



UNIVERSITY OF THESSALY
MSc PROGRAM “HOSMIC-Host-Microbe Interactions”

MSc Thesis

*Development of a fast-track screening test with
ammonia-oxidizing bacteria for the identification
of novel biological nitrification inhibitors*

Name: Alexandros Kanellopoulos

Supervisors: Dr. Evangelia S. Papadopoulou, Dr. Dimitrios G. Karpouzas

Tripartite committee:

Dr. Evangelia S. Papadopoulou

Dr. Dimitrios G. Karpouzas

Dr. Kalliope K. Papadopoulou

This thesis was conducted from March 2024 to September 2024 at the Laboratory of Plant and Environmental Biotechnology, Department of Biochemistry and Biotechnology, University of Thessaly, as part of the interdepartmental MSc Program “HOSMIC-Host-Microbe Interactions”

Supervising professors:

Dr. Evangelia S. Papadopoulou, Assistant Professor in of Environmental Microbiology,
Department of Environmental Sciences, University of Thessaly

Dr. Dimitrios G. Karpouzas, Professor of Environmental Microbiology and Biotechnology,
Department of Biochemistry and Biotechnology, University of Thessaly

Tripartite committee members:

Dr. Evangelia S. Papadopoulou, Assistant Professor in Environmental Microbiology,
Department of Environmental Sciences, University of Thessaly

Dr. Dimitrios G. Karpouzas, Professor in Environmental Microbiology and Biotechnology,
Department of Biochemistry and Biotechnology, University of Thessaly

Dr. Kalliope K. Papadopoulou, Professor in Plant Biotechnology, Department of
Biochemistry and Biotechnology, University of Thessaly

Acknowledgements

I would like to extend my thanks and sincere gratitude to my two supervisors, Dr. Dimitrios Karpouzas and Dr. Evangelia Papadopoulou. Their welcoming and helpful attitude, their guidance and supervision has provided me with the invaluable opportunity to discover new research fields, participate in different exciting projects and acquire a strong theoretical background and technical skillset, on which I can rely in my future scientific endeavours. I would also like to express my thanks to Dr. Kalliope Papadopoulou, for her eagerness to be part of the tri-partite committee and review this thesis, as well as for all the scientific discussions we have had since the start of this thesis.

I am grateful to Ms. Maria Kolovou, PhD student, working in the Plant and Environmental Biotechnology Laboratory, who was tasked to familiarise and train me when I started working in the group, and has contributed to provide invaluable advice ever since. My thanks go also to Mr. Dimitrios Dalkidis, PhD student, working in the Plant and Environmental Biotechnology Laboratory, with whom I have cooperated for the better part of my time in the group, and whose support and advice was of great importance. Great thanks go to Dr. Hugo Ribeiro, post-doctoral researcher in the group, alongside the Archaea Biology and Ecogenomics Unit (group of Dr. Christa Schleper) from the University of Vienna, people with whom we have cooperated for many important findings, not all of which are presented in this thesis. We are also grateful for the material provided to us by the Molecular Systems Biology unit from the University of Vienna (group of Dr. Wolfram Weckwerth).

Finally, my gratitude extends to the whole Plant and Environmental Biotechnology Laboratory. Each member, for the last 2.5 years, has been welcoming to me, and all people have generated an international environment, conducive for personal and professional growth and the pursuit of science. I am proud to have been part of this group and I wish all the best to everyone involved.

Table of Contents

Abstract.....	
1. Introduction	1
1.1. The Nitrogen Cycle-Ecology and functioning	3
1.1.1. The Nitrogen Cycle-Ecology and functioning: Processes in Nitrogen Transformation.....	3
1.1.2. The Nitrogen Cycle-Ecology and functioning: Nitrogen Fixation and Ammonification	4
1.1.3. The Nitrogen Cycle-Ecology and functioning: Nitrification at the core of Nitrogen Cycling.....	6
1.1.3.1. <i>Ammonia Oxidation by AOB</i>	7
1.1.3.2. <i>Ammonia Oxidation by AOA</i>	9
1.1.3.3. <i>Ecological niche differentiation between AOB and AOA</i>	9
1.1.3.4. <i>Nitrite Oxidation</i>	11
1.1.3.5. <i>Complete Ammonia Oxidation (comammox)</i>	11
1.1.4. The Nitrogen Cycle-Ecology and functioning: Denitrification	12
1.2. The Nitrogen Cycle-Environmental and agricultural impact in the 21st century. 13	
1.2.1. The Nitrogen Cycle-Environmental and agricultural impact in the 21 st century: Anthropogenic nitrogen fixation	14
1.2.2. The Nitrogen Cycle-Environmental and agricultural impact in the 21 st century: Loss of Nitrogen Use Efficiency in agriculture	15
1.2.3. The Nitrogen Cycle-Environmental and agricultural impact in the 21 st century: NO ₃ ⁻ leaching as an environmental concern	16
1.2.4. The Nitrogen Cycle-Environmental and agricultural impact in the 21 st century: Emission of N ₂ O, a highly potent greenhouse gas	18
1.3. Nitrification Inhibition-Mitigating agricultural NUE loss and GHG emissions ..19	
1.3.1. Nitrification Inhibition-Mitigating agricultural NUE loss and GHG emissions: Synthetic Nitrification Inhibitors (SNIs)	20
1.3.2. Nitrification Inhibition-Mitigating agricultural NUE loss and GHG emissions: Biological Nitrification Inhibitors (BNIs)	23

1.4. The assessment process of Biological Nitrification Inhibitors	27
1.5. Aims of the study	30
2. Materials and Methods.....	31
2.1 Experimental Design	31
2.2 AOM strains and growth conditions	32
2.3 Nitrification Inhibitors.....	32
2.3.1 Nitrification inhibitor analysis through High Pressure / High Performance Liquid Chromatography (HPLC)	33
2.4 AOM Fast-Track System generation.....	33
2.5 Liquid Culture Assays	35
2.5.1 Liquid Culture Assays: Validation of the Fast-Track system with known SNIs and BNIs	35
2.5.2 Liquid Culture Assays: Proof-of-concept of the fast-track testing system with wheat root exudates	35
2.5.3 Liquid Culture Assays: Proof-of-concept of the fast-track testing system with pearl millet root exudates	36
2.6 Nitrite determination	36
2.7 Cell density determination.....	37
2.8 Data analyses and statistics	37
2.8.1 Modelling of the routine AOM cultures growth.....	37
2.8.2 EC ₅₀ calculation	38
2.8.3 Ammonia Oxidation Inhibition % (AOI%).....	39
2.8.4 Statistical tests.....	39
3. Results	41
3.1 Fast-track system standardisation	41
3.1.1 <i>Nitrosospira multififormis</i>	41
3.1.2 <i>Nitrosomonas ureae</i> and <i>Nitrosomonas communis</i>	43
3.2 Fast-track system validation with known SNIs and BNIs	47

3.3 Fast-track system proof-of-concept with plant root exudates	51
3.3.1 <i>Wheat root exudates</i>	51
3.3.2 <i>Pearl millet root exudates</i>	55
4. Discussion.....	62
4.1 Evaluation of the proposed fast-track assay	62
4.1.1 <i>Time frame of the fast-track assay</i>	62
4.1.2 <i>Ecological relevance of the fast-track assay</i>	63
4.2 Determination of inhibition thresholds for pure NIs	65
4.3 Determination of BNI activity of plant root exudates.....	67
4.4 Perspectives on the discovery and applicability of BNIs	68
5. Conclusions	70
6. References	71

Abstract

The issue of increasing global food insecurity demands an elevation in food production, through the adoption of a sustainable agricultural intensification model. This model involves approaches aimed at increasing agricultural productivity, while avoiding conventional practices that are both ineffective and harmful to the environment. Since nitrogen availability is a critical factor influencing crop productivity, enhancing soil nitrogen cycling is of paramount importance for both agriculture and the environment. Nitrogen cycling involves a naturally occurring sequence of microbial nitrogen transformations, with nitrification being a crucial step. However, exacerbated nitrification, often triggered by excessive nitrogen fertilisation, poses significant environmental and agricultural concerns. It results in nitrogen loss and decreased Nitrogen Use Efficiency (NUE), while, also leading to nitrate pollution, eutrophication of aquatic systems, and the emission of nitrous oxide, a potent greenhouse gas. The inhibition of nitrification has garnered significant interest, with synthetic nitrification inhibitors (SNIs) showing promise in mitigating the negative effects of excessive nitrification. However, due to drawbacks like off-target effects, there is now a shift towards the adoption of biological nitrification inhibitors (BNIs). The discovery and evaluation of novel BNIs is a complex and demanding process. Conventional bioassays that use nitrifiers as reporter strains to study nitrification inhibition *in vitro* are often limited by low throughput. High-throughput versions of these bioassays tend to lack ecological and phylogenetic diversity among the reporter strains, while others rely on genetically modified strains or are not validated with established BNIs. In this context, this study presents the development of a refined fast-track bioassay, primed for BNI screening, utilizing three distinct, soil-relevant ammonia-oxidizing bacteria (AOB). The bioassay has been standardised by achieving consistent cell activity and density for each strain and validated by determining inhibition thresholds for established and novel SNIs and BNIs. As shown in previous studies, nitrapyrin was the most effective NI across all AOB strains, with results for established nitrification inhibitors (NIs) aligning with findings from the literature. Differences in NI efficacy and strain sensitivity were consistent and reflected observations from numerous prior studies. The bioassay was further applied for high-throughput screening of root exudates from a large number of wheat and pearl millet genotypes for BNI activity, and it successfully differentiated inhibitory patterns of the root exudates, revealing (a) a higher inhibitory potential for wheat root exudates, (b) strain-specific sensitivity that reflected the responses observed in pure SNIs and BNIs tests; and (c) a correlation between certain root exudates and increased inhibition of specific AOB strains. Overall, this refined

bioassay represents an improvement over previously reported versions, featuring a more soil-relevant and diverse selection of AOB reporter strains. The evaluation of established SNIs and BNIs further confirmed its applicability, while providing inhibition thresholds for pure SNIs and BNIs against *Nitrosomonas ureae* and *Nitrosomonas communis* for the first time. This fast-track bioassay holds significant potential to advance the discovery of novel BNIs and BNI-plant genotypes, helping to mitigate pressing agricultural and environmental challenges, such as NUE loss, nitrite pollution and greenhouse gas emissions that contribute to climate change.

1. Introduction

The 21st century marks a critical crossroads, as the balance between the agricultural advancement and planetary sustainability may soon be irreversibly decided. The global population, projected to reach 9 billion within the next 20 years (FAOSTAT 2020), threatens to exacerbate the current food insecurity crisis [Figure 1A]. As of 2024, 28.9% of the global population is experiencing moderate or severe food insecurity [Figure 1C], according to FAOSTAT (2023a). This ranges from insufficient quality and quantity of food required for a healthy diet, to complete lack of food (FAO 2009). In response to growing food demand, conventional agricultural intensification has long been a cornerstone of increasing crops yields. This approach involves enhancing agronomical inputs, via use of fertilisers, pesticides, and intensive tilling, to boost production (Kremen 2020), resulting in significant increase in the yields of many essential crops [Figure 1B]. However, despite their contribution to food security, these practices have a substantial environmental cost. The most significant consequences include Greenhouse Gas (GHG) emissions (Stein 2023), groundwater and soil pollution, and the loss of both aboveground and belowground soil biodiversity (Seppelt et al. 2020; Barros-Rodriguez et al. 2021), as well as disruptions to ecosystem functioning. Together with other human activities, modern agriculture and food systems have driven multiple planetary boundaries beyond safe limits, particularly with respect to climate change and nitrogen cycling (Webb et al. 2020; Stein 2023).

