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*Review*

# Deciphering the Interactions in the Root–Soil Nexus Caused by Urease and Nitrification Inhibitors: A Review

**Sneha Gupta [1,*], Sibel Yildirim [2], Benjamin Andrikopoulos [2], Uta Wille [2] and Ute Roessner [3]**

[1] School of BioSciences, University of Melbourne, Parkville, VIC 3010, Australia
[2] School of Chemistry, Bio21 Institute, The University of Melbourne, 30 Flemington Road, Parkville, VIC 3010, Australia; sibelcansuy@student.unimelb.edu.au (S.Y.); bandrikopoul@student.unimelb.edu.au (B.A.); uwille@unimelb.edu.au (U.W.)
[3] Research School of Biology, The Australian National University, Acton, ACT 2601, Australia; ute.roessner@anu.edu.au
* Correspondence: snehagupta14@gmail.com

**Abstract:** Optimizing nitrogen (N) availability to plants is crucial for achieving maximum crop yield and quality. However, ensuring the appropriate supply of N to crops is challenging due to the various pathways through which N can be lost, such as ammonia ($NH_3$) volatilization, nitrous oxide emissions, denitrification, nitrate ($NO_3^-$) leaching, and runoff. Additionally, N can become immobilized by soil minerals when ammonium ($NH_4^+$) gets trapped in the interlayers of clay minerals. Although synchronizing N availability with plant uptake could potentially reduce N loss, this approach is hindered by the fact that N loss from crop fields is typically influenced by a combination of management practices (which can be controlled) and weather dynamics, particularly precipitation, temperature fluctuations, and wind (which are beyond our control). In recent years, the use of urease and nitrification inhibitors has emerged as a strategy to temporarily delay the microbiological transformations of N-based fertilizers, thereby synchronizing N availability with plant uptake and mitigating N loss. Urease inhibitors slow down the hydrolysis of urea to $NH_4^+$ and reduce nitrogen loss through $NH_3$ volatilization. Nitrification inhibitors temporarily inhibit soil bacteria (*Nitrosomonas* spp.) that convert $NH_4^+$ to nitrite ($NO_2^-$), thereby slowing down the first and rate-determining step of the nitrification process and reducing nitrogen loss as $NO_3^-$ or through denitrification. This review aims to provide a comprehensive understanding of urease and nitrification inhibitor technologies and their profound implications for plants and root nitrogen uptake. It underscores the critical need to develop design principles for inhibitors with enhanced efficiency, highlighting their potential to revolutionize agricultural practices. Furthermore, this review offers valuable insights into future directions for inhibitor usage and emphasizes the essential traits that superior inhibitors should possess, thereby paving the way for innovative advancements in optimizing nitrogen management and ensuring sustainable crop production.

**Keywords:** inhibitors; nitrification; nitrogen; nitrogen cycling; smart agriculture; plant nitrogen uptake; sustainable management; soil–root nexus; urease



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## 1. Introduction

Nitrogen (N) is essential to the survival of all living organisms [1]. The global N cycle involves the flow of atmospheric nitrogen ($N_2$) into terrestrial and marine ecosystems through N fixation, a reductive process that produces ammonia ($NH_3$) or ammonium ($NH_4^+$), which is converted by microbiological processes into higher oxidized forms of nitrogen (nitrification) and finally returns to the atmosphere as reduced, gaseous forms of nitrogen (denitrification) [2]. In contrast to the unreactive atmospheric $N_2$, the various reduced or oxidized forms of N are often described as "reactive N" ($N_r$) [3]. Despite the abundance of $N_2$ in the environment, $N_r$ is a major limitation to global net primary

productivity and food production [4]. Increasing the availability of $N_r$ is therefore essential for plant growth and food production for the continuously growing world population.

The invention of the Haber–Bosch process in the early 20th century, which enabled the catalytic hydrogenation of $N_2$ under elevated pressure and temperature to produce biologically available $NH_3$, triggered the production of N fertilizers on a large industrial scale [5]. The Haber–Bosch process is considered as the most important industrial development of the 20th century, which was recognized by two Nobel Prizes in chemistry awarded to Fritz Haber in 1918 and Carl Bosch in 1931 [6]. Industrially fixed N fertilizers revolutionized agricultural productivity worldwide, mainly through an application on staple grain crops, wheat, rice, and maize [7]. Nowadays, about 80% of the total Haber–Bosch produced N goes to plant production [8,9].

With the expected world population to hit the 10 billion mark by 2050, the reliance on industrial N fertilization will significantly increase further. While the Haber–Bosch process itself is optimized and has nearly reached thermodynamic process efficiency [10], unfortunately, the application of Haber–Bosch N in agriculture is highly inefficient. Urea is the dominating N fertilizer worldwide [11], which has a N content of 46%. Unfortunately, a high proportion (50–70% of applied $N_r$) is not taken up by the plant roots and "lost" to the environment [12,13], leading to the accumulation of nitrate ($NO_3^-$) in terrestrial and aquatic ecosystems, resulting in the contamination of drinking water and eutrophication of water bodies, which has negative impacts for many ecosystems and significant consequences for the earth's climate and environmental health [8,14–16]. In addition, the emission of gaseous $N_r$ species, such as $NH_3$, which is a precursor to potentially harmful microscopic particulate matter ($PM_{2.5}$) [17–19], and the greenhouse gas nitrous oxide ($N_2O$) into the troposphere contributes to air pollution, global warming, and the depletion of stratospheric ozone [19]. Hence, the effectiveness of N fertilizers must be significantly increased to limit environmental degradation resulting from agricultural processes.

One of the current approaches to reduce unwanted N losses to the environment and increase plant uptake is by slowing down the rate of urea hydrolysis in soils using urease inhibitors and by slowing down the subsequent microbiological nitrification process using nitrification inhibitors. The objective of this review is to provide an overview of the current knowledge of the spatial and temporal interplay of urease and nitrification inhibitors with plants and N uptake by roots, with particular emphasis on the need to develop design principles for inhibitors with improved efficiency.

## 2. Nitrogen as an Important Plant Nutrient

Nitrogen (N) is the most significant macro-nutrient element, which is included in a considerable number of nutritional and energy substrates [20]. N is crucial for the growth and development of plants, as well as for metabolic activities, such as photosynthesis, energy production, biomass production, and yield [21,22]. The enzymatic activity, chlorophyll content, photosynthesis, respiration rate, and yield of crop plants are all significantly reduced by N deficiency. Therefore, sufficient N fertilizer along with inhibitors has been added to farmland soil all over the world for decades to ensure crop production. To optimize the performance of inhibitors in agricultural systems to improve N availability with plant demand, it is necessary to understand the mechanisms by which plants influence microbial N transformations to improve nutrition, identify the N forms that plants can take up, and how N compounds are influenced by the availability of various forms of N in 89 soils and by plant properties (Figure 1). Soluble N in the form of fertilizer in which nitrogen is easily accessible for plant uptake, in soil, varies greatly over space and time, and plants are capable of exploiting any ephemeral microscale patches of N [23]. Generally, plants take up N in their inorganic forms as $NO_3^-$ and $NH_4^+$ [24] but are also capable to use N bound in organic matter [25].

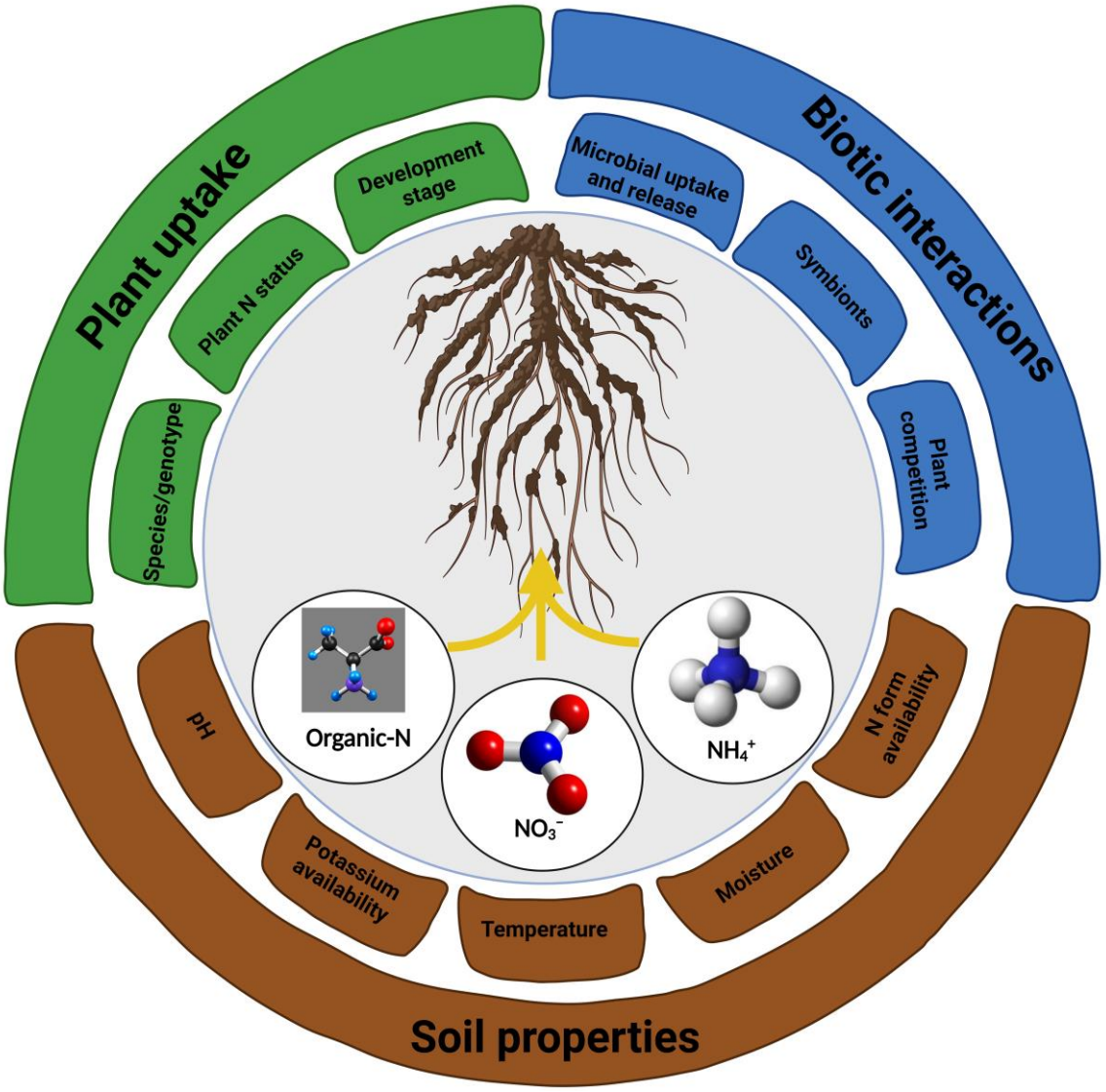



**Figure 1.** Factors affecting the nitrogen (N) forms uptake by plants.

Plant properties such as genotype act mainly via characteristics linked to transporters capable of transporting different forms of N [26] and those are susceptible to $NH_3$ toxicity [27]. Additionally, plant N status and plant growth stage can alter rates of both $NO_3^-$ and $NH_4^+$ uptake differentially [28]. Microorganisms can influence the uptake of N in plants, for instance, it has been found that competing neighboring plants [29] and competing soil microorganisms [30] may reduce the availability of certain N forms.

