rates and, consequently, reduced N_2O emissions (Figure 3C-D) [63,69,98]. Like AMO, the *nosZI*-encoded N_2OR enzyme utilizes Cu as a co-factor [99]. Therefore we speculate that NIs positively affect N_2O reducing bacteria due to reduced Cu competition with inhibited AOB populations (Figure 3C-D)[100]. This further implies that NIs not only reduce N_2O emissions by inhibiting nitrification but also by stimulating the conversion of N_2O to N_2.

Finally, increased temperature positively affects nitrification rates [3,101], presumably due to positive effects on the AOA community [77,84], which could explain a lower efficiency of both DMPP and DCD at higher temperatures (Figure 1) [78,83]. This again advocates for a co-application of AOA- and AOB-targeting NIs when temperatures are expected to increase due to global warming.

5. Concluding remarks and perspectives

Overall, the use of NIs is an important tool for sustainable agriculture, but different factors affect the effectiveness of NIs. Organic matter, N level, pH, Cu level and temperature are clearly main determinants of the nitrification rate or the nitrifying community composition and may affect NI efficiency. However, most available studies focus only on a limited set of soils and/or inhibitors which complicates the interpretation of correlations between different soil parameters and the efficacy of different NIs. Therefore, future research should be done testing larger sets of NIs on a wider range of soils, preferably under more controlled lab conditions (in contrast to more variable field conditions). As this implicates highly labor-intensive research, more efficient soil assays would be valuable. Combined with soil microbiome analyses, this could lead to the development of improved (combinations of) NIs, or enable site-specific management by selecting optimal NIs based on the site characteristics. It is for instance clear that AOA and heterotrophic nitrifiers significantly contribute to nitrification under certain conditions. Therefore, there is a need for new inhibitors that specifically target these different nitrifiers to ensure sustainable nitrogen management, and to be able to accurately quantify their relative contributions to nitrification.

Possibly more important however, there is also a need for a changing policy. Improved crop yield alone does not always justify the increased cost of a NI-containing fertilizer, and farmers do not receive direct financial benefits from greenhouse gas emission reductions. Moreover, NIs may also be useful to block nitrification in conditions where no crops are growing, for example during tillage or on grasslands, solely to reduce greenhouse gas emissions. The use of NIs in such scenarios could be an important sustainable agricultural practice, but would require policies, like financial incentives or subsidies (e.g., taxing N emissions, or reducing taxes on enhanced-efficiency fertilizers), encouraging farmers to adopt such practices. Likewise, policies can also encourage the development and use of new nitrification inhibitors that are more effective.

Also BNIs can be of great value in the future, in particular because their production in plants or even crops may facilitate alternative, possibly more efficient, application of NIs. But for this, it will be important to know which crops produce and release BNIs and under which conditions this occur. Intercropping or mixed cropping planting might be very valuable but awaits further validation and optimization research.

In summary, the optimal use or application of NIs requires a multifaceted approach, including continued research to better understand their mechanisms of action and interaction with soil microbial communities, policy support to promote sustainable agricultural practices, and the exploration of alternative NIs to reduce N pollution more efficiently.

Outstanding questions

- **About the long-term effects:** What are the long-term effects of nitrification inhibitors (NIs) application? Could nitrifying communities develop mechanisms to evade their action, leading to reduced efficacy in the future? Would it be advisable to alternate between different types of inhibitors in each season? How persistent are NIs in the environment and what are the effects on humans and animals?
- **About legislation:** How can farmers be incentivized to adopt NI practice? Should we legislate for the mandatory application of inhibitors?
- **About application:** Can we predict the most appropriate NI (combination) to be used in agroecosystems based on soil type and/or soil microbial community? How does soil copper content affect NI efficiency? Could we ensure/manipulate/stimulate biological NIs production to guarantee nitrification inhibition in crop systems?