In light of these challenges, numerous calls have been made to transition towards a more sustainable agricultural model, that can meet global food demands, while minimizing environmental impacts. The United Nations Sustainable Development Goal #2 (SDG2) (United Nations 2023) and the European Union European Climate Law, which seeks to reduce GHG emissions by 55% (European Commission), highlight the need to shift from conventional to ecological agricultural intensification. This new model focuses on improving yields by enhancing natural processes (Kremen 2020), while at the same time, promoting environmental sustainability and supporting ecosystem services (Kremen 2020). One such ecosystem service, deeply intertwined with modern agriculture, is the nitrogen cycle, a process that can both positively affect agriculture and be negatively affected by it in return. Therefore, it is no surprise that conventional agriculture relies heavily on nitrogen fertilisers to boost nitrogen availability and, consequently, plant performance. However, various sustainable agriculture approaches, such as the application of nitrogen-rich plant residues (Ammar et al. 2023) and

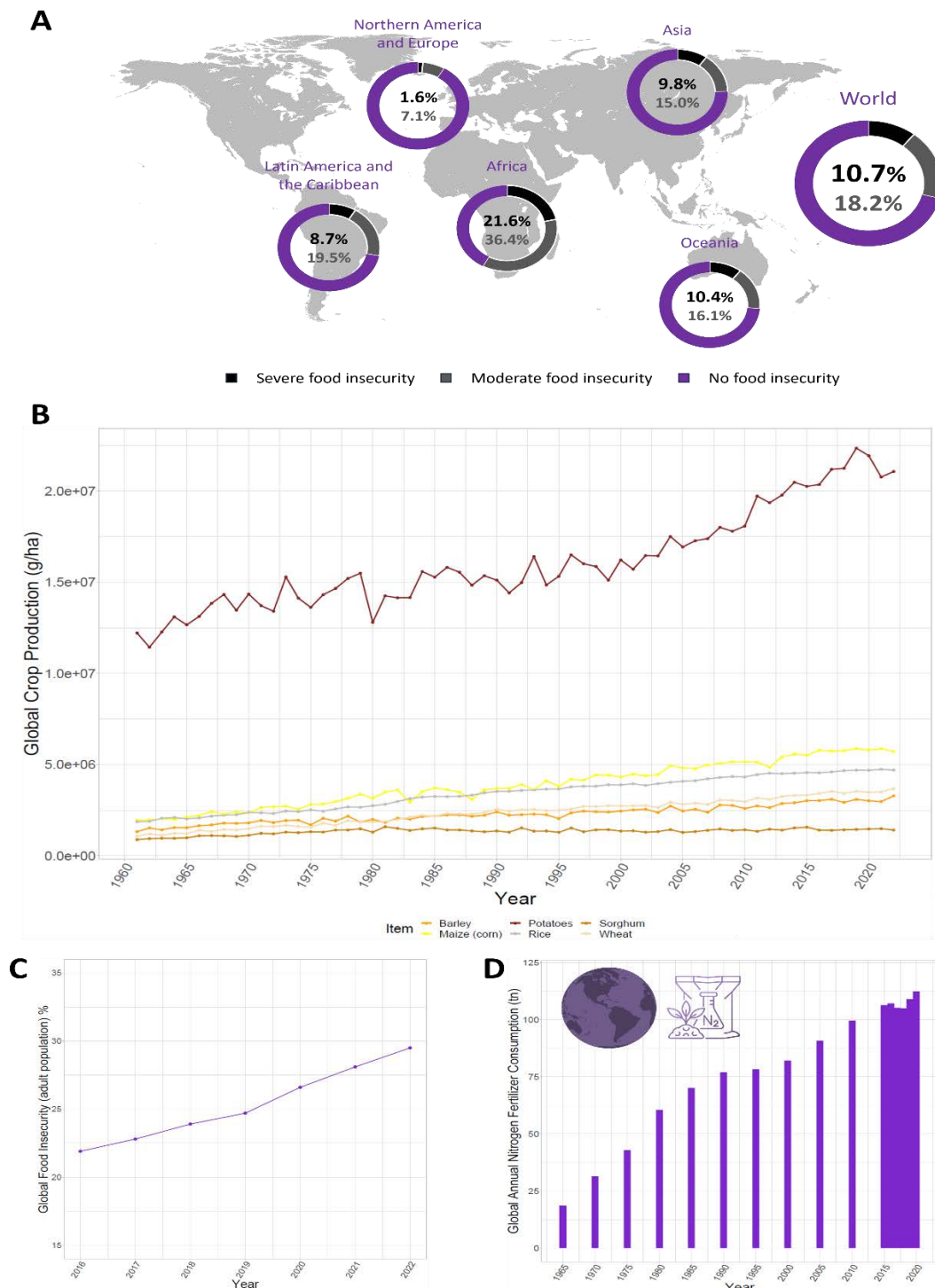


Figure 1. Overview of the food security and agriculture scene in the 21st century. (A) Estimates of global severe and moderate food insecurity based on 2024 data averaged from the last three years (FAOSTAT, 2023a). (B) Global production trends for six major crops (FAOSTAT, 2022). (C) Global trends in combined severe and moderate food insecurity with 2022 data averaged from the last three years (FAOSTAT, 2023a). (D) Global nitrogen fertiliser consumption from 1965-2020, based on data from the Statista Research Department (Statista Research Department, 2023a).

nitrogen-fixing bacteria (Ammar et al. 2023) also aim to enhance the nitrogen cycle by increasing bioavailable nitrogen for plants. Such approaches are complemented by the use of both synthetic nitrification inhibitors (SNIs), and the more recent, and still developing, biological nitrification inhibitors (BNIs) (Subbarao et al. 2012; Wang et al. 2021). Given its strong interconnection with agriculture, nitrogen cycling has become a primary focus in the transition towards sustainable farming practices.

1.1 The Nitrogen Cycle-Ecology and functioning

Nitrogen is one of the most essential elements for sustaining life, playing a crucial role in the biosynthesis of numerous biomolecules, including nucleic acids, and proteins (Kuypers et al. 2018), as well as structural glycoproteins. Additionally, nitrogen is integral to smaller functional molecules that are essential for oxidoreductive metabolic reactions, such as electron carriers like NADH and NADPH. Furthermore, nitrogenous secondary metabolites, such as alkaloids and glucosinolates, are important for plant health (Singh 2018). Nitrogenous antibiotics and lantibiotics, produced predominantly by *Streptomyces* species (Krysenko 2023), are key players in microbe-microbe interactions. Moreover, the availability of different nitrogen forms has been shown to influence critical symbiotic relationships in nature, such as the legume-rhizobia symbiosis (endophytic nitrogen fixating bacteria) (Valmas et al. 2023), as well as tri-partite interactions between legumes, rhizobia and AMF (Arbuscular Mycorrhizal Fungi) (Kafle et al. 2019). The immense agricultural significance of nitrogen lies in the fact that its limitation, along with phosphorus deprivation, is often a major cause of reduced crop productivity (Valmas et al. 2023). Therefore, monitoring nitrogen cycling in soil, along with interventions to enhance nitrogen availability for plants, is of high agricultural significance.

1.1.1 The Nitrogen Cycle-Ecology and Functioning: Processes in Nitrogen Transformation

The nitrogen cycle is a microbially-driven biogeochemical process, involving a wide range of bacteria, archaea and fungi (Bernhard 2010). The cycle encompasses various nitrogen forms, with different oxidation states [Figure 2C], and occurs in both oxic and anoxic conditions, relying on a diverse array of microorganisms and enzymes [Figure 2C]. Despite its designation as a cycle, this process is more accurately described as a dynamic assembly of distinct nitrogen transformation processes, each associated with varying nitrogen fluxes [Figure 2B] (Kuypers et al. 2018). These processes primarily include nitrogen fixation (the reduction of gaseous

dinitrogen (N_2) to ammonia (NH_3)), nitrification (a step-wise oxidation of ammonia (NH_3) to nitrite (NO_2^-) and, then, to nitrate (NO_3^-)), and denitrification (a step-wise reduction of nitrate (NO_3^-) back to dinitrogen (N_2)) [Figure 2A-B]. Ammonia is also generated through ammonification (a mineralisation process of organic nitrogenous biomass). Anammox, an anoxic process, also oxidizes ammonia to produce N_2 . Due to the metabolic versatility of the microorganisms involved in the nitrogen cycle, classifying them solely based on these processes has become increasingly difficult (Kuypers et al. 2018), as many microorganisms participate in more than one processes. Furthermore, many steps in the cycle can operate in reverse, such as respiratory nitrate reduction to nitrite, or Dissimilatory Nitrite Reduction to Ammonia (DNRA) (Kuypers et al. 2018).

1.1.2 The Nitrogen Cycle-Ecology and Functioning: Nitrogen fixation and Ammonification

The largest freely available nitrogen source is dinitrogen gas, with a global inventory of 3.9×10^9 Tg of nitrogen [Figure 2B] (Kuypers et al. 2018). In soil, however, organic nitrogen dominates the nitrogen pool, as 98% of total soil nitrogen is stored in organic sources (Soumare et al. 2020). Nevertheless, plant available forms of nitrogen are limited to ammonium nitrogen ($\text{NH}_4^+\text{-N}$) and nitrate-nitrogen ($\text{NO}_3^-\text{-N}$) (Soumare et al. 2020), making almost all free nitrogen inaccessible for direct plant uptake. Nitrogen fixation and ammonification are two processes that convert dinitrogen gas to ammonia and release ammonia from organic sources, respectively.

Nitrogen fixation [Figure 2B (1)] is performed by both bacteria and archaea through the nitrogenase enzymatic system, which consists of the dinitrogenase and dinitrogenase reductase enzymes (Stein and Klotz 2016) [Figure 2C (11)]. The most well-known case of nitrogen-fixing microorganisms are the rhizobia, alpha-proteobacterial genera such as *Rhizobium*, *Mesorhizobium* and *Bradyrhizobium* (Mahmud et al. 2020), which engage in symbiosis with plants through nodulation (Oldroyd et al. 2011). Other nitrogen-fixing bacteria live symbiotically with unicellular algae (Kuypers et al. 2018), contributing to marine nitrogen fixation. Additionally, free-living and associative nitrogen-fixing bacteria (Soumare et al. 2020), which do not form obligatory plant symbioses, can also perform nitrogen fixation. Examples include members of the genera *Azospirillum* (Mahmud et al. 2020), *Azotobacter* and *Pseudomonas* (Orr et al. 2011). Furthermore, some methanotrophic bacteria and cyanobacteria can fix atmospheric dinitrogen, using energy from methane oxidation and photosynthesis,