The capacity of plants to absorb certain forms of N can be enhanced by N-fixing symbiosis among α- and β-proteobacteria and legumes [31,32]. This symbiotic relationship between soil bacteria and legume roots is mediated by endosymbiotic interaction, where plants generate nodules (a new differentiated special organ) that help to fix atmospheric N through the action of the enzyme nitrogenase [33]. A review by Mahmud et al. (2020) [34] collates several studies on the mechanism of N fixation by legumes and suggests potential approaches to introduce these mechanisms into economically important crops, such as rice, maize, and wheat.

### *2.1. Acquisition and Assimilation of N by Plants*

In general, plants take up N in its inorganic forms such as $NO_3^-$ and $NH_4^+$ [24]. The $NH_4^+$ uptake is either active or passive, depending on its concentration in soils [35]. The

active uptake could be achieved by direct use of metabolic energy to carry a solute across a membrane toward a region of higher electrochemical potential. The passive uptake could be completed by solute flux across a membrane along the electrochemical potential gradient, in response to a favorable electrical gradient [36]. However, $NH_4^+$ is bound to negatively charged soil particles through electrostatic interactions, reducing its mobility in soils, while $NO_3^-$ is more mobile in such soil conditions and therefore principally more available for plant N uptake [24]. Uptake of $NO_3^-$ is an active, energy-demanding process as it is absorbed against an electrochemical gradient [37] and requires reduction to $NH_4^+$ for assimilation within the plant, which makes it less energetically favorable than $NH_4^+$ uptake.

Several inorganic and organic N transporters have been identified in roots with different substrate affinities and specificities. Detailed mechanisms of N root uptake with inorganic N transporters for $NO_3^-$ and $NH_4^+$ in several cereal crops can be found in the work of Tegeder and Masclaux-Daubresse [38]. In *Arabidopsis*, $NH_4^+$ acquisition by roots is accomplished by four ammonium transporters (AMTs): AMT1;1, AMT1;3, and AMT1;5 are involved in direct $NH_4^+$ uptake from the soil via the root epidermis, while AMT1;2 is expressed in cortical and endodermal cells and mediates apoplastic absorption of $NH_4^+$. Low- and high-affinity nitrate transporters mediate the $NO_3^-$ uptake in soil. The low-affinity transport system allows transport in high (>0.5 mM) external nitrate concentrations, whereas the high-affinity transport system provides a capacity for nitrate absorption at low (<0.5 mM) external nitrate concentrations [39]. The majority of the nitrate transport (NRT1) family (which is also called as the nitrate transporter1/peptide transporter family, NPF) is described as low-affinity systems [40]. In *Arabidopsis*, six transporters are involved in root nitrate uptake as shown in [38]. Of these, chlorate-resistance protein 1 (CHL1) and NPF4.6/NRT1.2 mainly operate under high nitrate supply, whereas NRT2.1, NRT2.2, NRT2.4, and NRt2.5 function under $NO_3^-$ starvation [41–48].

Plants can thrive on a wide range of dissolved organic N forms, ranging from simple, low-molecular-weight compounds, such as amino acids, nucleotides, and urea [49], to more complex polymeric material, such as proteins [50]. The uptake of organic N can differ between these simple and complex materials, and this difference can depend on various factors, such as accessibility, uptake mechanisms, and regulation and preferences [51]. These factors are elaborated here: (i) Accessibility—Simple, low-molecular-weight compounds such as amino acids are readily available in the soil solution and can be directly taken up by plant roots. They are easily absorbed through specific transporters in the root membranes [52,53]. In contrast, more complex polymeric materials such as proteins require additional steps for breakdown and assimilation. Proteins need to be enzymatically hydrolyzed into their constituent amino acids or smaller peptides before they can be taken up by plant roots [54]. (ii) Uptake mechanisms—The uptake of simple organic N compounds is often facilitated by specific transporters that recognize and transport these compounds across the root membranes [55,56]. Different transporters may have different affinities and specificities for different types of organic N compounds. In the case of polymeric materials such as proteins, specific extracellular enzymes secreted by plants or associated microbes are involved in the breakdown of proteins into smaller components. Once broken down, the resulting amino acids or peptides can be taken up by plant roots through the same transporters used for simple organic N compounds [51,57]. (iii) Regulation and preference—Plants possess regulatory mechanisms that can modulate the expression of transporters and enzymes involved in the uptake and utilization of different organic N forms. They can adjust their nutrient uptake strategies based on the availability and relative abundance of different organic N compounds in the soil. In some cases, plants may exhibit preferences for specific types of organic N compounds based on their metabolic requirements or environmental conditions [51,56,57].

Uptake of organic N is the least energy-consuming pathway for the plant, as no assimilation process is required [23]. Organic N can enter the roots through passive diffusion at high exogenous concentrations (>1 mM). Transport across the plasma membrane is generally driven by families of P-type proton ATPase ($H^+$-ATP-ase) fueled active transporters,

the genes for which appear to be constitutively expressed at high levels, irrespective of soil N supply [58]. Although these high-affinity transporters maintain cytoplasmic N concentrations at the mM level, demonstrating the efficiency of these N capture systems, as a consequence the external N concentration around the roots can be depleted to the nM level [59]. In turn, this steep concentration gradient across the plasma membrane results in the continual passive loss of organic N from the root back into the apoplast and soil, via rhizodeposition [60]. It has also been suggested that the expression of these transporters may be related to the exchange of signaling molecules between plant-growth-promoting microorganisms and roots or in the root sensing of their environment [61].

Despite evidence that roots can take up organic N, the ecological significance of this N-acquisition pathway remains controversial. While thousands of individual organic N compounds are present in the soil solution, many of these are composed of humic-rich by-products resulting from microbial breakdown and are largely unavailable to plants [62,63]. This is primarily because of the molecular complexity that these humic substances possess. They are composed of a complex mixture of aromatic and aliphatic compounds with diverse functional groups, including carboxyl, hydroxyl, and phenolic groups. This complexity makes it challenging for plants to directly utilize these compounds as a nitrogen source [64]. Another reason could be due to their size and molecular weight. Humic substances are typically large in size and have high molecular weights. Their large molecular size can limit their movement through soil pores and restrict their diffusion to plant roots. This physical limitation can make it difficult for plants to access and uptake these compounds effectively [65]. The binding and sorption capability of humic-rich by-products also reduce their availability for plant uptake, as humic substances have a strong affinity for cations and can form stable complexes with metal ions in the soil. These interactions can result in the binding and sequestration of organic nitrogen within the humic substances. Furthermore, although also smaller and less complex N-containing molecules are present in the soil, for example, glucosamine, which is likely a breakdown product of fungal cell wall chitin, they may not be widely taken up by roots or used in plant metabolism [66]. Numerous additional substances are present at very low concentrations (<1 μM), which may indicate that they have little bearing on plant N nutrition because they are so close to the net influx–efflux equilibrium point. However, it is their rate of replenishment and any potential reserves that may be present during the soil exchange phase that are crucial, not their concentration in the soil solution. For instance, it has been estimated that the soil solution amino acid pool is replenished more than 1000 times a day yet is maintained at low concentrations (ca. 20 μM) in all ecosystems [67]. This rate of cycling can be orders of magnitude faster than soil $NH_4^+$ and $NO_3^-$ production rates.

Overall, while there is a good qualitative understanding of the different N forms that can be taken up by plants, the quantitative importance of organic N uptake is not yet well understood and requires further investigation to enable a better understanding of the ecology, physiology, and molecular biology of plant N nutrition. Potential approaches could include the study of soil solution dynamics of inorganic and organic N compounds, mechanistic understanding of root uptake processes, and plant N uptake under field conditions [68].

### *2.2. Plants Influence Microbial N Transformations in Soils*

Plants respond differently to spatial and temporal variation in nutrient availability. This topic is of long-standing interest to ecology and has been addressed by recognizing the existence of an evolutionary trade-off between fast-growing exploitative and slow-growing conservative growth strategies. Trait-based approaches, such as phenotypic traits, are now commonly used to better categorize plant distribution along resource gradients in relation to these strategies. However, this focus has recently been shifted to below-ground root traits and associations with soil microbial communities and nutrient cycling processes [69]. It has been shown that soil N availability is strongly dependent on various microbial guilds that transform inert $N_2$ into $N_r$ species that can be taken up by plants and that plants

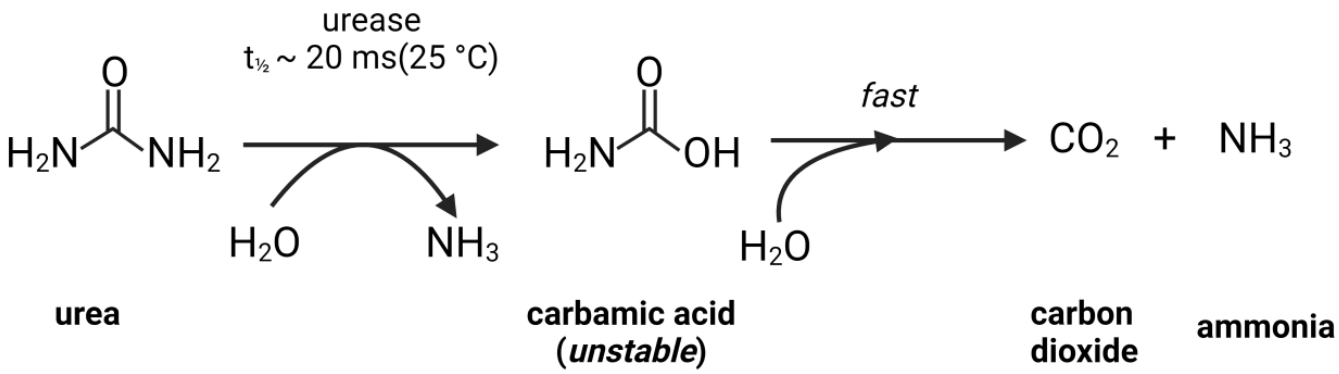
can shape the activity and composition of the soil microbial communities [70,71]. As a plethora of microbial guilds are of importance for N availability in soil, it seems reasonable to hypothesize that plants have also evolved multiple mechanisms to acquire $N_r$ by shaping and recruiting these N-cycling soil microbial communities. When examining the function of urease and nitrification inhibitors when administered as part of a fertilizer mix, it becomes especially critical to comprehend the interaction between N availability, plant N absorption, and microbial composition. We will elute to this below in more detail.