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Figures

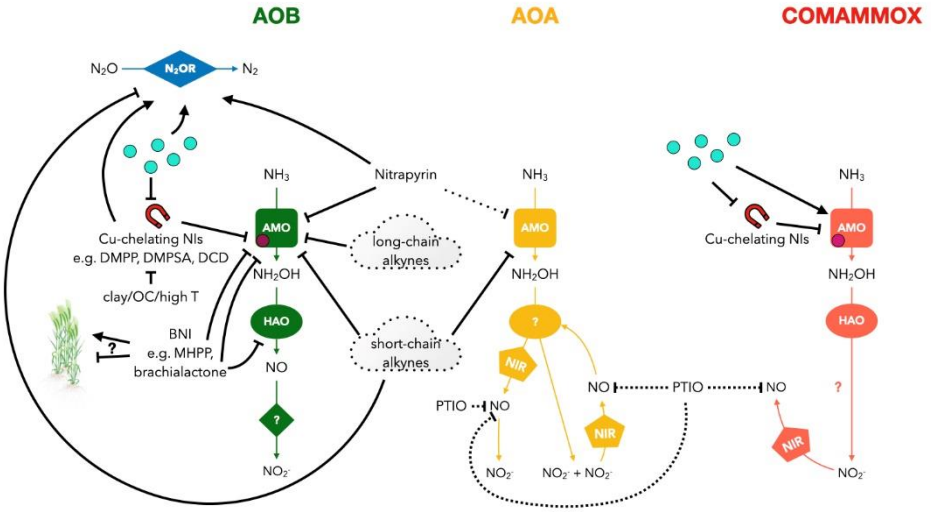

Figure 1: Targets and effects of nitrification inhibitors in different ammonia oxidation pathways. PTIO, an NO-scavenger, inhibits nitrification in AOA (in yellow) and comammox bacteria (in red), but not in soil (dashed lines). Cu (blue dots) in soil is utilized as co-factor (purple dot) by AMO. Nitrapyrin targets AOB (in green) and AOA *in vitro*, but not AOA in soil (dashed lines). BNIs inhibit AOB and/or AOA, but possibly also other (plant) pathways. Blunt arrows indicate an inhibitory effect, regular arrows indicate a stimulatory effect. Abbreviations: AOB, ammonia-oxidizing bacteria; AOA, ammonia-oxidizing archaea; comammox, complete ammonia-oxidizing; NIs, nitrification inhibitors; AMO, ammonia monooxygenase; HAO, hydroxylamine dehydrogenase; NIR, nitrite reductase; PTIO, 2-phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl 3-oxide; N_2OR, nitrous oxide reductase; OC, organic content; T, temperature; BNIs, biological nitrification inhibitors; SIAS, 4-[1,6-bis(propan-2-yl)-1H-pyrazolo[3,4-b]pyridine-4-carbonyl]thiomorpholine.

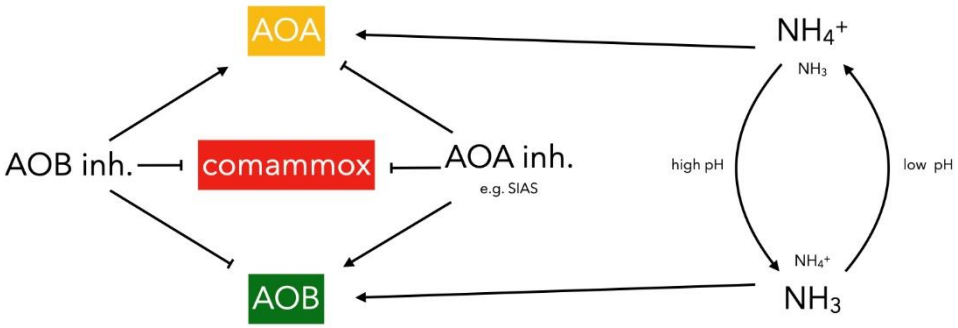

Figure 2: Potential effects of nitrification inhibitors and soil pH on abundances of different ammonia-oxidizers. A high NH_3 content favors growth of AOB over AOA and comammox bacteria, while reduced NH_3 levels (or an increased NH_4^+:NH_3 ratio), a low soil pH favors growth of AOA and comammox bacteria over AOB. Similarly, single use of a bacterial or archaeal NI reduces competition for the non-targeted ammonia-oxidizer. Blunt arrows indicate an inhibitory effect, regular arrows indicate a stimulatory effect. Abbreviations: AOB, ammonia-oxidizing bacteria; AOA, ammonia-oxidizing archaea; comammox, complete ammonia-oxidizing; SIAS, 4-[1,6-bis(propan-2-yl)-1H-pyrazolo[3,4-b]pyridine-4-carbonyl]thiomorpholine.