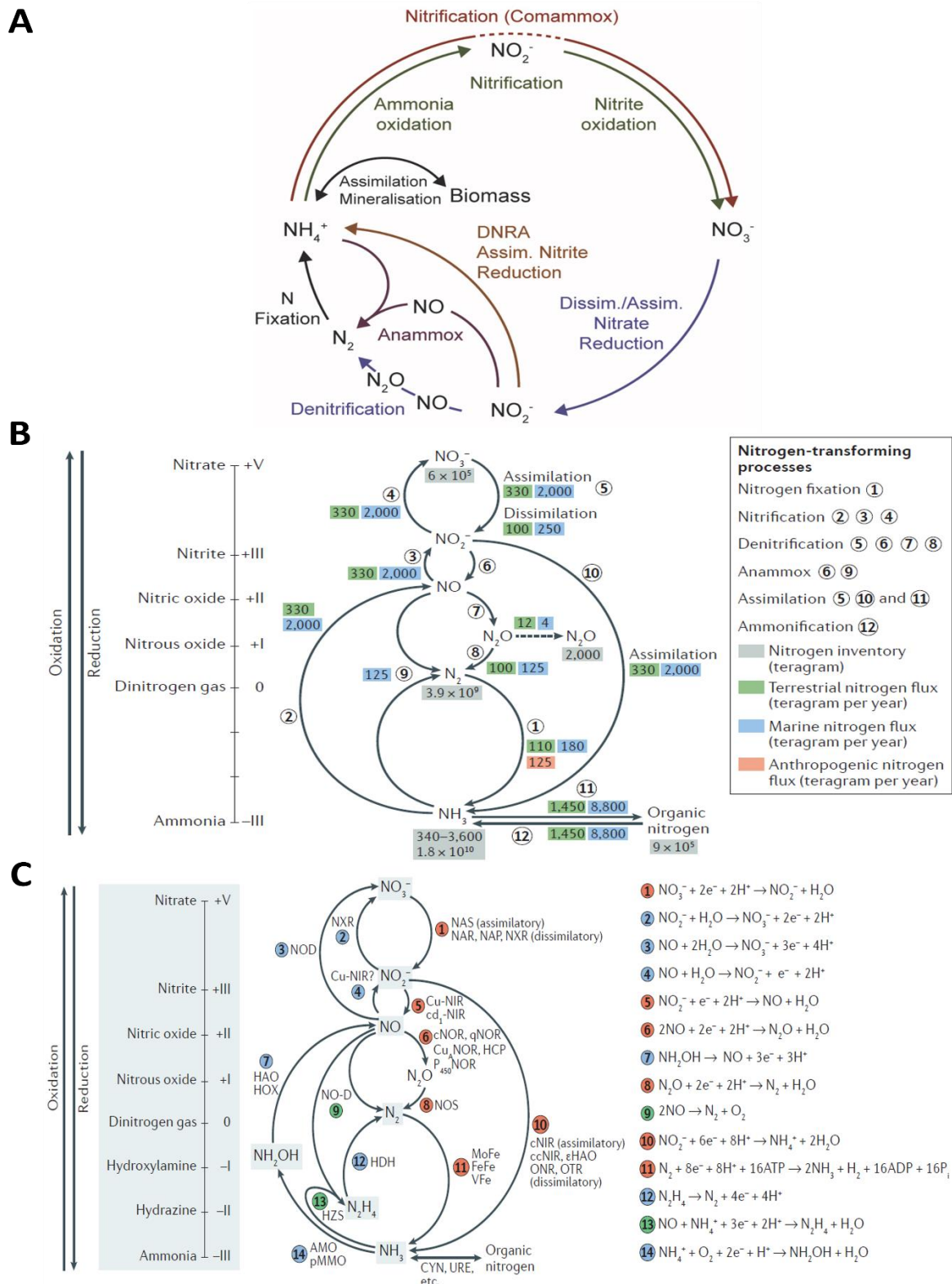


Figure 2. Overview of the Nitrogen Cycle. The figure illustrates the different steps of the Nitrogen Cycle. (A) A detailed depiction adapted from Daims et al. (2016). Note that organic nitrogen is produced during the assimilation of ammonia nitrogen. (B) An in-depth illustration, including estimated nitrogen fluxes for each process (Kuypers et al. 2018). (C) An overview of the enzymes catalysing the reactions in the different processes of the Nitrogen Cycle (Kuypers et al. 2018).

respectively (Cui et al. 2022; Massey and Davis 2023). Interestingly, nitrogen fixation is thought to have originated in bacteria and later transferred to archaea (Pi et al. 2022). However, only few archaeal species (373) carry at least one nitrogenase gene compared to bacteria (3485), and most archaeal nitrogenase genes are phylogenetically nested with bacterial ones, with the process primarily found in the *Euryarchaeota* phylum (Dos Santos et al. 2012).

Ammonification (Kuypers et al. 2018), also known as mineralisation (Stein and Klotz 2016) involves the enzymatic release of ammonia from organic matter [Figure 2B (12)]. This process involves a range of microbially produced extracellular enzymes that break down large nitrogenous polymers into monomers and ammonium (Ouyang and Norton 2020). These enzymes mainly target proteins, cell wall components and nucleic acids (Sieradzki et al. 2023). Generally, ammonification can be described as a two-step process: first, microorganisms use proteases and other polymer-degrading enzymes to break down polymers into monomers, such as amino acids. Then, enzymes like the nickel-dependent urease (Svane et al. 2020) [Figure 2C] release ammonia from the amine groups of simple compounds, such as urea (Strock 2008). The process results in the formation of a dissolved ammonia nitrogen pool (Schimmel and Bennett 2004).

1.1.3 The Nitrogen Cycle-Ecology and Function: Nitrification at the core of Nitrogen Cycling

Among all the processes within the nitrogen cycle, nitrification serves as the rate-limiting step of the entire cycle, with ammonia oxidation setting the pace for the nitrification process itself (Lehtovirta-Morley 2018). As a process, it is one of the most studied biological processes, with studies as early as 1890 (Frankland and Frankland 1890; Winogradsky 1890), and it is of high agricultural interest due to the nitrogen transformations it entails, and the relatively narrow phylogenetic range of microorganisms participating in agricultural soils. Traditionally, nitrification is viewed as a modular process with two main steps (Coskun et al. 2017). In the first step, ammonia oxidation, ammonia- produced via nitrogen fixation and ammonification- is oxidized to nitrite [Figure 3]. In the second step, nitrite is further oxidized to nitrate [Figure 3] (Coskun et al. 2017). However, nitrification is more complex than this simple two-step view suggests. It involves, several intermediates, such as hydroxylamine (NH₂OH) and nitric oxide (NO) (Lehtovirta-Morley 2018). Additionally, nitrogen fluxes may not follow a straightforward path, as intermediates can be recycled within the system [Figure 3B]. Furthermore, ammonia oxidation is supported by microorganisms with different lifestyles,

including anammox (Anaerobic Ammonia Oxidation), lithoautotrophic and heterotrophic ammonia oxidation (Nardi et al. 2020) and comammox (Complete Ammonia Oxidation) (Daims et al. 2015).

Initially, ammonia oxidation was thought to be mediated by ammonia oxidizing bacteria (AOB) (Prosser and Nicol 2012). However, after the discovery of ammonia oxidizing archaea (AOA), which, sometimes, dominate ammonia oxidation in certain ecosystems (Prosser and Nicol 2012), it became clear that both bacteria and archaea play roles in the first step of nitrification (Coskun et al. 2017). Additionally, heterotrophic ammonia oxidation has been observed in some fungi (Nardi et al. 2020). Ammonia oxidising microorganisms are ubiquitous in the environment (Lehtovirta-Morley 2018), having been discovered in garden soils (Winogradsky 1890; Tourna et al. 2011), agricultural soils (Watson et al. 1971), freshwater ecosystems (Ghimire-Kafle et al. 2023), marine habitats (Könneke et al. 2005), engineered ecosystems, like wastewater treatment plants (Rowan et al. 2003) and even extreme environments such as thermal springs (Abby et al. 2018) and permafrost cryosols (Sanders et al. 2019). Ammonia oxidisers have even been discovered on human skin (Lehtovirta-Morley 2018) and as symbionts in sponges (Moeller et al. 2019), where they may form unique ecosystems in cooperation with other nitrogen cycling microorganisms (Schleper and Nicol 2010). In agricultural soils, these ammonia oxidizers are of great significance, as they regulate nitrogen fluxes between different forms, affecting both biomass production and environmental impact.

1.1.3.1 Ammonia Oxidation by AOB

Soil-relevant AOB are represented by species of the beta- proteobacterial *Nitrosospira* and *Nitrosomonas* genera (Nardi et al. 2020). These microorganisms are chemolithoautotrophs, obtaining carbon by carbon dioxide (CO₂) fixation via the Calvin-Benson-Bassham (CBB) cycle (Lehtovirta-Morley 2018), although mixotrophy has also been reported (Wright and Lehtovirta-Morley 2023). Initial ammonia oxidation is catalysed by the membrane-bound, copper-dependent ammonia monooxygenase (AMO) enzyme [Figure 3A], which is typically encoded in multiple copies within an operon structure (Lehtovirta-Morley 2018; Nardi et al. 2020). Of the three subunits, the gene coding for the α -subunit (*amoA*) has traditionally been used as a hallmark phylogenetic marker gene for both AOB and AOA (Rotthauwe et al. 1997). This gene allows for the detection, quantification, and identification of ammonia oxidisers.

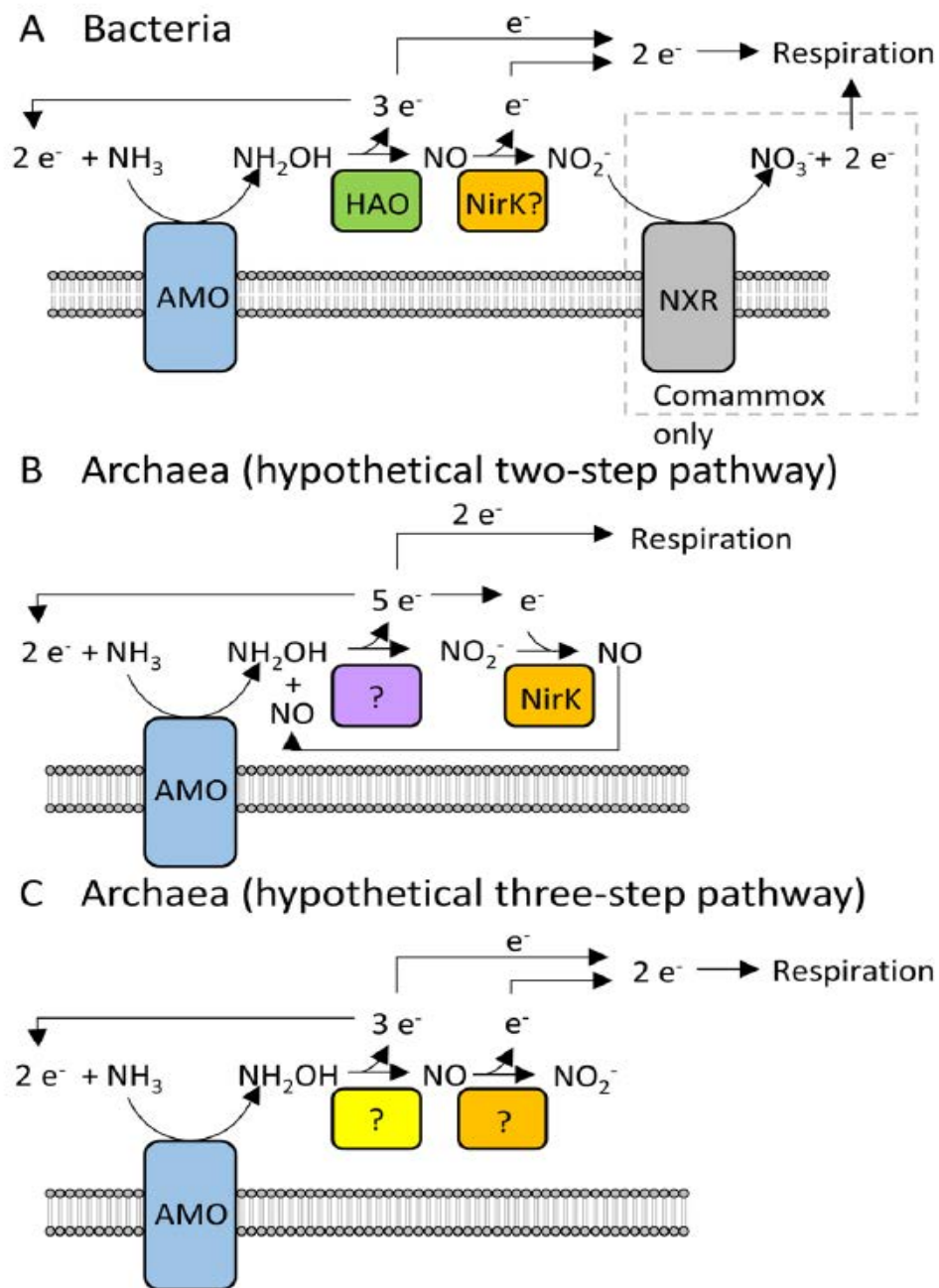


Figure 3. Pathways of ammonia oxidation in bacteria and archaea. (A) Ammonia oxidising bacteria and comammox bacteria. (B) Hypothetical two-step model in ammonia oxidizing archaea and (C) Hypothetical three-step model in ammonia oxidizing archaea (Lehtovirta-Morley 2018).

Rather than nitrite being the immediate product of ammonia oxidation, ammonia is first transformed into hydroxylamine by AMO and subsequently oxidized to nitric oxide by a hydroxylamine oxidoreductase (HAO) [Figure 3A]. Finally, nitric oxide is oxidized to nitrite by an- as of yet- unknown enzyme [Figure 3A], though candidates include the nitrite reductase (NirK) which is involved in the later stage of denitrification [Figure 3A], as well as the red

copper protein nitrosocyanin (NcyA) (Lehtovirta-Morley 2018). AOB are characterized by a low-affinity AMO enzyme and extensive intracytoplasmic membrane structures (Lehtovirta-Morley 2018). This structural adaptation allows them to express high levels of the enzyme, which works optimally at relatively high substrate concentrations. Furthermore, some AOB species are urease positive (Lu et al. 2012; Kerou et al. 2016; Wright and Lehtovirta-Morley 2023), enabling them to utilise urea as ammonia source for ammonia oxidation.