Plants can also limit microbial processes that would lead to loss of N through a range of mechanisms and traits. It has been shown that a relationship exists between plant growth strategies and/or plant traits, the activity of N-cycling microbes, and N retention and loss in the soil. Thus, de Vries and Bardgett [72] and Abalos et al. [73] demonstrated that, compared with slow-growing species, plants with exploitative growth strategies are associated with reduced N losses via leaching in soil and reduced microbial $N_2O$ emissions. Cantarel et al. [74] also showed that nitrification parameters were strongly driven by below-ground traits, such as specific root length (root length per unit of root biomass), root N, and plant affinity for $NH_4^+$, whereas structural equation modeling revealed that root length density (RLD) (root length per unit of soil volume) was a key trait regulating the effects of plants on $N_2O$ emissions as RLD has been shown to be important for N capture from nutrient patches, which are hotspots that trigger $N_2O$ emissions [73,74]. As an acquisitive strategy, plant traits such as high specific root length and/or high root length density [75] are commonly associated with high rates of soil resource acquisition [73,76]. Thus, it can be interpreted that plants and microbes compete for N based on the linkages between plant traits and the activity of N-cycling microbes. In fact, Moreau et al. [69] demonstrated that the abundance of bacteria capable of using $NO_3^-$ as an alternative electron acceptor, rather than carbon released by the plant in the rhizosphere, was inversely related to root N uptake rate. This finding indicates that microbes performing the first step of denitrification, i.e., $NO_3^- > NO_2^-$, can be outcompeted by plants with a high root N uptake rate. Therefore, the type of N fertilizer, as well as urease and nitrification inhibitors, is an important determining factor in N loss pathways as it directly affects the $NO_3^-/NH_4^+$ concentration ratio in soil [77].

## 3. Influence of Soil Nitrogen Conversions on Nr Loss Control

$NH_4^+$-based fertilizers, which include urea ($(H_2N)_2C=O$), anhydrous $NH_3$, ammonium sulfate ($(NH_4)_2SO_4$), and ammonium nitrate ($NH_4NO_3$), are the most commonly used forms of N fertilizers applied in agriculture [78]. As mentioned above, of these, urea is the most common synthetic N fertilizer used in the world [79,80], due to its high N content (46%), relatively low cost per N unit, high water solubility, and high foliar uptake compared with other solid N fertilizers [81]. Urea is hydrolyzed in the soil to $NH_3$ and carbon dioxide ($CO_2$) via the intermediate unstable carbamic acid [82,83] (Scheme 1), which is catalyzed by the enzyme urease, a nickel (Ni)-dependent enzyme. Carbamic acid spontaneously hydrolyzes at physiological pH to form carbonic acid and a second molecule of ammonia. The rate of $NH_3$ loss through volatilization is governed by fertilizer application rate, as well as soil pH, moisture, and temperature [5].

$NH_3$ can be lost to the environment at the soil surface through volatilization or following deprotonation of $NH_4^+$ supplied through ammonium-containing fertilizers (for example, $(NH_4)_2SO_4$ or $NH_4NO_3$) at high pH (Figure 2). Another loss channel for $NH_3$ is microbiological nitrification that involves the stepwise oxidation of $NH_3$ to $NO_3^-$. The latter can leach into the groundwater or be converted into various gaseous N species (such as $N_2O$, nitrogen oxide (NO), and $N_2$) by reductive denitrification processes, ultimately resulting in loss of $N_r$ from the soil–plant system [84].




**Scheme 1.** Hydrolysis of urea.

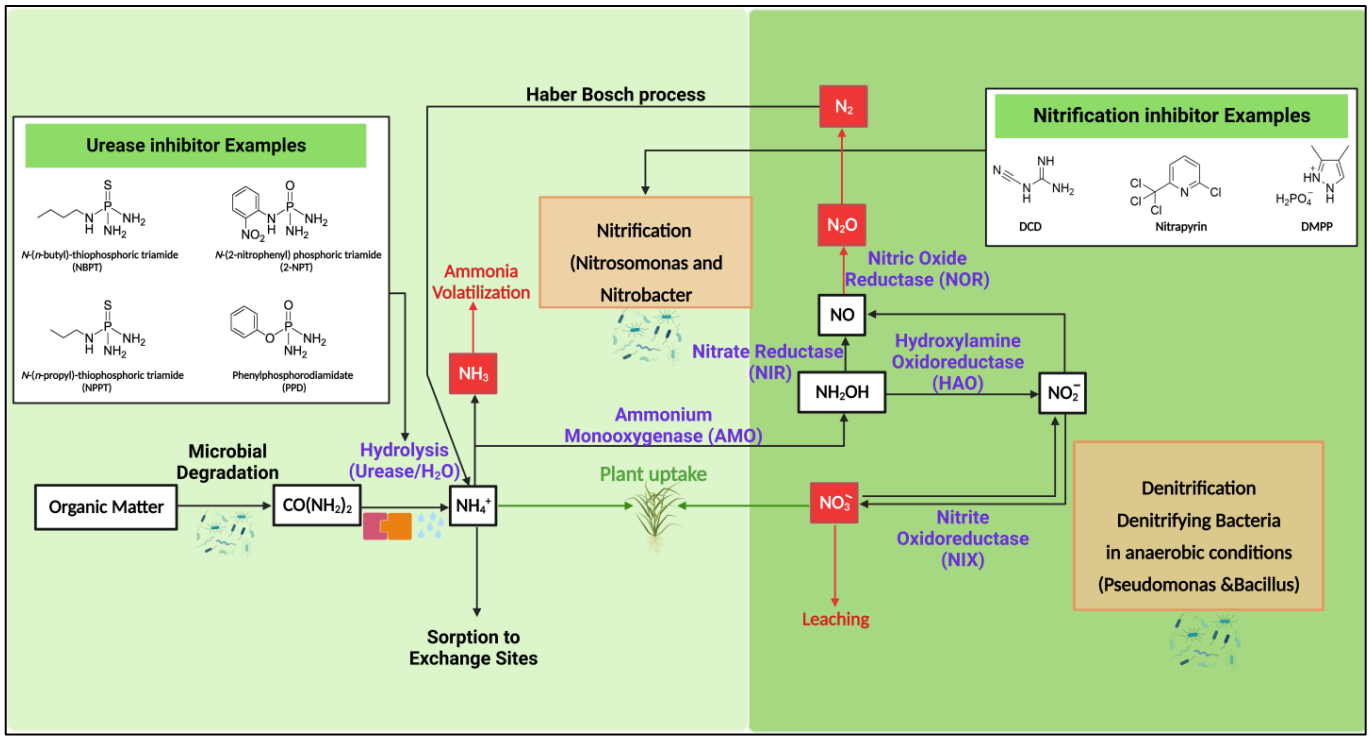



**Figure 2.** Primary pathways for nitrogen losses from the soil/plant system. Urea is hydrolyzed in the soil to $NH_4^+$ (left), catalyzed by the enzyme urease. This results in acidic micro-sites with high concentrations of $NH_4^+$ which drive the formation of $NH_3$ (given in red square). $NH_3$ is lost to the environment through volatilization. Nitrification involves the oxidation of $NH_3$ to $NO_3^-$ (left light green background), which can be leached into the groundwater or converted into various gaseous N forms by the process of denitrification (right dark green background). This results in the loss of N from the soil–plant system. Examples of nitrification and urease inhibitors are given in two white boxes (urease on the left side and nitrification on the right side) that help in the slowdown of the catalytic hydrolysis of urea to $NH_4^+$ and delaying the oxidation of $NH_4^+$ to $NO_3^-$, respectively. Figure adapted from Byrne et al. [85]. Although archaeal and fungal nitrifiers are known microbial players [86,87], nitrification has long been considered as the domain of two groups of chemoautotrophic bacteria: the ammonia (for example, *Nitrosomonas* spp. or *Nitrosococcus* spp.) and nitrite-oxidizing bacteria (for example, *Nitrobacter* spp.). The process involves three steps (Equation (1)).

$$NH_3 \xrightarrow[\text{(AMO)}]{\text{ammonia monooxygenase}} NH_2OH \xrightarrow[\text{(HAO)}]{\text{hydroxylamine oxidoreductase}} NO_2^- \xrightarrow[\text{(NIX)}]{\text{nitrite oxidoreductase}} NO_3^- \quad (1)$$

Nitrification occurs in three steps, where, in the first step, $NH_3$ is oxidized to hydroxylamine ($NH_2OH$) by ammonia-oxidizing bacteria (AOB). This step is rate-limiting and catalyzed by the enzyme ammonia monooxygenase (AMO), which is encoded by the amoA gene found in both ammonia-oxidizing archaea (AOA) and AOB. In the second step, oxidation of $NH_2OH$ to nitrite ($NO_2^-$) occurs, catalyzed by hydroxylamine oxidoreductase (HAO). In the final step, nitrite-oxidizing bacteria containing the enzyme nitrite oxidoreductase (NIX) transform $NO_2^-$ to $NO_3^-$ [86,88,89]. It was recently shown that a group of bacterial species, i.e., comammox *Nitrospira*, can accomplish $NH_3$ oxidation to $NO_3^-$ within the same bacterial cell [89,90].

The reverse process of denitrification, where $NO_3^-$ is reduced to $NO_2^-$, NO, $N_2O$, and $N_2$, is also catalyzed by a diverse cast of bacterial and fungal species [86,91] (Equation (2)).

$$NO_3^- \xrightarrow[\text{(NR)}]{\text{nitrate reductase}} NO_2^- \xrightarrow[\text{(NIR)}]{\text{nitrite reductase}} NO \xrightarrow[\text{(NOR)}]{\text{nitric oxide reductase}} N_2O \xrightarrow[\text{(NOS)}]{\text{nitrous oxide reductase}} N_2 \quad (2)$$

The latter three products are gases, which are lost to the atmosphere. Of particular concern is the greenhouse gas $N_2O$, which has a 273 times higher global warming potential than carbon dioxide ($CO_2$) [92].

Attempts to fully understand and mitigate agricultural $N_r$ losses have become the focus of intensive research initiatives. Numerous strategies have been put forward, such as coordinating fertilizer application methods with plant growth patterns, improving cropland management practices, and the use of synthetic urease and nitrification inhibitors [93,94]. The latter are typically applied as a formulation with the respective fertilizer.