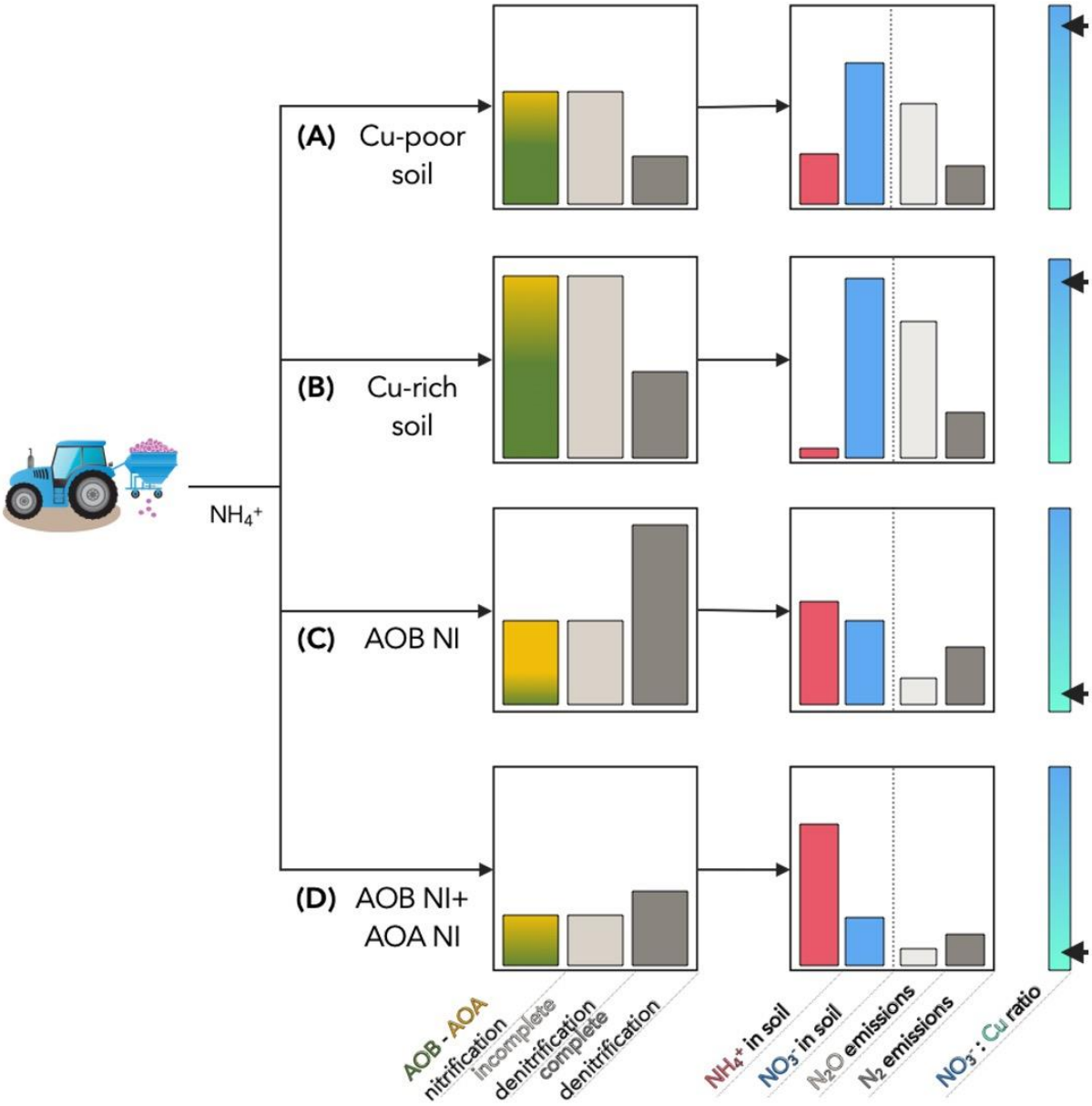

Figure 3: Speculated effect of fertilization on nitrification,(in)complete denitrification and N-compounds in different conditions. (A-B) Without inhibitor, fertilization results in both a high nitrification (mainly due to an increase of AOB as indicated in green) and incomplete denitrification (indicated in light grey) rate. The nitrification rate is expected to be lower in Cu-poor soils (A) because of Cu-limitation, and higher in Cu-rich soils (B), resulting in higher NO_3^-levels (indicated in blue), and as a result generally more N-emissions from incomplete (light grey) or complete (dark grey) denitrificationIn both situations, N_2O reducers, contributing to complete denitrification, compete with AOB for Cu and encounter a high NO_3^-:Cu ratio (as indicated in the blue to cyan gradient bar). This results in a balance towards incomplete denitrification and a high N_2O:N_2 emission ratio. (C) With a (Cu-chelating) bacterial nitrification inhibitor, nitrification will be inhibited, but AOA will increase (as indicated in yellow) and more Cu will be available for N_2O reduction due to less competition with AOB (as indicated in the blue to cyan gradient bar). As a result, less N_2O will be produced and the N_2O:N_2 ratio is expected to decrease compared to A and B. (D) The use of two types of NIs will more efficiently inhibit nitrification resulting in less NO_3^- that is available for denitrification. Abbreviations: Cu, copper; AOB, ammonia-oxidizing bacteria; AOA, ammonia-oxidizing archaea; AOB NI, bacterial nitrification inhibitor; AOA NI, archaeal nitrification inhibitor.