1.1.3.2 Ammonia Oxidation by AOA

AOA in soil environments are mainly members of the *Nitrososphaera* and *Nitrosocosmicus* genera (Lehtovirta-Morley 2018). In soils, they often dominate over AOB (Prosser and Nicol 2012), sometimes by orders of magnitude (Lehtovirta-Morley 2018). Like AOB, AOA are chemolithoautotrophic, and they fix bicarbonate (HCO_3^-) via the 3-hydroxypropionate/4-hydroxybutyrate cycle (Lehtovirta-Morley 2018). From a biochemical perspective, ammonia oxidation in archaea proceeds similarly to that in bacteria. However, certain differences exist, primarily due to gaps in our current understanding. For instance, hydroxylamine and nitric oxide are intermediates in both bacterial and archaeal ammonia oxidation. However, archaeal genomes lack homologues of the HAO enzyme found in bacteria, suggesting that a novel enzyme may be responsible for hydroxylamine oxidation in archaea (Lehtovirta-Morley 2018) [Figure 3B-C]. Alternatively, abiotic transformation of hydroxylamine is also a possibility. Similarly to AOB, the transformation of nitric oxide to nitrite in archaea remains debated, with two different hypothetical pathways proposed (Lehtovirta-Morley 2018) [Figure 3B-C]. The archaeal AMO exhibits much higher substrate affinity compared to its bacterial counterpart, while soil-dwelling archaea also show relatively higher specific growth rates than AOB (Prosser and Nicol 2012). Ureolysis has also been reported in AOA, similar to AOB (Lehtovirta-Morley et al. 2016; Wright and Lehtovirta-Morley 2023).

1.1.3.3 Ecological niche differentiation between AOB and AOA

AOB and AOA are considered to compete for the substrate of ammonia oxidation (Prosser and Nicol 2012). The ecological niches of these different ammonia oxidisers in soil are influenced by environmental factors, with ammonia concentration and pH being the two key drivers of the diversity, abundance and activity of these microbes (Lehtovirta-Morley 2018). Other factors proposed to affect niche differentiation include temperature (Lehtovirta-Morley 2018), oxygen availability and light (Ghimire-Kafle et al. 2023), salinity (Rowan et al. 2003), and the presence of urea and metals (Lehtovirta-Morley 2018).

Ammonia oxidation is the primary, and sometimes the sole source of energy and reducing power for ammonia oxidizing microorganisms (Wright and Lehtovirta-Morley 2023), with ammonia (NH_3) rather than ammonium (NH_4^+) being the preferred substrate for AMO (Wright and Lehtovirta-Morley 2023). Niche differentiation may occur due to variations in ammonia affinity and tolerance to high ammonia concentrations (Prosser and Nicol 2012). Initially, it was suggested that AOB would dominate in high ammonia soils, due to their higher ammonia affinity and tolerance, while AOA would prevail in low-ammonia soils, respectively (Prosser and Nicol 2012). This has been observed in various environments (Lehtovirta-Morley 2018) and microcosm studies (Prosser and Nicol 2012; Hink et al. 2018). However, specific AOA, such as “*Ca. Nitrosocosmicus franklandianus*”, have been found to grow in ammonia concentrations characteristic of the AOB niche (Lehtovirta-Morley et al. 2018; Hink et al. 2018), indicating a more complex competition. In addition, AOB are considered to prefer inorganic over organic ammonia sources, leading to higher activity in fertilised soils (Stein 2023).

Ammonia concentration is also tightly linked to environmental pH, as ammonia and its protonated form, ammonium, exist in a pH-dependent equilibrium (Wright and Lehtovirta-Morley 2023), with ammonia concentrations being extremely low in acidic soils (Lehtovirta-Morley 2018). This, theoretically, favours AOA dominance in acidic soils, supported by the fact that no acidophilic AOB had been isolated (Prosser and Nicol 2012), whereas several acidophilic AOA has been cultured, such as *Nitrosotalea devanaterrea* (Lehtovirta-Morley et al. 2011) and “*Candidatus Nitrosotalea sinensis Nd2*” (Lehtovirta-Morley et al. 2014). However, this notion was challenged by early observations of ammonia oxidation by bacteria in acidic soils (De Boer et al. 1991) and the eventual isolation of “*Candidatus Nitrosoglobus terrae TAO100*”, an acidophilic ammonia oxidizing bacteria species (Hayatsu et al. 2017). This suggests that niche differentiation based on pH is more complex than previously thought. In acidic soils with both AOA and AOB present, AOA often dominate functionally (Nardi et al. 2020). Nevertheless, low ammonia concentrations in acidic soils do not entirely exclude AOB activity. Mechanisms such as ureolysis, aggregate and biofilm formation (Lehtovirta-Morley 2018) and bacterial occupation of neutral pH microsites in acidic soils (Lin et al. 2021) may enable AOB to compete in these environments. Furthermore, AOB have been shown to grow in acidic conditions after urea amendment and selective inhibition of AOA (Zhao et al. 2020). Nitrification in alkaline soils is less studied, as an obligate alkaliphilic ammonia oxidizer has yet to be isolated (Lehtovirta-Morley 2018).

1.1.3.4 Nitrite Oxidation

The process of nitrite oxidation is carried out by nitrite-oxidizing bacteria (NOB), primarily from the genus *Nitrobacter* and *Nitrospira*, among others (Nardi et al. 2020). This leads to the oxidation of nitrite (NO_2^-) to nitrate (NO_3^-) [Figure 2B (4)] through the membrane-bound nitrite oxidoreductase (NXR) enzyme [Figure 2C (2)]. Similar to the *amoA* gene, the genes coding for the α -subunit (*nxrA*) and the β -subunit of NXR (*nxrB*) have been used as phylogenetic markers (Vanparys et al. 2007; Kop et al. 2024), with *nxrB* providing higher resolution within the *Nitrospira* lineage compared to *nxrA* (Pester et al. 2014; Daims et al. 2015). In soil environments, NOB are mainly represented by *Nitrobacter* and *Nitrospira*-like species (Nardi et al. 2020). NOB are predominantly autotrophs, obtaining energy through nitrite oxidation (Kuypers et al. 2018), although some *Nitrobacter* species exhibit mixotrophy, capable of growth on alternative energy sources like cyanate and formate (Nardi et al. 2020). Initially, nitrite oxidation was thought to be unidirectionally dependent on ammonia oxidation, as nitrite is produced from the latter process. However, observations that ammonia, released from cyanate degradation, can be utilized by ammonia oxidisers, suggests a potential interdependent trophic relationship between these microorganisms (Nardi et al. 2020). Similar to ammonia oxidisers, a niche specialization can be inferred for NOB. *Nitrobacter*-like NOB are considered r-strategists, characterized by low substrate affinity and a relatively high growth rate, similar to AOB. In contrast, *Nitrospira*-like NOB are K-strategists, with low growth rates, and better adaptation to low substrate concentrations, paralleling the ecological behaviour of AOA (Nardi et al. 2020).

1.1.3.5 Complete Ammonia Oxidation (comammox)

Ammonia oxidation was traditionally believed to be a stepwise process, mediated by two distinct groups of microorganisms (Kuypers et al. 2018), following the division originally proposed by Winogradsky (1890) and supported by the theory of the optimal pathway length (Costa et al. 2006). Contrary to this belief, it has been suggested that “comammox”, complete ammonia oxidation in a single pathway combining both ammonia and nitrite oxidation, could be energetically possible. This pathway results in lower growth rates but higher overall yield (Costa et al. 2006). Since 2015, bacteria belonging to the well-known nitrite-oxidizing *Nitrospira* genus (Lehtovirta-Morley 2018), have been isolated and found to possess the enzymatic arsenal (AMO and HAO) of their AOB counterparts (Nardi et al. 2020) [Figure 3A]. These bacteria are capable of performing complete ammonia oxidation within a single cell

[Figure 2A]. The genetic repertoire of the first isolated comammox, “*Candidatus Nitrospira inopinata*”, is believed to have been acquired through horizontal gene transfer, supported by the presence of upstream transposase genes and a distinct tetranucleotide pattern in the relevant genes (Daims et al. 2015). Similar to other ammonia oxidisers, the *amoA* gene can be used as a phylogenetic marker for comammox bacteria (Nardi et al. 2020). Comammox bacteria fix carbon from CO₂, like AOB, but utilize the reductive tricarboxylic acid (rTCA) cycle, in contrast to the CBB cycle (Lehtovirta-Morley 2018) employed by AOB. These bacteria appear well-adapted to environments with low ammonia concentrations (Kuypers et al. 2018), suggesting that their ecological niche is similar to that of AOA (Nardi et al. 2020.). The initial notion was that comammox bacteria may outcompete most cultured ammonia-oxidizing microorganisms in such environments (Kuypers et al. 2018), but not in ammonium-rich habitats (Li et al. 2023). Additionally, and unlike AOB, their growth is believed to be inhibited by inorganic nitrogen amendments (Nardi et al. 2018). Interestingly, however, there have also been reports of comammox *Nitrospira* being the active nitrifiers in high-ammonia soil environments (Li et al. 2023), including a patty soil with neutral pH (Zhou et al. 2020), though other studies observed lower comammox activity in soil compared to canonical nitrifiers (Xu et al. 2020).

1.1.4 The Nitrogen Cycle-Ecology and Functioning: Denitrification

Denitrification is a major nitrate (NO₃⁻) reduction pathway, carried out by facultative anaerobic bacteria that ultimately produce dinitrogen (N₂) via intermediate gaseous species (Suri et al. 2021) [Figure 2A]. The process is modular, with a range of reductases transforming nitrate to nitrite (NO₂⁻), nitric oxide (NO), nitrous oxide (N₂O) and, finally, dinitrogen gas [Figure 2B (5-8)]. Denitrification occurs strictly under anaerobic conditions, where nitrogen compounds act as terminal electron acceptors (Knowles 1982). Unlike nitrification, denitrification involves a broad range of biochemically and phylogenetically distinct microbial groups with varying lifestyles (Knowles 1982). Denitrifying bacteria include *Pseudomonas*, *Achromobacter*, *Bacillus*, *Paracoccus denitrificans*, *Thiobacillus denitrificans* and *Halobacterium* among many others (Knowles 1982). Additionally, denitrification has been observed in halophilic and hyperthermophilic archaea (Zumft 1997), as well as fungi (Pang and Wang, 2021), though bacteria are considered the primary contributors (Pang and Wang, 2021). Interestingly, an alternative form of denitrification, termed nitrifier denitrification, is also observed in nitrifiers. In this process, nitrite acts as a substrate and provides a distinct

pathway for N₂O production, using the same enzymes as the canonical denitrification pathway (Van Groenigen et al. 2015). Denitrification can be classified into heterotrophic and autotrophic types, depending on the electron donors used by the denitrifiers. Heterotrophic denitrification generally exhibits higher rates (Pang and Wang 2021). Limitation of electron donors or the presence of higher oxygen concentrations are key inhibitors of the denitrification process (Zumft 1997). The genes responsible for denitrification include those for the reduction of nitrate (*nar*), nitrite (*nir*), nitric acid (*nor*), and nitrous acid (*nos*) [Figure 2C (1,5,6,8)]. These genes are often clustered together in the genome (Zumft 1997). Additional genes for electron transfer, such as cytochromes and azurins are also important (Zumft 1997). Denitrifying microorganisms are typically believed to possess the full genetic repertoire required for complete denitrification (Zumft 1997). However, in some cases, missing or mutated genes (Mefferd et al. 2022), together with enzyme inhibition, such as inhibition of the *nosZ* enzyme by methanobactin released by methanotrophs (Chang et al. 2022), can lead to incomplete or truncated denitrification pathways. Incomplete denitrification can result in the accumulation of intermediates like nitric oxide or nitrous oxide (Mefferd et al. 2022).