## 4. Use of Inhibitors

Urease inhibitors (UIs) are synthetic chemicals that can be added to the urea-based fertilizer to slow down the catalytic hydrolysis of urea to $NH_4^+$ by preventing its binding to the enzyme urease (Figure 2). Urea is highly soluble in water and is often applied in agricultural systems through top-dressing. By slowing down its hydrolytic degradation, urea can travel deeper into the soil after rainfall or watering so that less $NH_3$ escapes from the soil, leaving more nitrogen available for plant uptake through the growth cycle [95,96]. Furthermore, a slowed-down urea hydrolysis leads to a gradual $NH_4^+$ production (rather than one big burst), which enables a more efficient $NH_4^+$ uptake by plants, thereby reducing the extent of nitrification and the undesired loss of $NO_3^-$ through leaching into the ground and surface water [96].

While many compound classes with the potential to inhibit urease activity in soils are known, phosphoric and thiophosphoric triamides, which can be considered as urea analogs, are the most promising synthetic urease inhibitors so far (Figure 2). In particular, *N*-(*n*-Butyl) thiophosphoric triamide (NBPT, trade name Agrotain) is effective in a wide range of soils at low concentrations of 0.01% of applied nitrogen [97]. The active inhibitor species is believed to be *N*-(*n*-butyl) phosphoric triamide (NBPTO), which is a urea analog that results in the hydrolysis of NBPT by soil microorganisms (Figure 3). The reason why in practice NBPT—and not NBPTO—is applied onto soils is due to the latter's high susceptibility to hydrolysis, whereas the less nucleophilic P=S moiety increases the shelf-life, making the handling of this compound easier. A mixture of NBPT with the homolog *N*-(*n*-propyl) thiophosphoric triamide (NPPT) is marketed under the trade name Limus (BASF) [98]. Recently, NBPT has been co-formulated with Duromide, an NBPT derivative

containing a covalently linked urea moiety (trade name ANVOL, Koch, Wichita, KS, USA), which increases NBPT efficiency by reducing $NH_3$ volatilization.

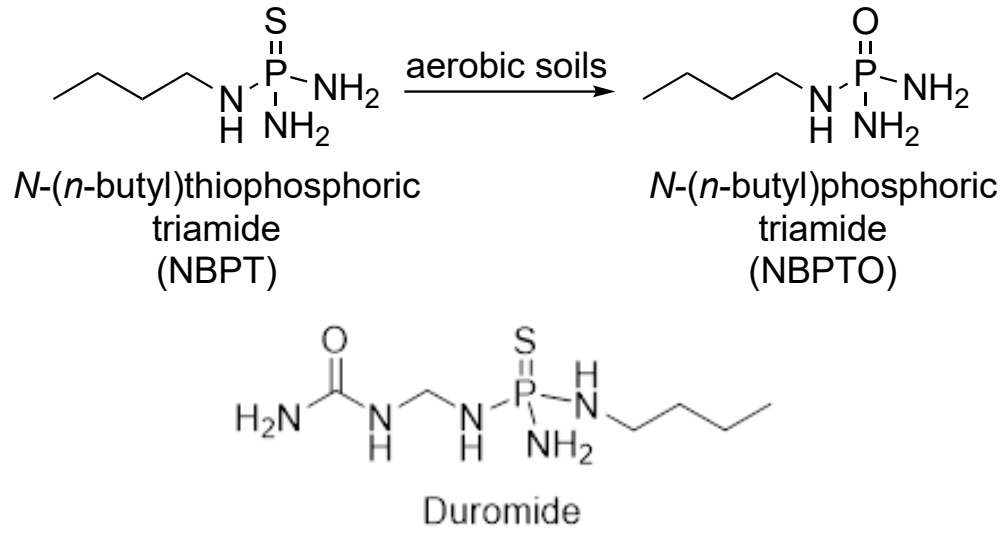



**Figure 3.** Hydrolysis of *N*-(*n*-butyl) thiophosphoric triamide (NBPT) to *N*-(*n*-butyl) phosphoric triamide (NBPTO) in soils (left). Structure of Duromide (ANVOL, Koch) (right).

Similarly, nitrification inhibitors (NIs) are intended to increase the residence time of $NH_4^+$ by delaying its oxidation to $NO_3^-$ (Equation (1)) by inhibiting the AMO enzyme of nitrifying microbes in the soil responsible for this process. Many of the compounds with known nitrification-inhibitory properties are aromatic heterocycles containing two or more N, O, or S atoms, suggesting that NIs target the first step of the ammonia oxidation process, i.e., $NH_4^+ \rightarrow NH_2OH$, that is catalyzed by AMO. The second nitrification step, where $NH_2OH$ is oxidized to $NO_2^-$ catalyzed by HOA, is normally not influenced [99].

It should be noted that plant roots can secrete compounds that can also slow down the rate of $NH_4^+$ oxidation by blocking both the AMO and HAO-catalyzed steps of the nitrification cascade [100–102]. For example, a study on *Brachiaria* pasture [101] led to the discovery of brachialactone as a new biological nitrification inhibitor (BNI), which contributed up to 90% of the inhibitory activity released from the roots of this tropical grass. Brachialactone blocks both AMO and HOA enzymatic pathways in *Nitrosomonas* [101], and within three years of establishment, *Brachiaria* pastures have suppressed soil nitrifiers, such as archaeal and bacterial ammonia-oxidizing microorganisms. A similar decrease was reported for two forage crops, *Medicago sativa* and *Dactylis glomerata* [103]. It was suggested that the release of BNIs is triggered by high $NH_4^+$ concentrations in the root environment [101,102]. This proposal was supported by a study, where half of the root system was exposed to $NH_4^+$ and the other half to $NO_3^-$. Only the part exposed to $NH_4^+$ triggered the release of brachialactone, indicating a localized release process. Brachialactone is a tetracyclic diterpene with a unique 5-8-5-membered ring system, a γ-lactone ring, and seven stereogenic centers.

From the highly diverse (and complex) frameworks of these compounds, different modes of action could be suggested, although mechanistic details are not yet known. There is some controversy in the literature about whether the use of BNIs could be a viable strategy to modulate nitrification processes in agricultural soils [104]. From the viewpoint of synthetic organic chemistry, access to these BNIs would require multi-step processes, which is not feasible for the large-scale synthesis required for applications in broad-acre agricultural systems. On the other hand, genetic engineering approaches [105] might potentially provide an opportunity to create plants with the ability to excrete higher concentrations of NBIs. In this overview, we will focus only on synthetic small-molecule inhibitors and refer the reader interested in BNI to explore recent reviews in [5,106–108].

2-Chloro-6-(trichloromethyl)-pyridine (Nitrapyrin or N-Serve®, Dow Chemical Co., Midland, MI, USA) is the first synthetic nitrification inhibitor that has been brought to the market [109]. Since then, several compounds have been developed to control nitrification in soil (see Figure 2). The most important examples are dicyandiamide (DCD), 2-amino-4-chloro-6-methylpyrimidine, and 3,4-dimethylpyrazole phosphate (DMPP). Amongst these, the properties and performance of DCD and DMPP have been most widely studied [110]. The active site in DMPP is 3,4-dimethyl-1*H*-pyrazole phosphate (DMP), but because of its volatility, this inhibitor is usually applied as a salt with phosphoric acid.

### *4.1. Mechanism of Urease Inhibition (UI)*

The mechanism by which NBPT (or NBPTO) inhibits urease activity can be understood from the interactions between urea or inhibitor, respectively, and the enzyme's active site. Figure 4 shows that urea coordinates with the two Ni centers in the enzyme's active site via the carbonyl oxygen and one of the amino groups, which activates the carbonyl group for nucleophilic attack by the OH bridge between the two Ni atoms. NBPTO is a tridentate ligand that forms a complex with both Ni atoms and the oxygen atom of the urea-derived carbamate, which mimics the transition state of urea hydrolysis. The lateral *N*-(*n*-butyl) amine substituent blocks access of urea to the active site. Unfortunately, in the NBPTO-urease complex, NBPTO is also activated for hydrolysis, which leads to the loss of the *n*-butyl amine substituent, which results in the deactivation of the inhibitor [111].

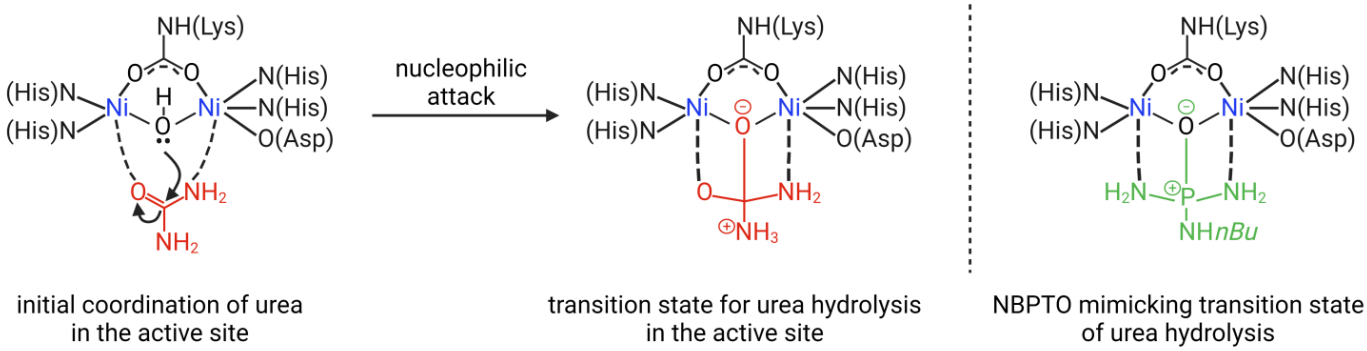



**Figure 4.** Mechanism of urea hydrolysis by urease and mode of action of NBPTO (Adopted from Mazzei et al. [111]).

While NBPT can delay urea hydrolysis for 3–14 days, depending on soil and environmental conditions [112], the impact on the $N_2O$ emissions is highly variable, ranging from no effect to reduced or even increased $N_2O$ emissions [113]. Likewise, the benefits of NBPT for crop yield, N-uptake, and NUE are quite variable and span from some increase to no significant effect [114].

Many other compound classes with urease-inhibiting properties are known, which act through different mechanisms [115,116], for example, by preventing urea access through targeted "space-filling" of the active site involving noncovalent interactions with neighboring amino acid residues, typically histidine, aspartate, and lysine [117] (Figure 5a). Through analysis of the crystal structure of the active site and molecular docking studies, inhibitor compounds can be designed for specific applications [118].

Inhibition can also be achieved through modification of the cysteine-rich mobile flap, which allows access to the active site, through covalent [83] (and refer to Figure 5b (bottom)) or π-interactions [121]. Data from biochemical and mechanistic studies show the formation of a covalent C–S bond with 1,4-benzoquinone, a known inhibitor, which reduces the flexibility of the mobile flap and slows down or even prevents access of urea to the active site of the enzyme (Figure 5b). However, in the context of inhibition of urease in soils, the behavior of new inhibitor compounds in the complex soil matrix also needs to be considered: Mobility, as defined by molecule size, lipophilicity, hydrophilicity, and charge, as well as stability towards degradation are important factors [122].