1.2 The Nitrogen Cycle-Environmental and agricultural impact in the 21st century

The nitrogen cycle plays a crucial environmental role by converting inert dinitrogen into reactive forms that can be used for biomass production, and by recycling nitrogen from dead or extracellular organic matter back into the environment. However, the anthropogenic activities have disrupted the nitrogen cycle, leading to alterations in nitrogen fluxes (Kuypers et al. 2018) [Figure 2B], and surpassing thresholds for human and ecosystem health (Melillo 2021; Stein 2023), which are exceeded particularly due to environmental pollution from excessive reactive nitrogen compounds like nitrate [Figure 4 (2)]. Soil ammonia oxidisers also contribute to significant nitrogen losses in agriculture, particularly by converting fertiliser ammonium into nitrate, which is more prone to leaching (Wang et al. 2020) [Figure 4 (1)]. This contributes to low Nitrogen Use Efficiency (NUE) in agriculture, meaning that a large proportion of the nitrogen in fertilisers is not assimilated by crops. Finally, both denitrifiers

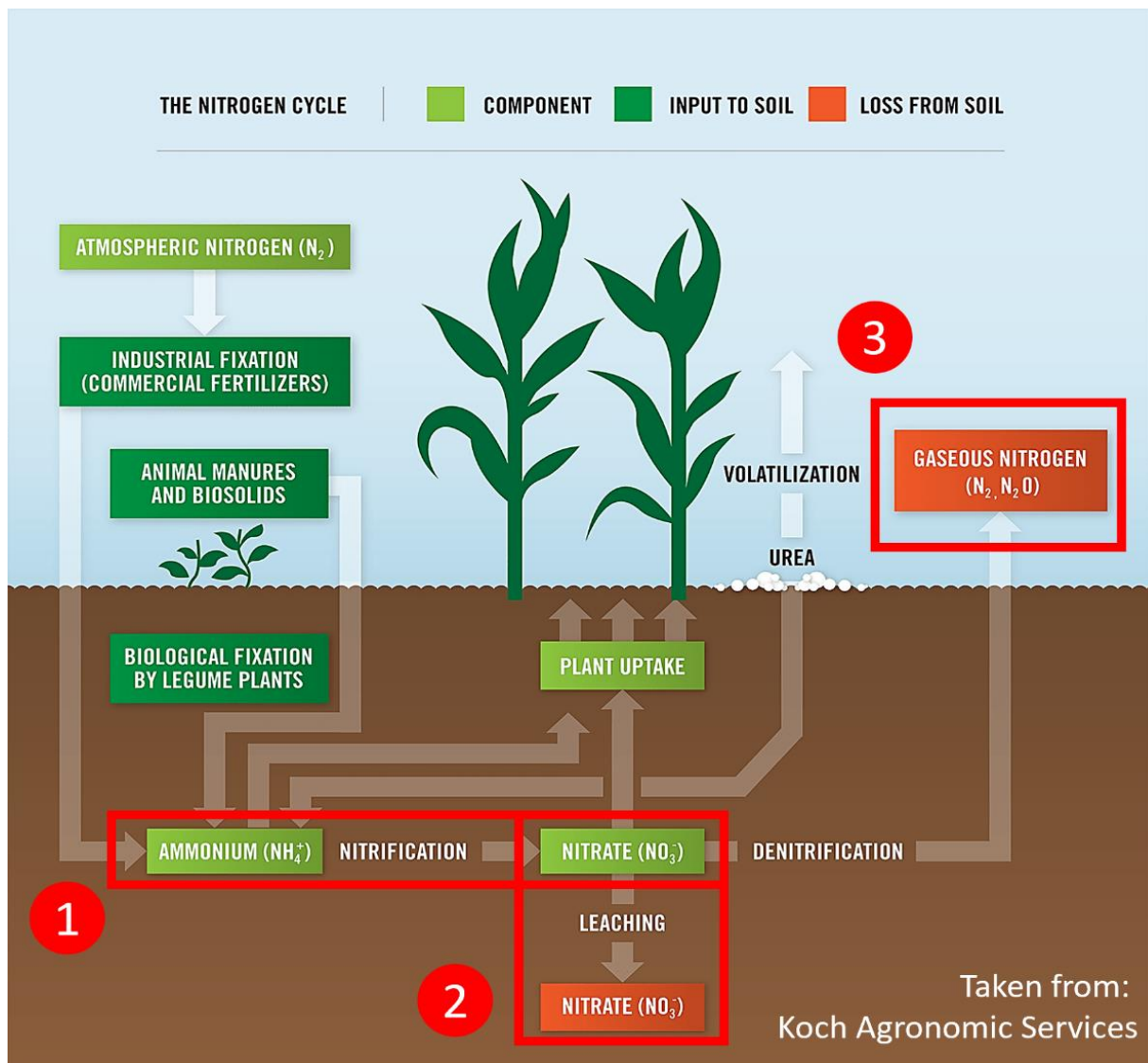


Figure 4. Graphical representation of the effects of nitrogen cycle processes in both environmental and agricultural contexts. The graph illustrates (1) the conversion of fertiliser ammonium to nitrate, (2) nitrate leaching from soil, and (3) the production of gaseous nitrogen forms, including greenhouse gases.

and ammonia oxidisers contribute to GHG emissions (Wang et al. 2020, Nardi et al. 2021) [Figure 4 (3)], further amplifying the environmental impact of nitrogen cycle disruption.

1.2.1 The Nitrogen Cycle-Environmental and agricultural impact in the 21st century: Anthropogenic nitrogen fixation

The disruption of the nitrogen cycle is largely due to human activities, particularly anthropogenic nitrogen fixation and excessive ammonia input into soils. Natural nitrogen fixation, which includes biological nitrogen fixation, alongside a small contribution of about

5 Tg year⁻¹ from lightning (Fowler et al. 2013), result in an estimated 110 Tg of nitrogen fixed annually in terrestrial ecosystems (Kuypers et al. 2018). In contrast, human-driven nitrogen fixation has surged dramatically, from 5 Tg year⁻¹ in the 1900s (Melillo 2021) to 125 Tg year⁻¹ in the 21st century (Kuypers et al. 2018) [Figure 2B]. This increase is primarily due to the excessive use of the industrial Haber-Bosch process (Stein et al. 2023). The Haber-Bosch process, which requires high temperatures and pressures to fix nitrogen, is responsible for 1.4% of global carbon dioxide emissions and consumes 1% of the world's total energy production (Capdevilla-Cortada 2019). Additionally, its widespread use is considered to have exceeded planetary sustainability limits (Stein et al. 2023). The financial cost of this process is also significant, ranging from \$300 (Nature Synthesis Editorial 2023) to \$800 (Nosherwani and Neto 2021), and in some cases even reaching \$1,600 per ton of ammonia produced (Ofori-Bah and Amanor-Boadu 2022). As a direct result, the global consumption of synthetic nitrogen fertilisers, previously sourced mainly from mining nitre deposits (Nature Synthesis Editorial 2023), has risen sharply [Figure 1D], dominating fertiliser markets in many countries [Figure 5A]. This heavy reliance on industrial nitrogen fixation and the overuse of synthetic fertilisers has led to a doubling of the reactive nitrogen entering Earth's terrestrial ecosystems each year (Kuypers et al. 2018; Melillo 2021). Importantly, this increased fixed nitrogen input intensifies nitrogen cycle processes, driving higher rates of nitrification and denitrification [Figure 4], resulting in a threefold impact.

1.2.2 The Nitrogen Cycle-Environmental and agricultural impact in the 21st century: Loss of Nitrogen Use Efficiency in agriculture

The first significant impact of nitrogen cycle disruption is seen in increased microbial activity, particularly in nitrification, and the ammonia oxidation process [Figure 4 (1)]. When nitrogen fertilisers containing urea are applied, urea is hydrolysed to release ammonia. Nitrification then converts this ammonia into nitrate (Wang et al. 2021). While both ammonia and nitrate are forms of nitrogen that plants can use for biomass production (Zayed et al. 2023), ammonia is more efficiently retained in soil compared to nitrate (Beeckman et al. 2024). Two important points illustrate why ammonia is preferred in soil. Firstly, ammonia binds well to negatively charged soil particles, which helps retain it in the soil (Wang et al. 2021). On the other hand, nitrate is highly mobile and does not bind as effectively, making it more prone to leaching, one of the primary pathways for nitrogen loss (Huang et al. 2017). Secondly, during the nitrification process, two intermediates-hydroxylamine and nitrite-are formed before ammonia is fully

converted to nitrate [Figure 3]. Both intermediates can generate N₂O as a byproduct. Hydroxylamine can be spontaneously oxidized to N₂O during the nitrification process (Van Groenigen et al. 2015), while nitrite is the substrate for nitrifier denitrification, another process that produces N₂O (Van Groenigen et al. 2015). In both cases, N₂O escapes into the atmosphere (Van Groenigen et al. 2015), reducing the nitrogen available for plant use. These processes contribute to approximately 30% of the total N₂O emissions from the nitrogen cycle.

Since 1961, the global average nitrogen use efficiency has consistently remained below 60%, dropping to as low as 39.8% in 1989 (FAOSTAT 2023b). This inefficiency leads to significant financial losses for farmers due to higher production costs (Nardi et al. 2020). Furthermore, heavy fertilisation, not only fails to prevent nitrogen loss, but exacerbates it. Studies show that NUE loss increases proportionally to the amount of synthetic nitrogen fertiliser applied (Lassaletta et al. 2014) [Figure 5B]. This is supported by research (Martin et al. 2017; Hu et al. 2022) and meta-analyses (Lei et al. 2022) indicating that AOB dominate N₂O emissions, especially in fertilised soils. AOB are more abundant than AOA in agricultural soils treated with inorganic fertilisers (Stein 2023). As a result, heavy use of synthetic fertilizers, which promotes AOB-driven nitrification, worsens NUE loss, though nitrate leaching and increased N₂O production as a by-product.

1.2.3 The Nitrogen Cycle-Environmental and agricultural impact in the 21st century: NO₃⁻ leaching as an environmental concern

Nitrification is responsible for approximately 50% of nitrogen loss from fertilisers, primarily through nitrate leaching into deeper soil layers (Beeckman et al. 2024), groundwater, and surface water, leading to significant pollution (Singh et al. 2022). Heavy fertilisation increases nitrate levels, making it a major environmental pollutant (Wang et al. 2023), second only to pesticides, in terms of surface and groundwater pollution (Singh et al. 2022). This pollution contributes to issues like eutrophication (Wang et al. 2021) and contamination of drinking water (Lin et al. 2023). Increased nitrifier activity, often due to soil acidification, can also leach essential metals like calcium and magnesium, (Nardi et al. 2020), reducing soil fertility.

Nitrate, along with phosphorus, plays a key role in eutrophication (Li et al. 2022), which affects both the diversity and stability of ecosystems (Hautier et al. 2020). Eutrophication occurs when excess nutrients cause a massive algal bloom (Akinnawo 2023) in water bodies such as rivers,

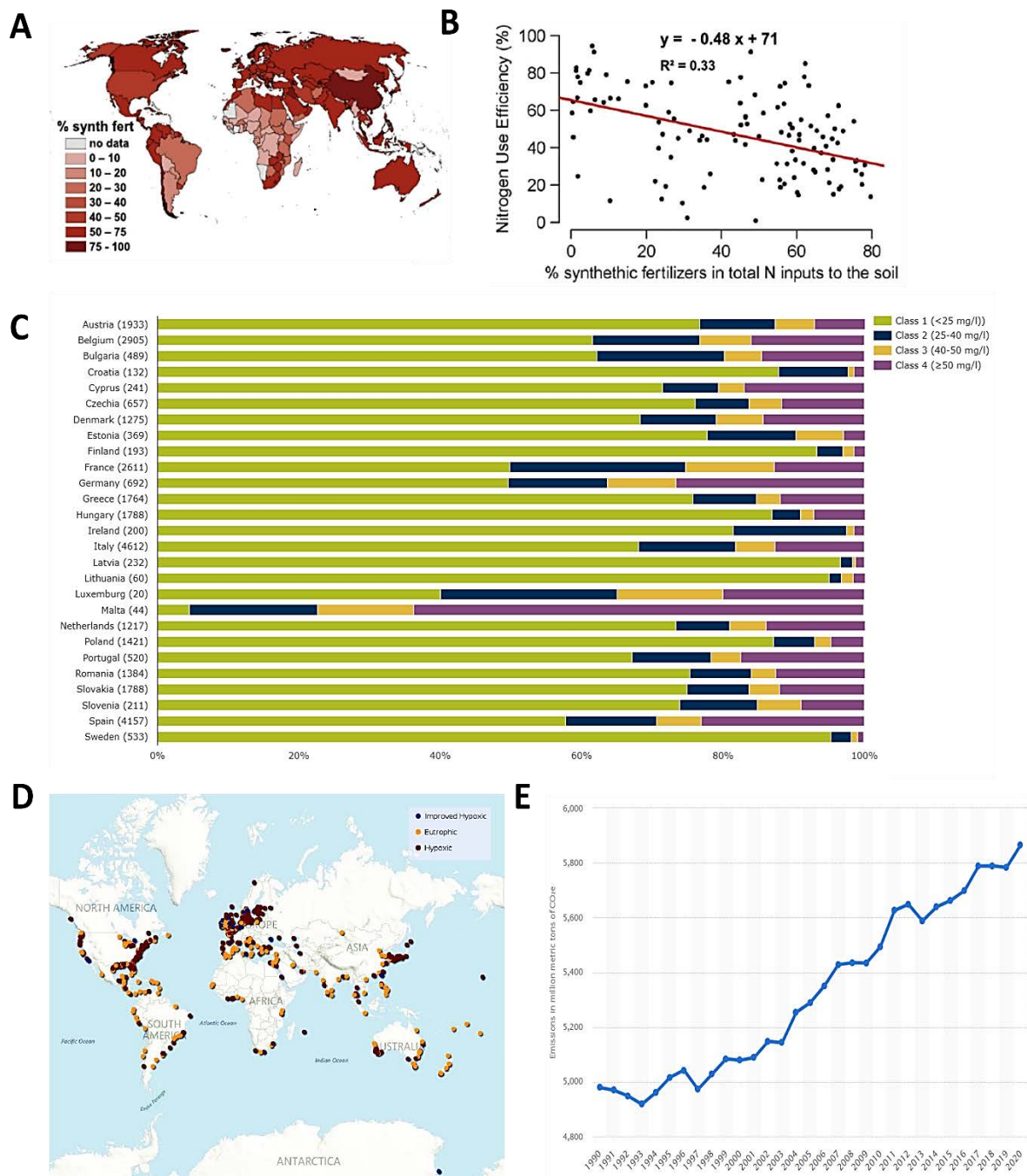


Figure 5. Summary of the effects of the Nitrogen Cycle processes in environmental and agricultural contexts. (A) Percentage of synthetic fertilisers relative to total fertiliser consumption per country (Lassalle et al. 2014). (B) Linear relationship between Nitrogen Use Efficiency and the percentage of synthetic fertilisers used (Lassalle et al. 2014). (C) Nitrate concentrations in groundwater samples from EU member states (2016-2019) (EEA 2023). (D) Global distribution of eutrophic, hypoxic and improved hypoxic sites, classified by (Jessen 2013). (E) Trend in greenhouse gas emissions from agriculture expressed in million tons of CO₂ equivalents from 1990 to 2020 (Statista research Department 2023b).

lakes, and coastal waters (Bonsdorff 2021) and is considered a global issue, with many coastal areas affected by eutrophication and hypoxia [Figure 5D]. In the European Union, around 30% of surface waters and 80% of marine waters are affected by eutrophication (EEA 2023). The economic cost of eutrophication was estimated at \$2.2 billion annually in the USA in the 2010s (Chislock et al. 2013), and it is expected to worsen GHG emissions due to increased methane (CH₄) production from affected ecosystems (Beaulieu et al. 2019).

Elevated nitrate levels in drinking water are also a health concern, as approximately 80–90% of the world's freshwater comes from groundwater (Lin et al. 2023). Nitrate contamination affects both livestock (Wang et al. 2023) and humans, especially infants where it can cause methemoglobinemia, or "blue baby syndrome" (Lin et al. 2023; Wang et al. 2023). The World Health Organization (WHO) has set a maximum of 50 mg L⁻¹ for NO₃⁻ in drinking water (Singh et al. 2022), and the European Union, through different Directives, aims to reduce high nitrate levels in groundwater (EEA 2023). While the average nitrate level in EU groundwater is about 21 mg L⁻¹, 14.1% of stations exceed the 50 mg L⁻¹ limit, with several countries reporting numerous cases of contaminated groundwater (EEA 2023) [Figure 5C]. Nitrate contamination of drinking water is also a significant concern in the USA (Wang et al. 2023), China (Wang et al. 2023), South Korea (Singh et al. 2022), and India, where approximately 110 million people consume water with nitrate levels exceeding 100 mg L⁻¹ (Singh et al. 2022).

1.2.4 The Nitrogen Cycle-Environmental and agricultural impact in the 21st century: Emission of N₂O, a highly potent greenhouse gas

From 1990 to 2020, global GHG emissions have steadily increased, with agriculture representing one of the main contributors to anthropogenic emissions (Statista Research Department 2023) [Figure 5E], accounting for 10-14% of total global emissions (Shakoor et al. 2020). While CO₂ dominates anthropogenic emissions, non-CO₂ gases, such as CH₄ and N₂O, are also significant contributors, with projections indicating future increases (Montzka et al. 2011). CH₄ is a more potent greenhouse gas than CO₂ but has a shorter atmospheric lifespan (Balcombe et al. 2018). In contrast, N₂O has nearly 300 times the warming potential of CO₂, a large atmospheric lifespan of over 100 years, and is a leading contributor to stratospheric ozone depletion (Lawrence et al. 2021). These properties have led to its inclusion in the Environmental Protection Agency's (EPA) key climate indicators (Bruhwiler et al. 2021).

Currently, agriculture is the primary anthropogenic source of N₂O, mainly due to the intensive use of synthetic nitrogen fertilisers (Lawrence et al. 2021). The atmospheric growth rate of nitrous oxide has accelerated significantly over the past decade (Harris et al. 2021). Nitrification and denitrification are the most important microbial processes contributing to N₂O emissions (Shakoor et al. 2020). Approximately 30% of total N₂O emissions are generated as a by-product of nitrification. However, the majority comes from denitrification. Incomplete denitrification, or the partial reduction of nitrate to N₂, can lead to the accumulation of nitrous oxide (Mefferd et al. 2022). This runoff of N₂O is exacerbated by high levels of NO₃⁻ produced through ammonia oxidation, especially with heavy nitrogen fertilisation. Due to the growing demand for agricultural products, atmospheric N₂O levels are projected to continue rising (Lawrence et al. 2021).

1.3 Nitrification Inhibition-Mitigating agricultural NUE loss and GHG emissions

Mitigating nitrogen loss has garnered significant attention, with current approaches focusing on controlling nitrogen release from fertilisers and utilizing fertiliser synergists (Wang et al. 2021). The first strategy involves the application of controlled-release (Lawrencina et al. 2021) or slow-release fertilisers (Wang et al. 2021). Despite the promise of this approach, challenges remain such as the lack of standardization in quantifying released nitrogen and discrepancies between laboratory results and the actual nutrient release rates (Lawrencina et al. 2021). The second approach involves combining fertilisers with synergists, specifically urease or Nitrification Inhibitors (NIs) (Wang et al. 2021). Urease inhibitors slow down the hydrolysis of the urea, reducing available ammonia, while NIs delay the oxidation of ammonia. Urease inhibition can be achieved using metals like silver, copper and zinc, as well as organic coatings containing neem plant (*Azadirachta indica*) extracts (Cantarella et al. 2018; Ghafoor et al. 2022) and N-(n-butyl) thiophosphoric triamide (NBPT), a highly effective (da Fonseca et al. 2023) and commonly used (Cantarella et al. 2018) urease inhibitor.

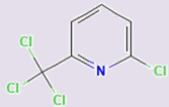
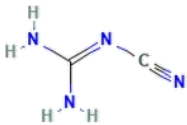
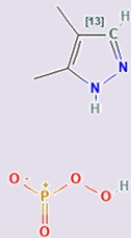
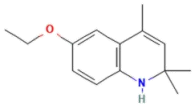
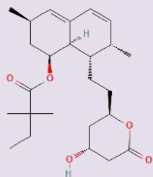
Nitrification inhibition is an area of immense scientific interest, as many compounds have been identified to facilitate this process (Papadopoulou et al. 2020). As previously mentioned, nitrification contributes to NUE loss and nitrate pollution, while also stimulating denitrification which results in N₂O emissions. Therefore, targeting this step of the nitrogen cycle is crucial for addressing these issues. NIs primarily inhibit the activity of nitrification-related enzymes (Wang et al. 2021), particularly the archaeal and bacterial AMO enzyme (Kolovou et al. 2023),

effectively delaying the oxidation of NH_4^+ to NO_3^- (Wang et al. 2021). The market of nitrification and urease inhibitors is projected to reach \$1.85 billion by 2027, with NIs holding the largest share as of 2021 (IndustryARC 2021). Industrially synthesized compounds, termed “Synthetic Nitrification Inhibitors” (SNIs) are routinely incorporated into fertilisers, leading to the development of N-stabilised fertilisers (Mateo-Marín et al. 2020). Recently, NIs of biological origin, known as “Biological Nitrification Inhibitors” (BNIs), have also shown great potential (Wang et al. 2021).

1.3.1 Nitrification Inhibition-Mitigating agricultural NUE loss and GHG emissions: Synthetic Nitrification Inhibitors (SNIs)

The concept of nitrification inhibition has been employed in agriculture since 1974 (Woodward et al. 2021), with the introduction of the first synthetic inhibitor, 2-chloro-6-(trichloromethyl) pyridine (nitrapyrin) to agricultural use [Table 1]. Since then, two additional SNIs, dicyandiamide (DCD) and 3,4-dimethylpyrazole (phosphate) (DMPP), have gained importance for agricultural use [Table 1]. While nitrapyrin is used widely in the United States, DCD and DMPP are more widely used in Europe (Woodward et al. 2021). For a SNI to be considered as agriculturally important, it must demonstrate consistent effectiveness over time and across varying soil and environmental conditions (Taggert et al. 2021). It should also target a wide range of ammonia oxidizing microorganisms and be suitable for applications in a non-gaseous state (Papadopoulou et al. 2020). Despite promising results in laboratory studies, some compounds, such as simvastatin (Zhao et al. 2020) [Table 1] and PTIO (2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide) have proven unsuitable for use in agricultural settings (Papadopoulou et al. 2020). Newly identified SNIs, including ethoxyquin, a fruit packaging antioxidant that inhibits ammonia oxidation (Papadopoulou et al. 2016) [Table 1], as well as substituted triazoles (Taggert et al. 2021), azoles, and diazoles (Beeckman et al. 2023), have shown potential as NIs, theoretically expanding the available portfolio of SNIs. Interestingly, some SNIs express inhibitory activity through their transformation products rather than the parent compound, similar to certain synthetic pesticides, whose transformation products are equally or more toxic to microorganisms (Mercurio et al. 2018). This is true, for DMPP and its transformation product DMP (Beeckman et al. 2023), and nitrapyrin, which acts through its metabolite 6-chloropicolinic acid (Nardi et al. 2020). In case of ethoxyquin both the parent compound and its metabolite, quinone imine, share inhibitory capacity (Papadopoulou et al. 2020). Mechanistically, SNIs are considered to act on the AMO enzyme, inhibiting ammonia

Table 1. Overview of the most common Synthetic Nitrification Inhibitors. The chemical structure and molecular weight data were obtained from PubChem, while the references cited first describe the inhibitory activity of each compound. Specificity information was sourced from Papadopoulou et al. (2020) and Kolovou et al. (2023).