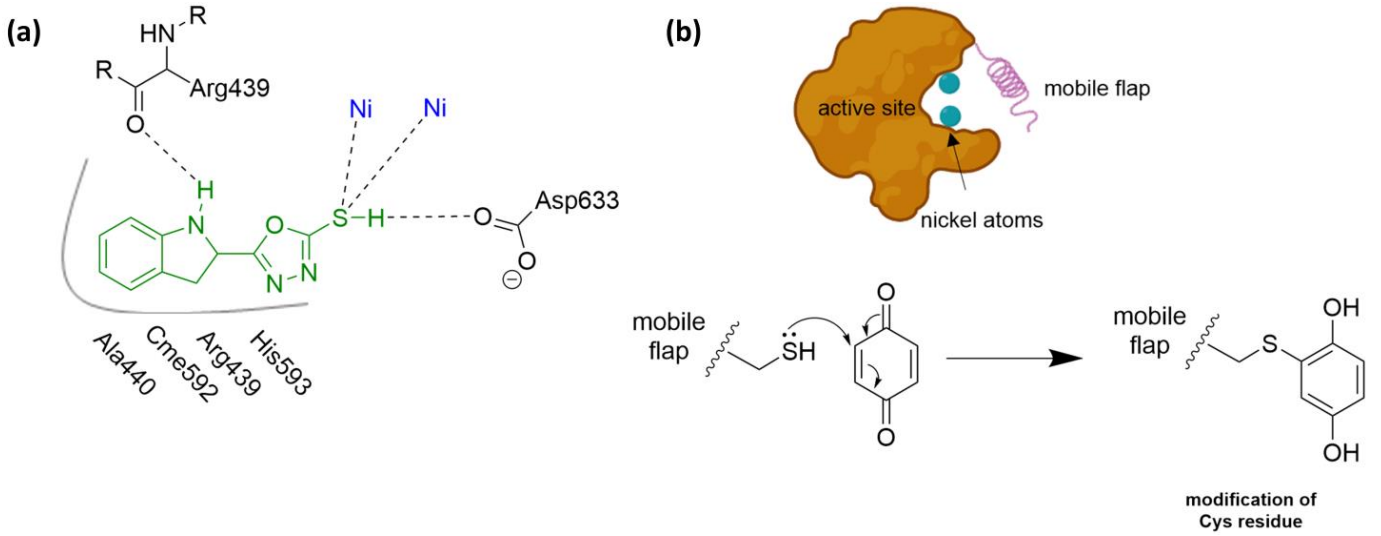



**Figure 5.** (**a**) Example of a "space-filling" 1,3,4-oxadiazole urease inhibitor with key interactions with neighboring residues. Adopted from Hanif et al. [119]. (**b**) Location of the mobile flap of urease relative to the active site (**top**) and a known inhibitor 1,4-benzoquinone which covalently modifies the Cys-containing mobile flap (**bottom**). Adopted from Mazzei et al. [120].

### *4.2. Mechanism of Nitrification Inhibition (NI)*

The biochemical mechanisms of nitrification inhibition and the effects of nitrification inhibitors on reducing nitrification rates have not been confirmed or verified [109]. However, based on the inhibitory effects of several compounds, it is suggested that NIs target the first step of the nitrification process, specifically the metalloenzyme enzyme AMO [123]. AMO contains copper(II) (Cu(II)) in its active site, and the essential role of Cu(II) in the catalytic process was discovered already in the 1940s [124]. However, the mechanism by which commercial inhibitors, for example, DMPP, inhibit AMO, is still unclear, which is likely due to the lack of knowledge of the structure of the active site in AMO. It has been recently revealed that DMP and DCD act as reversible NI and do not compete for the same binding site as $NH_3$ [125], confirming the potential chelator properties of DMP and DCD. As a membrane-bound enzyme, its activity decreases significantly after isolation [126], hampering direct enzymatic studies so far. The discovery of the evolutionary similarity between AMO and the particulate methane monooxygenase (pMMO) has been an important milestone in gaining a better understanding of AMO, which emphasized the significant role of copper in its activity [127]. As an alternative to enzymic studies, strategies involving pure bacterial cultures have been employed to screen potential nitrification inhibitors [125]. Most commonly, *Nitrosomonas europaea* (NE) has been used as the model organism because it can be grown in a laboratory setup in high cell numbers [128]. The nitrification rate can be determined by a colorimetric assay (Griess Assay), where $NO_2^-$ production is monitored over time [129]. More recently, archaea have been increasingly utilized in these assays as they are believed to be the more predominant species in soils [129–132]

The chemical transformations mediated by AMO have been explored with small molecules, including linear and cyclic alkanes, alkenes, aromatic compounds, as well as $NH_3$ (Figure 6) [133,134]. Thus, AMO in NE catalyzes the insertion of oxygen into C-H bonds, enabling the conversion of alkanes or aromatic compounds to the corresponding alcohols. In the reaction with alkenes, the formation of epoxides occurs, whereas alcohols can be further oxidized to carbonyl compounds. $NH_3$ is oxidized to $NH_2OH$ through the insertion of O into an N-H bond.

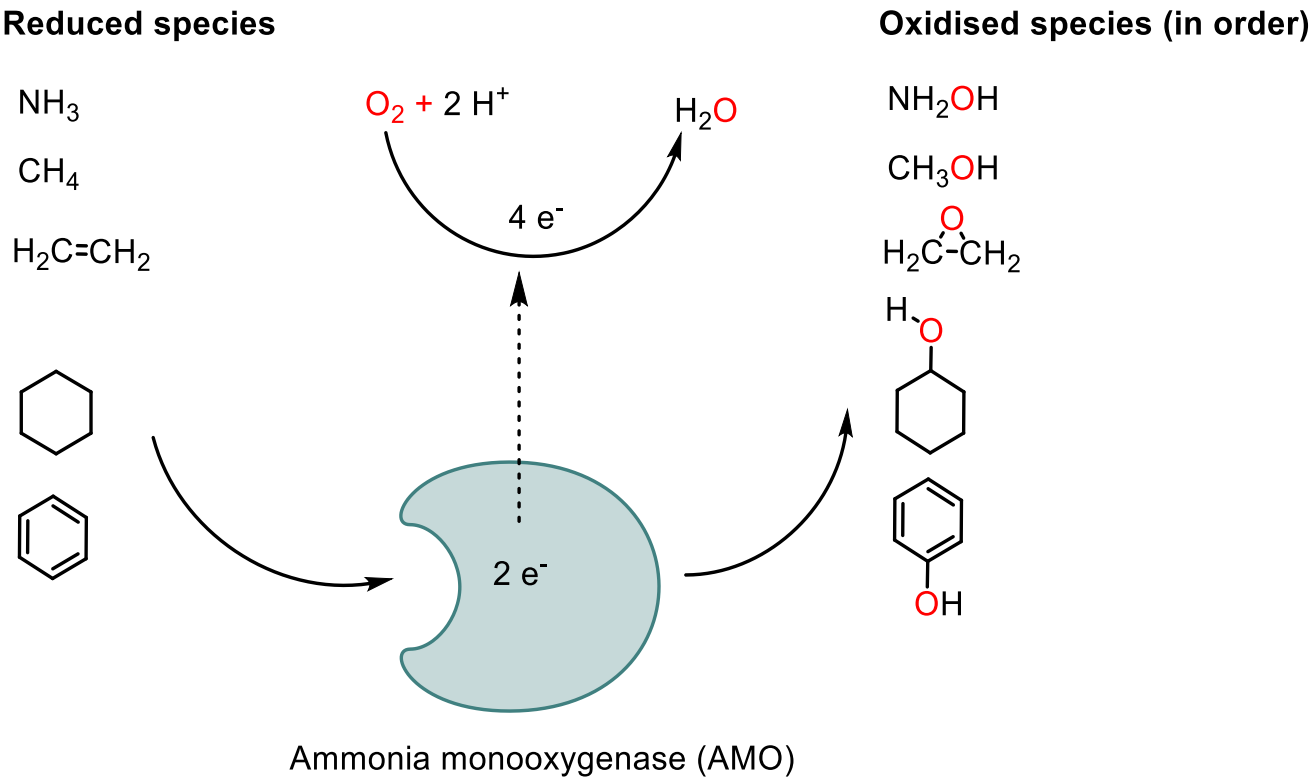



**Figure 6.** Exemplary substrates oxidized by the ammonia monooxygenase (AMO), adopted from Bédard and Knowles [133], Hubley et al. [135], Hyman and Wood [136], Hyman and Wood [137], Stirling and Dalton [138], Voysey and Wood [139], Ward [140], and Hyman et al. [141]. AMO catalyzes the oxidation of various structural motifs including ammonia, methane, ethylene, cyclohexane, and benzene, which releases 2 electrons (2 $e^-$). Simultaneously, $O_2$ is reduced, which requires 4 $e^-$. The missing 2 $e^-$ are provided from the set of electrons released during the subsequent oxidation processes catalyzed by HAO. The schematic overview is shown in Figure 7.

Importantly, the growth of nitrifying bacteria can only be maintained when $NH_3$ is present [142]. This finding can be understood by examining the flow of electrons during the first two nitrification steps (Figure 7). Thus, the AMO-catalyzed oxidation of $NH_3$ (or $NH_4^+$, respectively) to $NH_2OH$ releases two electrons, which are taken up by $O_2$. The reduction $O_2 \rightarrow 2\ O^{2-}$ requires four electrons. The other two electrons are provided by HAO, which supplies a total of four electrons through the oxidation $NH_2OH \rightarrow NO_2^-$. The remaining two electrons are transferred to *Cytochrome C554*, membrane cytochrome $C_m552$, and Ubiquinone-8 (QH2). HAO is, therefore, a crucial enzyme in the nitrification process that fuels the AMO reaction [143,144]. In other words, if the compound produced by AMO is not a substrate for HAO, the entire oxidation cascade cannot be energetically maintained.

Based on this, AMO inhibition could be achieved via two pathways: (i) by substrate inhibition, where the inhibitor binds to AMO but its oxidation product is not a substrate for HAO and (ii) by ligand binding to Cu(II) in the enzyme's active site, which maintains its catalytic function but prevents binding of $NH_3$. The crystal structure of the N-terminus of the amoB subunit in AMO shows that the periplasmatic cupredoxin-like domain is formed by three histidine residues, which coordinate with Cu(II) [126,145]. These data suggest that the existing commercial NIs bind to Cu(II) in a similar fashion, thereby slowing down nitrification rates. This proposition is supported by crystallographic analyses of model systems, which revealed that four DMP molecules and two $Cl^-$ ions can form an octahedral complex with Cu(II) [146,147], demonstrating the ability of DMP to act as a ligand for Cu(II). The findings from Corrochano-Monsalve et al. [146] further show that the growth of $N_2O$-reducing bacteria appears to be controlled by Cu availability. These bacteria benefit

from the use of DMPs, which could be attributed to a decrease in competence when AOB growth is inhibited. This opened up the possibility to induce $N_2O$ reduction to $N_2$ through Cu fertilization.