Name	Chemical structure	Molecular weight (g/mol)	Polarity	Specificity	Application	Reference
Nitrapyrin; 2-chloro-6-(trichloromethyl)pyridine		230.90	Hydrophobic	Both AOA and AOB (Papadopoulou et al. 2020)	Applied in agriculture	Goring 1962
DCD; Dicyandiamide		84.08	Hydrophilic	AOB targeted (Papadopoulou et al. 2020)	Applied in agriculture	Solansky 1982
DMPP; 3,4-dimethylpyrazole (phosphate)		193.10	Hydrophilic	AOB targeted (Papadopoulou et al. 2020)	Applied in agriculture	Zerulla et al. 2001
Ethoxyquin; 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline		217.31	Hydrophobic	AOA targeted (Papadopoulou et al. 2020)	Novel, proposed	Papadopoulou et al. 2016
Simvastatin		418.60	Hydrophobic	AOA specific (Kolovou et al. 2023)	Used for research purposes	Zhao et al. 2020

Abbreviations: AOA-Ammonia Oxidising Archaea, AOB-Ammonia Oxidising Bacteria

oxidation (Nardi et al. 2020). Although the exact mechanism is poorly understood, all SNIs used in agriculture are proposed to be potent copper chelators (Papadopoulou et al. 2020) [Table 1], depriving the AMO enzyme of its essential co-factor. Other mechanisms have also been proposed. For example, nitrapyrin may alternatively function as a “suicide” inhibitor of the AMO enzyme (Vannelli and Hooper 1992), while DCD has been suggested to impair NH_3 uptake (Nardi et al. 2020), and DMP, the active metabolite of DMPP, is believed to bind zinc, another AMO co-factor (Corrochano-Monsalve et al. 2020). PTIO, though not used in agriculture, inhibits ammonia oxidation by scavenging NO, an obligatory intermediate in nitrification (Zhao et al. 2020). Additionally, some SNIs exhibit specificity towards AOA or AOB. For instance, simvastatin, an AOA-specific semi-synthetic NI, inhibits 3-hydroxy-3-methylglutaryl-CoA (HGM-CoA) reductase (Zhao et al. 2020), a key enzyme in the mevalonate pathway for archaeal membrane biosynthesis, which is absent in bacteria (Zhao et al. 2020; Kolovou et al. 2023) [Table 1].

Despite the strong inhibitory capacity of compounds like nitrapyrin, DMPP and ethoxyquin (Papadopoulou et al. 2020), these SNIs can negatively impact non-target soil microorganisms, which are not directly, or entirely, involved in the nitrification process (Nardi et al. 2020). However, their effects on NOB, denitrifiers, and microorganisms unrelated to the nitrogen cycle, remained understudied (Papadopoulou et al. 2022), making it difficult to predict potential consequences due to limited knowledge of their cellular-level mode of action (Bachtsevani et al. 2022). Soil studies have provided some insights, though with varied outcomes. For example, nitrapyrin has been found to reduce cellulolytic fungal diversity in one study (Zhang et al. 2022), while fungal diversity remained unaffected in another (Papadopoulou et al. 2022). DMPP, has been shown to increase *Fusarium* abundance, a genus that includes plant pathogenic fungi (Bachtsevani et al. 2022). Although some studies have suggested that SNIs cause no significant off-target effects on soil microbiota, particularly bacteria (Kong et al. 2016; Zhang et al. 2017, Wang et al. 2022), others argue that observed shifts in the soil microbial community exist but are unlikely to have a detrimental impact on soil health (Corrochano-Monsalve et al. 2021). It should be noted, however, that the Phospholipid Fatty Acid (PLFA) analysis often used to detect microbial community responses offers lower resolution than 16S rRNA-based metataxonomics (Lewe et al. 2021), suggesting that metataxonomic analyses should be incorporated to validate PLFA findings (Lewe et al. 2021). Additionally, studies focusing on broad microbial groups might miss significant toxicity effects (Papadopoulou et al. 2016). In conclusion, the lack of data on the off-target effects of

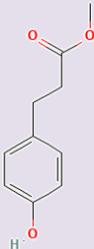
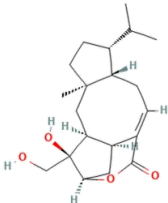
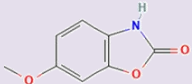
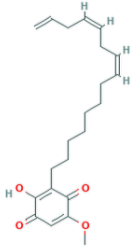
SNIs, combined with the high cost of synthesis, difficulties in application, degradation in soil, and potential groundwater pollution or entry into the food system (Coskun et al. 2017), highlights the need to explore alternative strategies for mitigating NUE loss and GHG emissions, despite the effectiveness of SNI-stabilised nitrogen fertilisers (Nardi et al. 2020).

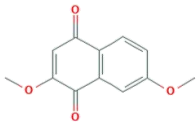
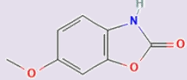
1.3.2 Nitrification Inhibition-Mitigating agricultural NUE loss and GHG emissions: Biological Nitrification Inhibitors (BNIs)

The shift toward sustainable agricultural intensification has promoted the replacement of synthetic compounds with biological alternatives, such as biopesticides (Fenibo et al. 2021; Pedrinho et al. 2024), biofertilisers (Kumar et al. 2021), and biostimulants (Castiglione et al. 2021). These biological products are considered cost-effective, environmentally friendly, sustainable, and specific in their mode of action, with no associated soil residues or the release of GHG emissions (Ayilara et al. 2023). In alignment with this trend, BNIs have emerged as alternatives to SNIs (Nardi et al. 2020, Wang et al. 2021). The idea of plants exuding nitrification inhibiting compounds via their root systems was first suggested over 100 years ago (Nardi et al. 2020). This concept was later confirmed through a combination of -omic approaches and *in vitro* bioassays, leading to the discovery of BNIs from various plants (Nardi et al. 2020) [Table 2]. These compounds, derived from root exudates, span several chemical categories (Coskun et al. 2017), including phenolics, aliphatic and fatty alcohols, acids and terpenes (Nardi et al. 2020), flavonoids, and aromatic derivatives, such as quinones [Table 2]. Notably, some of the plants that produce BNIs are major agronomical crops. For instance, sorghum (*Sorghum bicolor*) yields sorgoleone (Subbarao et al. 2013), sakuranetin (Subbarao et al. 2013), and methyl 3-(4-hydroxyphenyl) propionate (MHPP) (Zakir et al. 2008). Rice (*Oryza sativa*) produces 1,9-decanediol (Sun et al. 2016) and syringic acid (Lu et al. 2022), while maize (*Zea mays*) exudes 2,7-dimethoxy-1,4-naphthoquinone (zeanone) (Otaka et al. 2022), and 6-methoxy-2(3H)-benzoxazolone (MBOA) (Otaka et al. 2023). Signal grass (*Brachiaria humidicola*), exudes brachialactone, which is considered the most important BNI of this plant (Subbarao et al. 2009; Coskun et al. 2017).

There remains debate over whether BNIs have evolved specifically to inhibit nitrification and improve nitrogen uptake, or if they serve other functions in plants (Coskun et al. 2017). For example, the exudation of MHPP from corn is induced by the presence of NH_4^+ (Zakir et al. 2008), and the exudation of 1,9-decanediol in rice is triggered by the presence of NH_4^+ and nitrifying bacteria (Zhang et al. 2019), while 1,9-decanediol and sorghum BNIs are also

Table 2. Overview of the most commonly referenced Biological Nitrification Inhibitors. Data for chemical structures and molecular weights were obtained from PubChem, while the references listed first describe the inhibitory activity of each compound. Specificity data were sourced from Kaur-Bhambra et al. (2022) and Kolovou et al. (2023).

Name	Chemical structure	Molecular weight (g/mol)	Polarity	Specificity	Origin	Reference
MHPP; methyl 3-(4-hydroxyphenyl) propionate		180.2	Hydrophobic (from hydrophilic fraction)	AOB targeted (Kaur-Bhambra et al. 2022); Kolovou et al. 2023)	<i>Sorghum bicolor</i>	Zakir et al. 2008
Brachialactone		334.4	Hydrophobic (from hydrophobic fraction)	Unverified	<i>Brachiaria humidicola</i>	Subbarao et al. 2009
Sakuranetin; 4',5-Dihydroxy-7-methoxyflavanone		286.28	Hydrophobic	AOA targeted (Kolovou et al. 2023)	<i>Sorghum bicolor</i>	Subbarao et al. 2013
Sorgoleone		358.50	Hydrophobic	Unverified	<i>Sorghum bicolor</i>	Subbarao et al. 2013

1,9-decanediol; Decane-1,9-diol		174.28	Hydrophobic	Both AOA and AOB (Kaur-Bhambra et al. 2022)	<i>Oriza sativa</i>	Sun et al. 2016
Aliphatic, fatty alcohol						
Zeanone; 2,7-dimethoxy-1,4-naphthoquinone		218.21	Hydrophobic (from hydrophobic fraction)	Both AOA and AOB (Kolovou et al. 2023)	<i>Zea mays</i>	Otaka et al. 2022
Quinone						
MBOA; 6-methoxy-2(3H)-benzoxazolone		165.15	Hydrophobic (from hydrophilic fraction)	Unverified	<i>Zea mays</i>	Otaka et al. 2023
Aromatic						

Abbreviations: AOA-Ammonia Oxidising Archaea, AOB-Ammonia Oxidising Bacteria

preferably produced under acidic pH (Zhang et al. 2019; Wang et al. 2021). Furthermore, for some plants, BNI exudation seems to be limited only to parts of the root exposed to high NH_4^+ , where nitrification is expected to be at its maximum (Coskun et al. 2017). On the other hand, BNI activity has also been attributed to compounds, such as terpenes, which are usually involved in other plant functions, such as plant defence (Coskun et al. 2021), while sakuranetin, an identified BNI, is a phytoalexin important for responses to biotic and abiotic plant stress (Zhao et al. 2024). Interestingly, in the case of *Brachiaria humidicola*, BNIs have also been identified in the shoot (Wang et al. 2021) and have shown significant inhibitory activity (Kaur-Bhambra et al. 2022). Overall, this could form an argument that BNI exudation could employ compounds with already existing intracellular functions, with the difference that now these compounds are actively exuded by roots rather than released through more stochastic processes, such as litter decomposition and membrane leakiness (O'Sullivan et al. 2016).

Indeed, there are indications linking BNIs with active transport through root cell membranes (Coskun et al. 2017; Zhang et al. 2021), including correlation of BNI exudation with the expression levels (Zhu et al. 2012) and post-translational modifications (Afzal et al. 2020) of the membranic H⁺-ATPase, as well as a putative role of ATP-binding cassette (ABC) transporters (Coskun et al. 2017). After the exudation, polarity is an important factor determining BNI potency, as hydrophobic compounds are considered to play a role in the rhizosphere, due to adsorption to soil minerals or organic particles and diffusion limitations (Wang et al. 2021). In contrast, hydrophilic BNIs are more likely to inhibit nitrification in a larger area of soil (Wang et al. 2021), or in the more oxic upper soil layers (Kaur-Bhambra et al. 2022), due to increased motility.

Similarly to SNIs, BNIs inhibit ammonia oxidation through inhibition of the AMO pathway (Wang et al. 2021). However, some BNIs, such as sakuranetin, sorgoleone and brachialactone have also been shown to inhibit the HAO pathway (Wang et al. 2021). Additionally, mechanisms such as outer membrane permeabilization, disruption of the inner membranes and proton motive force, as well as NO scavenging, have been proposed (Nardi et al. 2020) but have not been investigated in detail. Consistent with what is also true for SNIs, specificity in the inhibitory activity of BNIs can be observed, with BNI exudation being induced in acidic pH values. Thus, the identified BNIs generally inhibit AOA more easily than AOB (Kaur-Bhambra et al. 2022; Kolovou et al. 2023). Despite the high variety of identified BNIs, *in vitro* assays have shown that, compared to the high inhibitory capacity of the currently established SNIs (nitrapyrin and DMPP) (Papadopoulou et al. 2020), most BNIs seem to be less efficient (Kaur-Bhambra et al. 2022; Kolovou et al. 2023). An exception to this could be zeaxone and its derivative 2-methoxy-1,4-naphthoquinone (Otaka et al. 2022; Kolovou et al. 2023), with both characterized by significantly low inhibitory concentrations for both AOA and AOB. Nevertheless, studies have revealed the great potential of BNI-producing plants to reduce NO₃⁻ production, nitrifier populations and GHG emissions in agricultural settings (i.e. greenhouse pot and field experiments) (Subbarao et al. 2009; Nuñez et al. 2018; Villegas et al. 2020). Overall, studies repeatedly report novel plant-derived BNIs, though knowledge of their inhibitory capacity remains unknown (Kolovou et al. 2023), limited or discrepant. Thus, two fundamental drawbacks with the identification process for BNIs have arisen: a lack of standardization and a low throughput in BNI identification and potency assessment.