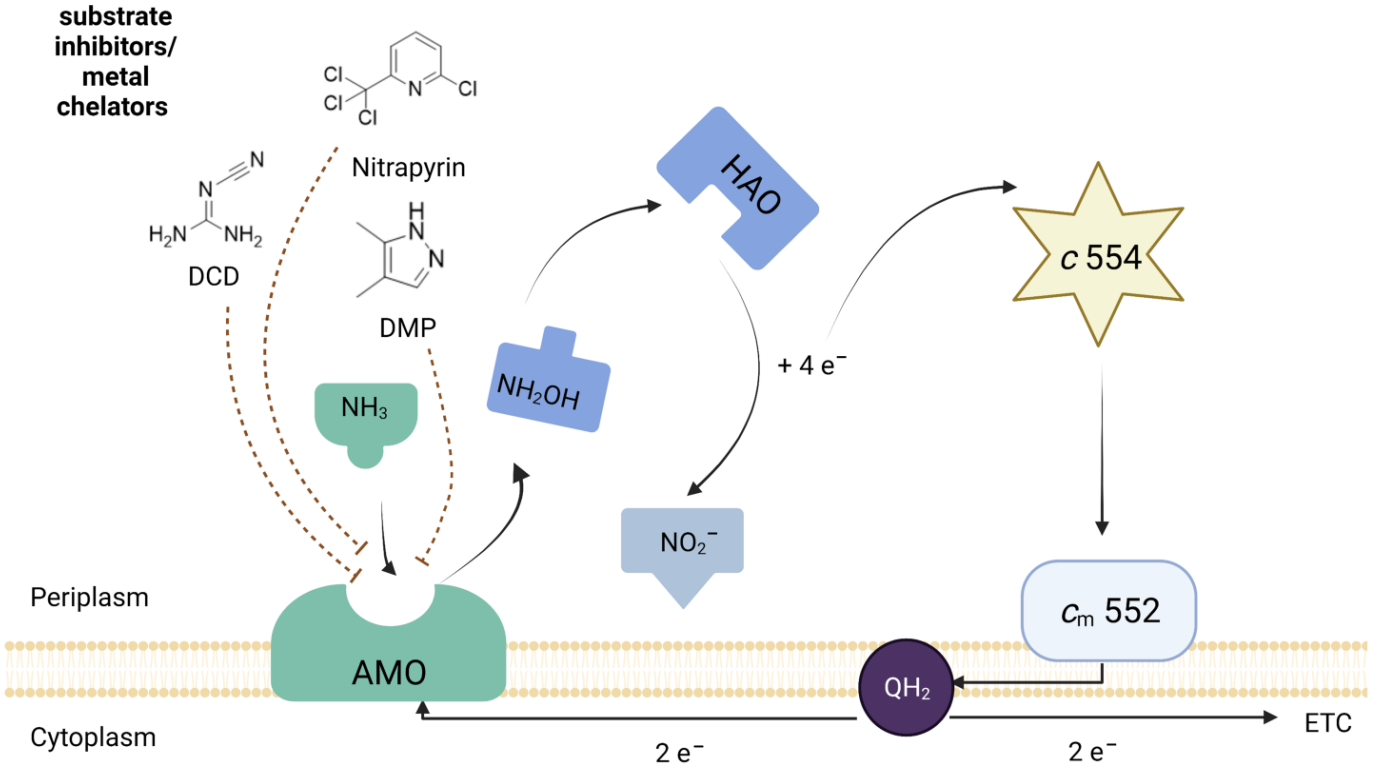



**Figure 7.** Membrane-bound AMO oxidizes $NH_3$ to $NH_2OH$, which is further oxidized to $NO_2^-$ by HAO. The four electrons generated during these processes are subsequently transferred to cytochrome C554, membrane cytochrome C*m*552, and ubiquinone-8 (QH2), from where two electrons are fed into the electron transport chain (ETC) and two electrons are transferred back to the AMO to deliver electrons for the reduction of oxygen. In the presence of nitrification inhibitors, such as DCD, Nitrapyrin, and DMP, the first step of ammonia oxidation is inhibited.

Recent innovations in the development of new NI compounds are mainly based on the modification of existing inhibitors to increase their effectiveness. The most recent modification for DMPP is 3,4-dimethylpyrazole succinic acid (DMPSA), showing (reduces $N_2O$ flux rates) in trial field experiments [148]. DMPSA is believed to be stable in basic conditions and can be formulated with calcium ammonium nitrate [149] but is not (yet) commercially available. Another approach is slow-release inhibitors, which are intended to inhibit nitrification over a longer time. For example, a recent soil incubation study where the amount of volatilized $NH_3$ was measured revealed that a copolymer of DMPP with acrylic acid (AA) can slow down nitrification over a duration of 24 h [131,150]. In this regard, it should be noted that many approaches to control the release of fertilizers (with or without inhibitors) have been made. This concept is outside the scope of this overview, and the interested reader is referred to a recent review by Vejan et al. [151].

### *4.3. Factors Affecting the Effectiveness of UIs and NIs*

Temperature, soil pH, clay content, organic matter, and water-filled pore space (WFPS) are among the most important factors influencing the effectiveness of urease and nitrification inhibitors [152–158].

#### 4.3.1. Temperature

A soil incubation study by Carmona et al. [159] found an increase in $NH_4^+$ production by 50% at 32 °C than at 18 °C, suggesting that higher application rates of NBPT would be required at higher temperatures to enable inhibition of urea hydrolysis to a similar extent than at cooler temperatures. Suter et al. [113] showed that 55% of urea was still present in the soil at temperatures between 5 and 15 °C two weeks after NBPT/urea treatment, while only <2% of urea remained at 25 °C. The decreased inhibitory efficacy at higher temperatures is likely due to an increased rate of hydrolytic degradation of NBPT, which can become a significant factor in the field during periods of temperatures > 35 °C that can occur during summer in certain places of the world. An additional challenge to inhibitor activity arises from the fact that the activity of urease also increases with increasing temperatures [160]. However, it should be noted that limited studies have specifically investigated the effects of lower soil temperatures on the performance of NBPT, indicating a need for further research in this area.

A decreased effectiveness of nitrification inhibitors with increasing temperature has also been found. A field study by McGeough et al. [161] revealed that the measured half-lives ($t_{1/2}$) of DCD across different mineral soils decreased with increasing soil temperature, suggesting that the efficacy of DCD depends not only on soil temperature but also on soil composition, such as clay content and organic matter. Kelliher et al. [162] showed in field trails that $t_{1/2}$ of DCD was approximately half of that measured at the same temperature under laboratory conditions, whereas the relationship between $t\frac{1}{2}$ mean soil temperature is: ($t\frac{1}{2}$ (days) = 54 − 1.8 T (°C). Similarly, the inhibitory activity of DMPP is also inversely related to temperature, with a significant drop in activity observed over a relatively small temperature change [163]. For example, it has been shown that at a soil temperature of 35 °C, which can occur during fertilization application in some South Asian regions, DMPP remains effective for only one week [164]. Using DMPP to mitigate $N_2O$ emissions from sheep urine patches revealed a poorer efficiency over summer [165].

#### 4.3.2. Soil pH

Deprotonation of $NH_4^+$ readily occurs in alkaline soils, which could lead to higher $NH_3$ volatilization. Thus, the contribution of urease inhibitors to reducing such unwanted $NH_3$ losses is relatively higher in alkaline than in neutral or acidic soils [156]. As mentioned previously, NBPT and NBPTO are both prone to hydrolytic degradation, in particular at low pH (<5) [166,167], whereas even at pH ~ 7, abiotic chemical hydrolysis of NBPT still occurs to some extent. Thus, a reduction in urea hydrolysis of about 17–86% by NBPT was reported by Tao et al. [168] in acidic soils compared to a 53–92% reduction in alkaline soil [166,167]. At higher pH (>8), microbial activity is likely responsible for the observed increase in the degradation rate of NBPT [167,168], as shown by the higher $t\frac{1}{2}$ of NBPT in sterilized compared to nonsterilized soil [165]. The significance of microbial degradation is further highlighted by a slightly longer $t\frac{1}{2}$ of NBPT in light compared to dark conditions (5.9 vs. 4.5 days, respectively), which could be attributed to the light-inhibiting microbial growth and therefore a slower degradation of NBPT [168].

Similarly, several studies [169–173] have shown that the distribution and abundance of ammonia oxidizers and therefore the efficacy of nitrification inhibitors are influenced by soil pH. The generally poor inhibitory performance of DMPP at low pH could result from the switch from the autotrophic AOB, which are targeted by DMPP, to AOA, which are not inhibited by DMPP [87,174]. AOA have a lower metabolic concentration threshold to using $NH_3$ and contribute to the nitrification process more than AOB in very low pH soils, where $NH_3$ availability is low [175,176]. While the mechanism of DMPP inhibition is not well understood [125], it has been shown that DMPP decreases the abundance and metabolic activity of AOB in alkaline and, to a lesser extent, in acidic soils (due to the lower AOB populations in these soils) but does not have an inhibitory effect on the AOA population in either soil type [177].

#### 4.3.3. Clay Content and Organic Matter

The inhibitory performance of NBPT and other urease inhibitors is also affected by soil organic matter (OM), where higher OM content results in increased adsorption and reduced effectiveness due to lower mobility [178]. Furthermore, in soils with a higher OM, the microbial activity is also increased, resulting in faster inhibitor degradation and therefore reduced efficacy. Christianson et al. [179] reported that NBPT changed soil pH and $NH_4^+$ concentration around the reaction site in loamy silt soil with 20% clay content, resulting in the diffusion of urea from the placement site. On the other hand, at a site with 46% clay soil, the inhibitory activity of NBPT was low, as revealed by an increased $NH_4^+$ concentration around the fertilized site.

Similarly, McGeough et al. [161], Zhang et al. [180], Wu et al. [181], and Barth et al. [182] reported that the specific composition of organic matter and clays affects DCD and DMPP efficacy. The lower recovery rates of DMPP from clay soil have been rationalized to be caused by greater interaction with the organic matter [177]. Another study by Chen et al. [183] was performed on brown Vertosol soil with clay loam texture under varying moisture and temperature conditions, where it was shown that DMPP reduced cumulative $N_2O$ emission over 42 days by more than 65% in clay loam soil.

### *4.4. Use of Combined UIs and NIs*

In principle, by using both urease and nitrification inhibitors together, increased N use efficiency should be achieved, as these inhibitors target the major N loss mechanisms through $NH_3$ volatilization, $NO_3^-$ leaching, and $N_2O$ emissions. However, although the NUE of crops can be increased by minimizing N losses, minimizing losses through one pathway could increase losses through the other [184]. For example, it has been shown that when NBPT is used alone, urea hydrolysis is slowed down, but once $NH_4^+$ is produced, it is exposed to nitrification and denitrification losses [153]. In particular, increases in $N_2O$ emissions with the use of UIs have been reported [185–187]. Similarly, when NIs are used alone, the prolonged retention of $NH_4^+$ in the soil can increase the extent of $NH_3$ volatilization [186–191], indicating that the use of NIs may not be beneficial in areas with concerns about $NH_3$ emissions. In fact, some NIs were found to increase $NH_3$ loss [23,24,188,192,193]. Thus, an approach that simultaneously targets all N pathways for an overall environmental benefit is required. Dual-action inhibitors could potentially provide a solution, where UIs, NIs, and ammoniacal ($NH_3$-N) fertilizers are applied in combination to minimize losses from the N-cycling pathways of hydrolysis, nitrification, and denitrification [189,192,194].

However, while such an approach seems to be "logical", several studies have revealed that the combination of both inhibitors with urea does not necessarily give the expected beneficial effect. For example, a soil incubation study where urea was applied together with NBPT and DCD revealed that DCD could even offset the effect of NBPT in reducing the loss of $NH_3$ through volatilization (Figure 8) [191]. As the inactivation of NBPT by DCD could be excluded (and vice versa), these data suggest that the slowed-down nitrification process leads to the accumulation of $NH_3/NH_4^+$ in the soil, allowing volatilization losses to continue.

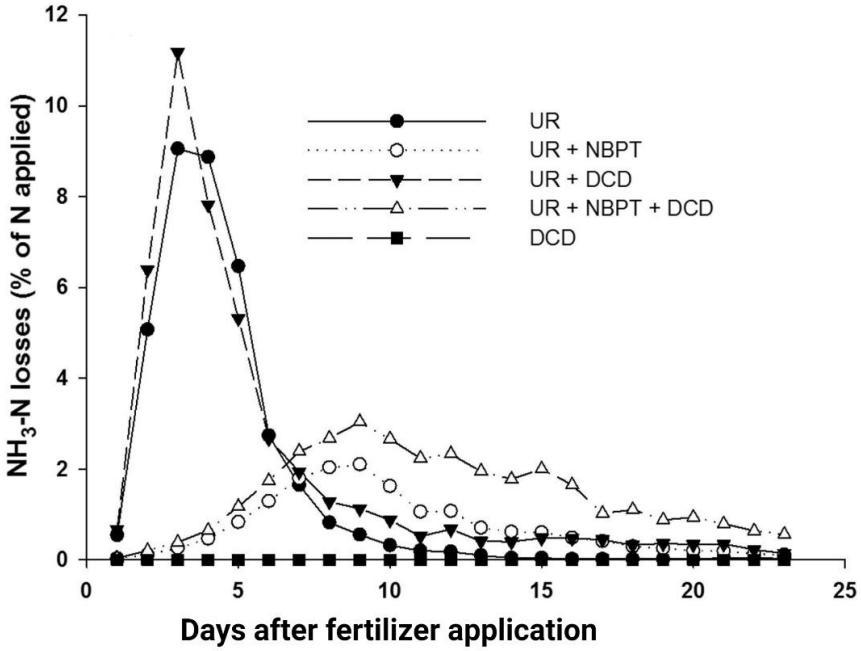



**Figure 8.** Loss of NH3 through volatilization after application of urea (UR) and urease (NBPT) and nitrification (DCD) inhibitors. Figure taken from Soares et al. [192] with permission from Soil Biology and Biochemistry.

## 5. Use of Nitrification and Urease Inhibitors in Different Plant Genotypes—What Is Known?

Slowing down urea hydrolysis in the soil through the use of UIs is also beneficial for crops, as it reduces the toxic effect of $NH_3/NH_4^+$ concentration spikes in the area surrounding fertilizer granules on seed germination, resulting in higher crop yields (barley and canola) and increased root growth (rice) [195–197].

However, NBPT may be taken up by plant roots, as has been demonstrated in maize seedlings through a reduced net urea uptake and lower ureic-N accumulation [198]. NBPT absorption by plants can lead to changes in metabolic pathways associated with N assimilation, such as a reduction in plant urease and glutamine synthetase [199,200]. A study by Cruchaga et al. [201] revealed that NBPT absorbed by pea plants caused inhibition of urease activity in the leaves and roots, which resulted in urea build-up and therefore necrosis of the plant at leaf margins. Other studies also found that NBPT applied at high concentrations of urea (0.5% *w/w*) and concentrations of 10 µg/g of soil was associated with leaf tip scorch. This effect is likely dose-dependent [202,203], with the leaf tip scorch believed to result from a toxic build-up of urea at the leaf tip rather than a cytotoxic effect exerted by NBPT itself. Cruchaga et al. [201] also found that NBPT taken up by pea and spinach roots is translocated to the leaves, potentially inhibiting the activity of endogenous leaf and root urease. Thus, NBPT can cause transient yellowing of leaf tips caused by urea toxicity soon after application. However, it has been reported that plants recover quickly and no effects on growth have been reported [199,204].

Similar to urease inhibitors, nitrification inhibitors added to N fertilizers can temporarily reduce the populations of nitrifying bacteria in the soil which are responsible for converting $NH_3$ to $NO_2^-$ (*Nitrosomonas*) and $NO_2^-$ to $NO_3^-$ (*Nitrobacter*). This process is important to plants as it leaves an extra stash of $NH_4^+$ that can be absorbed by the plants through their root systems. Few studies have reported the uptake of nitrification inhibitors by plants and their movements in plant tissues. The $K_{ow}$ (a relative indicator of an organic compound's ability to bind to soil) value of −1 for the nitrification inhibitor DCD shows that this is a polar compound, which should be able to translocate in the trans-vascular system of plants. Likewise, it has been found that plants have the capacity to take up

DMPP and DMPSA [205]. This study was conducted on clover plants, which revealed that DMPP (applied at 100 mg/kg soil) accumulated in clover leaves where it caused necrosis on the leaf margins. On the other hand, DMPSA (applied at 100 mg/kg soil) accumulated in the roots with no harm to the root tissue. As the application rates in this study were significantly higher than the estimated maximum amount used in agriculture (0.5 mg/kg soil), both inhibitors pose an extremely low risk of phytotoxicity [205]. Similarly, some studies are available that identified Nitrapyrin residues in food crops, providing evidence that plants also take up and accumulate this nitrification inhibitor. For example, a gas–liquid chromatographic study under field conditions by Kallio et al. [206] revealed that the amount of Nitrapyrin residues in red beetroots was strongly correlated with the application rate of this inhibitor.

Recently, in two separate field studies, it was shown that urea with Nitrapyrin alone or in combination with the potassium salt of gibberellic acid (GA-K salt) has the potential to enhance NUEs and yields of maize and wheat [207,208]. Thus, it was found that maize plant biomass, grain yield, and total N uptake were highest and increased significantly by 27%, 36%, and 25%, respectively, in the treatment with urea, Nitrapyrin, and GA-K salt compared to urea treatment alone or urea with Nitrapryrin. Similarly, wheat plant biomass, grain yield, and total N uptake were enhanced by 31, 37, and 44%, respectively, when urea was applied with Nitrapyrin and GA-K salt as compared to urea alone or used in combination with Nitrapyrin.

Meta-analyses have also been widely performed on various crops to study the effect of a series of inhibitors on plant productivity. Linquist et al. [209] explored the effect of several N fertilizers amended with either NIs or UIs on yield and N uptake in rice systems. The study found that, on average, the use of these fertilizers led to a 5.7% increase in yield and an 0.8% increase in N uptake. Abalos et al. [210] integrated available results to quantitatively evaluate the effect of commonly used NIs and UIs on crop productivity. A meta-analysis was conducted to characterize the response of crop productivity with the application of nitrification (DCD, DMPP), urease (NBPT), and combined use of both inhibitors (DCD + NBPT). This study demonstrated that co-application of NIs or UIs is generally higher under conditions that favor high drainage and if high inputs of fertilizer were applied [210]. Another meta-analysis by Yang et al. [211] compared the effectiveness of DCD and DMPP across maize field trials, revealing that both inhibitors were equally effective in altering soil inorganic N content, reducing inorganic N leaching and nitrous oxide ($N_2O$) emissions. However, DCD was more effective than DMPP in increasing plant productivity (6% increase in grain yields).

## 6. Future Outlook/Perspectives

Global cycling of N has reached its peak—the amount of atmospheric $N_2$ industrially converted into $NH_3$ to produce fertilizers now exceeds the amount that is cycled through all of the Earth's terrestrial processes [15]. One of the strategies to increase N-use efficiency in agricultural systems is through the use of enzyme inhibitors that modulate the microbial conversion of fertilizer N in soils. Thus, the hydrolysis of urea to $NH_4^+$ by the enzyme urease present in soils can be delayed by the use of urease inhibitors. Likewise, the oxidation of $NH_3$ to $NO_3^-$ and its accumulation during the cropping phase can be regulated by nitrification inhibitors. Urease inhibitors have been on the market for more than 20 years, with growing acceptance by farmers [192]. While NBPT, currently the most important commercial UI, can reduce (but not eliminate) $NH_3$ volatilization [192], serious limitations, such as the short period of effective inhibition in conjunction with a limited shelf life, should stimulate much-needed fundamental research towards the development of improved UIs that delay hydrolysis of surface-applied urea until the full dissolution in the soil body. Similarly, several nitrification inhibitors are currently on the market, such as Nitrapyrin, DMPP, and DCD, which slow down $NH_3$ oxidation and reduce N loss through $NO_3^-$ leaching and denitrification. However, there is inconsistency in both environmental and agronomic benefits of the use of inhibitors due to the complex interactions between soil,

crop, and agricultural management in conjunction with climate (and climate changes) occurring in cropping systems.

This review provides an evaluation of the current knowledge of the available inhibitor technology and its shortcomings. This assessment reveals that the development of novel inhibitors with consistent performance across many different agricultural settings requires an improved mechanistic understanding of the spatial and temporal interplay of urease and nitrification inhibitors with plants and N uptake by roots.

Various approaches have been suggested to increase the use efficiency of nitrogenous fertilizers with inhibitors, such as (i) improved agricultural practices, for example, increased frequency of crop rotation or farmers implementing best management practices with regards to the 4 Rs Nutrient Stewardship—the "right" rate, source, timing, and placement of nitrogen fertilizers to match plant needs; (ii) using coatings, which include rapid, low-cost, ideally one-step coating methods for fertilizers, for example, metal–ligand coordination complexes or biodegradable/compostable polymers that enable nutrient release upon environmental triggers, for example, a rainfall or pH changes [212,213]; or (iii) reducing $NH_3$ volatilization arising from urea hydrolysis by using inexpensive additives which are readily available as natural minerals or as industrial by-products, and through chemical and physical modifications. For example, zeolites or surface-modified coal tailings can be used to reversibly capture $NH_3$ [214], which could reduce the risk of N loss through runoff or denitrification. However, these approaches are only useful if the crop can indeed take up and use the saved N.

The benefits of using inhibitors to mitigate N losses have been explored in several field trials. For example, Meng et al. [215] investigated the effect of DMPP and DCD on $N_2O$ emissions and inorganic-N losses through leaching and runoff in a mixed pasture field. Although this study explored only one inhibitor concentration, positive effects of these NIs on greenhouse gas reduction, water quality, and improved soil health were found. Another field trial compared DCD and DMPP on altering soil inorganic N content in two soils with different pH and found that DCD is more effective than DMPP in increasing plant productivity [216]. However, one general disadvantage of these experimental approaches has been that only one variable was evaluated to test the efficiency of the inhibitors, such as soil pH, different inhibitor concentrations, or different crops. It is known that interactions among these variables can have a significant impact on system responses and therefore studying components in isolation is not necessarily sufficient to draw conclusions about the entire cropping system.

The development of new inhibitors requires a collaborative approach involving synthetic chemists, biochemists, microbiologists, plant nutritionists, and agronomists. Thus, to understand the overall benefit of the existing and newly developed inhibitor compounds, studies addressing their interactions with the soil components, in particular, the targeted enzymes in the soil (i.e., urease and AMO) and the root–soil system in dependence on concentration as well as their fate in the soil (i.e., identification of degradation products and degradation mechanism) in the absence and presence of plant roots are required. Factors such as microbial population, organic matter, and clay content are some examples of other variables that could affect inhibitor performance and degradation in soil. In other words, a better understanding of the chemical and microbiological processes that are triggered when an inhibitor is added to the soil is urgently required. For example, some known urease inhibitors, such as benzohydroxamic acid, perform well within in vitro assay experiments but poorly in soil incubation tests, possibly due to soil–metal ion interaction. This poses a problem as many urease inhibitors aim to chelate to the nickel centers within the active site and cannot perform inhibition if they are trapped by metal ions present in the soil. Therefore, understanding more about the fate of inhibitors and the effect of various soil variables by analyzing the inhibitor concentration–time profile in soils and their degradation products is essential for the design of new inhibitors with reliable performance.

This research could be further assisted by simulations using predictive models to create an artificial biological system, which will allow to link soil properties and biomass

production in silico [217]. For example, dynamic system simulation models (DSSMs) describe the changes in system states in response to external drivers, such as management practices, weather, and soil properties, and how these changes are affected by other components in the system. This approach can be used for all types of crop models and farming system models [217].

The development of novel inhibitor compounds will benefit from approaches used in the pharmaceutical drug discovery process, in particular, structure–activity relationship (SAR) studies, which assess the performance of different compounds in dependence on their molecular structure. SAR studies require large libraries of compounds, with their synthesis often becoming the rate-limiting step. One way to facilitate this design process is through using artificial intelligence (AI) [218]—a "deep learning" computer program that produces blueprints for the sequences of reactions needed to create new inhibitors. Pharmaceutical companies have been using AI to streamline the process of discovering new medicines for years [219]. For example, Segler and his team [220] used deep-learning neural networks to imbibe nearly all known single-step organic-chemistry reactions—approximately 12.4 million of them. The AI tool employs these neural networks repeatedly in the planning of a multi-step synthesis, deconstructing the desired molecule until it reaches the available starting reagents. The program-generated reaction pathways were validated in a double-blind trial, where the quality of the machine-devised synthetic route was compared to that designed by synthetic chemists. This example shows that the use of AI could be a promising approach to streamline the development of new inhibitor compounds.

Given the scale of production required for use in agriculture, new inhibitor compounds should be accessible in a few steps from readily available precursors. Furthermore, they need to be safe in handling, nontoxic to other soil organisms, aquatic life, crops and safe to the environment, efficient, and economical in use, as well as chemically stable over the duration of the application and decomposing to environmentally unproblematic products. The SAR-guided design of inhibitors based on the enzyme's active site is a useful approach for the design of urease inhibitors, as the active site has been widely studied in many species and remains largely conserved among them. Detailed crystallographic data are readily available that enable docking studies to assess binding modes and affinity of substrate–ligand complexes to guide inhibitor design before embarking on the synthesis [97,221]. Successful inhibitors later in the developmental process may be able to be co-crystallized with urease to obtain an understanding of the mode of binding. Additionally, prior to lengthy soil incubation experiments, inhibitor candidates can be screened for potential urease inhibition properties through an in vitro assay with Jack-Bean urease. Through this assay, $NH_3$ production can be quantified using a colorimetric technique developed by Weatherburn [222]. This approach is quick, reproducible, and allows for the rapid screening of a large library for inhibitory activity.

In the case of AMO, similar inhibitor–enzyme studies are hampered due to the lack of structural information on the active center. In this case, SAR studies could provide novel information about its structure. Rather than performing time-consuming soil incubations as the first performance screen, whole cell assays using commercially available AOB can be used to determine biochemical parameters in the absence of soil. For instance, a quick 60 min nitrification assay can determine the % inhibition and $IC_{50}$ value of new compounds in vitro. Once compounds with promising inhibitory effects have been identified more complex studies should be performed. One important tool is real-time kinetic studies, with a Clark-type electrode to distinguish between irreversible from reversible inhibitors of AMO [125,134]. In this experiment, the oxygen respiration rate of AOB is measured in situ prior to and after the addition of the NI to monitor the real-time kinetic order of the reaction [125,223]. Additionally, in-depth binding studies, such as Michaelis–Menten kinetics, reversibility of binding, and relative toxicity, are substantial experiments that can be incorporated into the pipeline to the development of new NIs [125,131].

Following the enzymatic studies using the JBU assay for UIs or the bacterial assay for NIs, promising candidates will be further evaluated in soil incubation studies without and with plants before moving to field trials.

A major consideration for the selection of UIs and NIs is their high effectiveness at the lowest possible application rate with a minimum of undesirable side effects. Uniform interaction of the substrate with the inhibitor can be achieved by either coating fertilizer granules with the inhibitor or by incorporating it into granules, as suggested by Slangen and Kerkhoff [224]. However, optimizing the inhibitor/N-fertilizer application rate is a complex task since it involves consideration of several variables, for example, different crops and cropping systems, soil properties, water availability, and climate. Using a bottom-up approach, where the complexity of the system is successively increased, could be the best strategy to address these problems. Plant–root interactions could be explored using simpler plant growth systems, such as Root-TRAPR [225] and EcoFAB [226]. These systems facilitate the exploration of root morphology and root exudate under controlled conditions and at a smaller scale. The devices are easy to build and can be used to study plant responses to elicitor challenges. Furthermore, to measure and validate the agronomic and environmental benefits of the new fertilizer formulations (with NIs and UIs), glasshouse and field trials for a range of production systems including vegetables and dairy pasture need to be carried out. This will help to evaluate the nitrogen loss pathways and yield benefits of the newly developed compounds. Measurements of $N_2O$ emissions and evaluation of potential ecotoxic effects will provide insight into the environmental impact of these new compounds. It is apparent that this program requires the cooperation of researchers from various disciplines.

One of the considerations for field trials could be the method of partial ammonium nutrition provision to crops. This means that ammonium can be a direct source of nitrogen for crops instead of nitrate. It is poorly translocated to the rhizosphere, unlike nitrate, which prevents its rapid uptake by plant root–soil systems. Ammonium nutrition to crops also improves the uptake of phosphorus. Thus, protons ($H^+$) are excreted to maintain charge equilibration in the roots when plants' roots take up $NH_4^+$, the resulting pH decrease in the rhizosphere supports the mobilization of phosphorus in the soil [227] and also increases the uptake of some micronutrients, such as manganese (Mn) [228]. Thus, by increasing the residence time of $NH_4^+$ in soil by applying a nitrification inhibitor, the effect on phosphate and micronutrients mobilization for plant nutrition can be intensified.

Another strategy to optimize N inputs is using the split application of N fertilizers in the soil. The split application involves applying smaller amounts of fertilizer at various times throughout a crop's growth cycle [229] (Halvorson et al., 2008). Rozas et al. [230] and Burton et al. [231] found that $N_2O$ emissions were reduced by delaying part of the application of N fertilizer from irrigated maize and in a warm wet year, respectively. In another study by Zhang et al. [232], it was revealed that the use of split nitrogen fertilizer application improved the antioxidant enzyme activity and the remobilization of photosynthate after anthesis in crops, thereby improving wheat grain yield. The use of nitrification and urease inhibitors along with the split application method could further reduce $NO_3^-$ concentrations, thereby reducing leaching and $N_2O$ emissions, providing a greater yield, high-quality characteristics, and balanced nitrogen management [233].

## 7. Conclusions

In conclusion, optimizing N availability to plants is crucial for maximizing crop yield and quality. However, the challenge lies in managing the various pathways through which nitrogen can be lost, including volatilization, emissions, leaching, runoff, and immobilization. While synchronizing N availability with plant uptake could potentially reduce N loss, it is influenced by both controllable management practices and uncontrollable weather dynamics. In recent years, the use of urease and nitrification inhibitors has emerged as a promising strategy to mitigate nitrogen loss and synchronize N availability with plant uptake. Urease inhibitors delay the hydrolysis of urea to $NH_4^+$, thereby reducing nitro-

gen loss through ammonia volatilization. Nitrification inhibitors temporarily inhibit the conversion of $NH_4^+$ to $NO_2^-$ by soil bacteria, slowing down the nitrification process and minimizing nitrogen loss as $NO_3^-$ or through denitrification.

This review has provided a comprehensive understanding of urease and nitrification-inhibitor technologies and their profound implications for plant growth and root nitrogen uptake. It emphasizes the need for developing inhibitors with enhanced efficiency and design principles. These inhibitors have the potential to revolutionize agricultural practices and play a significant role in optimizing nitrogen management for sustainable crop production. Looking ahead, future directions for inhibitor usage should focus on harnessing innovative advancements that prioritize the essential traits superior inhibitors should possess. By doing so, we can pave the way for more efficient and sustainable nitrogen management in agriculture.

In summary, the utilization of urease and nitrification inhibitors offers a promising approach to optimize nitrogen availability to plants, reduce nitrogen loss, and enhance crop production. Continued research and development in this field will contribute to the advancement of sustainable agricultural practices, ensuring improved nitrogen management and meeting the growing global demand for food while minimizing environmental impacts.

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