1.4 The assessment process of Biological Nitrification Inhibitors

The discovery process of compounds with putative BNI activity relies on two major approaches. Culture-dependent approaches aim to test pure compounds or plant exudates on axenic AOM cultures, utilizing dose-response schemes to determine AOM activity endpoints like ammonia oxidation Effective Concentration 50% (EC₅₀) (Shen et al. 2013; Papadopoulou et al. 2020; Kolovou et al. 2023) and Inhibitory Concentration 80% (IC₈₀) (Sun et al. 2016; Kaur-Bhambra et al. 2022) to assess NI potency. On the other hand, soil-based approaches, such as soil microcosm studies, aim to study the efficacy of NIs in a more agriculturally relevant context, employing endpoints such as *amoA* copy number (Subbarao et al. 2009), potential nitrification rate (Nuñez et al. 2018; Bachtsevani et al. 2022) and general NH₄⁺ and NO₃⁻ levels (Bachtsevani et al. 2022).

In vitro liquid batch culture experimental setups have been the go-to method for assessing the inhibitory effect of different compounds on monocultures of ammonia oxidizer and nitrite oxidizer strains. Such approaches have ranged from using a single strain, like *Nitrosomonas europaea* (Sun et al. 2016) or *Nitrosotalea devanattera* (Lehtovirta-Morley et al. 2013) to a combination of AOM strains, such as the soil-representative species *Nitrospira multififormis* and *Nitrososphaera viennensis* (Shen et al. 2013) to assess the potency of different SNIs and, later, BNIs. Others have utilized a more expanded set of reporter strains (Belser and Schmidt 1981; Wright et al. 2020), though those studies either included only AOB (Belser and Schmidt 1981) or relied on soil-representative AOA but non-representative AOB strains (Wright et al. 2020). In contrast, recent studies have used a broad range of soil-representative and phylogenetically diverse AOA and AOB to assess the efficacy of SNIs (Papadopoulou et al. 2020) and BNIs (Kaur-Bhambra et al. 2022; Kolovou et al. 2023). Overall, inhibition thresholds for agriculturally relevant SNIs and many candidate BNIs have been determined thanks to this approach. However, these studies have also revealed differences in the resistance of various AOM to different NIs, stressing the need to use a broad range of reporter strains (Kaur-Bhambra et al. 2022). Despite the capacity to determine a more accurate spectrum of NI activity using bioassays employing different strains (Papadopoulou et al. 2020), there is still a severe lack of standardization for the selected endpoints and the representation of ecologically diverse, agriculturally relevant ammonia oxidizer strains. Specifically, discrepancies in the results of different studies have been attributed to the use of different AOM strains, for example different *N. europaea* strains tested in Kolovou et al. (2023) (ATCC 25978) compared to Kaur-

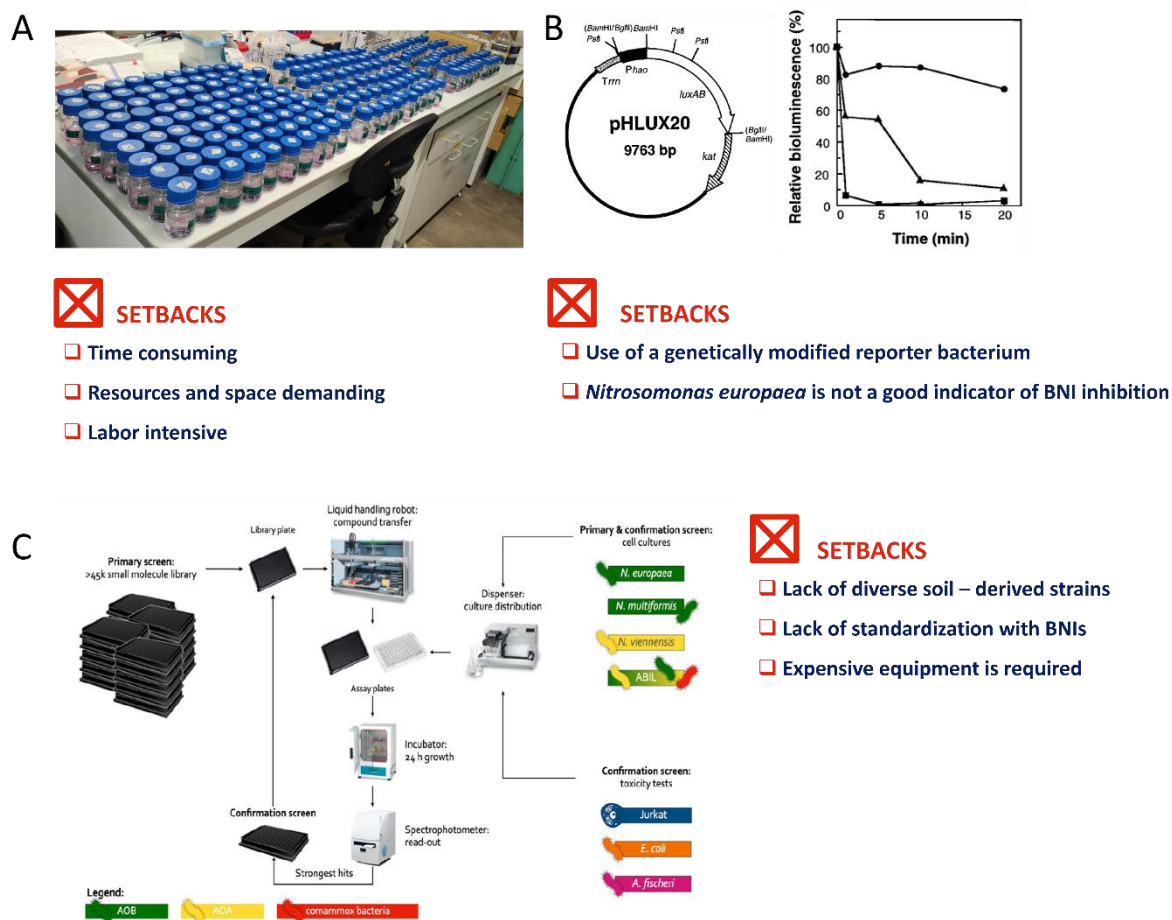


Figure 6. Summary of indicative bioassays for NI discovery. The summary provides an overview of different bioassays and highlights important limitations. (A) Liquid batch culture approach, (B) *N. europaea* bioluminescence approach (Iizumi et al. 1998) and (C) Drug-based setup for NI discovery (Beeckman et al. 2023).

Bhambra et al. (2022) (ATCC 19718). Another source of inconsistency is the different approaches for determining activity endpoints. EC₅₀ estimations based on nitrite production are more frequently utilized (Shen et al. 2013; Sun et al. 2016; Papadopoulou et al. 2020; Kolovou et al. 2023), though EC₅₀ values have also been determined based on the shift in the maximum specific growth rate ($\mu_{\max}$) (Kaur-Bhambra et al. 2022), a difference that further contributes to inconsistent inhibition thresholds between studies (Kolovou et al. 2023). Finally, it should be noted that, regardless of its precision, the liquid batch culture approach is burdened by large requirements in space, biomass, labour and cultivation time, making it a low-throughput method [Figure 6A].

Generally, *in vitro* tests employing AOM are primed for fast-track versions (Karpouzas et al. 2022). To combat the low throughput of conventional liquid batch cultures, a series of bioassays have been developed to facilitate fast-track and high-throughput BNI discovery and efficacy assessment. One of the most referred methods is the bioassay developed by Iizumi et al. (1998), which relies on a single *Nitrosomonas europaea* strain, genetically modified with a plasmid containing the luciferase-encoding genes *luxAB* from *Vibrio harveyi* (Iizumi et al. 1998) [Figure 6B]. This method couples the electron production that occurs through ammonia oxidation with the formation of NADPH⁺ and FMNH₂ carriers necessary for luciferase activity. Thus, nitrification inhibition correlates with a reduction in luciferase bioluminescence (Iizumi et al. 1998). Originally designed for detecting nitrification inhibition in wastewater treatment plants, this bioassay has been applied to detect BNI activity in *Brachiaria humidicola* (Subbarao et al. 2006) and *Leymus racemosus* wildrye (Subbarao et al. 2007), as well as for isolating compounds such as brachialactone (Subbarao et al. 2009), sakuranetin (Subbarao et al. 2013) and sorgoleone (Subbarao et al. 2013), by combining the assay with plant cultivation in hydroponics (Nardi et al. 2020). The approach has been highly praised for accurately quantifying BNI activity in plants and compounds with very low time requirements (Wang et al. 2021). However, two main points of controversy have risen [Figure 6B]. First, the use of the genetically modified AOB strain requires operation with specialized protective biochemical laboratory (Wang et al. 2021), which restricts potential outsourcing (Kaur-Bhambra et al. 2022). Second, the system uses *N. europaea* as its only reporter strain, despite not being a representative of soil AOB, in contrast to strains like *Nitrospira multiformis* (Norton et al. 2008; Lehtovirta-Morley et al. 2018). This neglects the relative composition and complexity of AOB consortia in agricultural soils and may not reflect the actual activity of microorganisms in nature (Wang et al. 2021), especially with respect to AOA (Coskun et al. 2017). Furthermore, discrepancies in results across studies (Kolovou et al. 2023), have been attributed to the fact that the inhibition threshold in the bioluminescent *N. europaea* system is conventionally derived from a single luminescence measurement in the span of 30 min after NI application (Subbarao et al. 2006) instead of employing sequential time point measurements over longer incubation periods.

Several studies have attempted to adapt the fast-track approach developed by Iizumi et al. (1998) for use with non-genetically modified AOM strains to test the BNI capacity of wheat and rice plants (O'Sullivan et al. 2016; Sun et al. 2016). However, the limited range of AOM reporter strains, particularly the absence of AOA, remains significant. These approaches relied

either on *N. europaea* (Sun et al. 2016) or on a combination of *N. europaea* and *N. multiformis* (O’Sullivan et al. 2016). Recently, a different approach was described by Beeckman et al. (2023), which tested a vast array of synthetic compounds for NI activity. This method combined conventional toxicity tests on AOM monocultures and an AOM assemblage, with confirmation toxicity tests on *Escherichia coli*, *Aliivibrio fischeri* and Jurkat cells, all under a fast-track system (Beeckman et al. (2023)). Despite its potential for NI discovery, several issues remain [Figure 6C]. Firstly, the bioindicator arsenal used misrepresents the composition of AOM in agricultural soils, as it includes *N. europaea* but omits representatives of the *Nitrosotalea* or *Nitrosocosmicus* AOA lineages. Notably, *Nitrosotalea* species would serve as crucial bioindicators, as this abundant, globally distributed AOA genus thrives in acidic soils (Lehtovirta-Morley et al. 2014), where AOA outnumber (Schleper and Nicol 2010; Lehtovirta-Morley 2018) and even outperform (Zhao et al. 2020) their AOB counterparts. Additionally, the repeatability of the system concerning call activity was not standardized, and the bioassay was validated only with known SNIs, which limits its immediate applicability for BNI discovery.

1.5 Aims of the study

The limitations of current liquid batch culture methods, including low-throughput and lack of standardization, alongside the challenges of existing high-throughput assays, highlight the need for a standardized, valid fast-track system, tailored for BNI discovery and potency assessment. This study, conducted in cooperation with the Archaea Biology and Ecogenomics Unit, University of Vienna, aimed to develop a rapid, miniaturised approach, with a result turnaround time of less than a day, utilized a simple 96-well plate format, to enable high-throughput, systematic screening of multiple candidate BNIs [Figure 7]. A protocol was designed to generate concentrated AOB cultures, and its behaviour was standardised. To validate the accuracy of the system inhibition thresholds for known and novel BNIs and SNIs were determined and the resulting EC₅₀ values were compared with inhibition data from the literature based on traditional liquid batch culture methods. Finally, root exudates from a wide range of wheat and pearl millet genotypes were screened for BNI activity, to assess the applicability of the developed fast-track assay. Complementary work was performed with AOA by the collaborators in the University of Vienna.

- ✓ **Rapid and miniaturised**
1 – day result turnaround time with minimal requirements
- ✓ **High – throughput**
Systematic screening of multiple candidate compounds
- ✓ **Standardized**
Behaves similarly each time
- ✓ **Valid**
Results similar with the established liquid batch culture approach
- ✓ **Ecologically relevant**
Use of diverse and highly abundant lineages of soil AOB

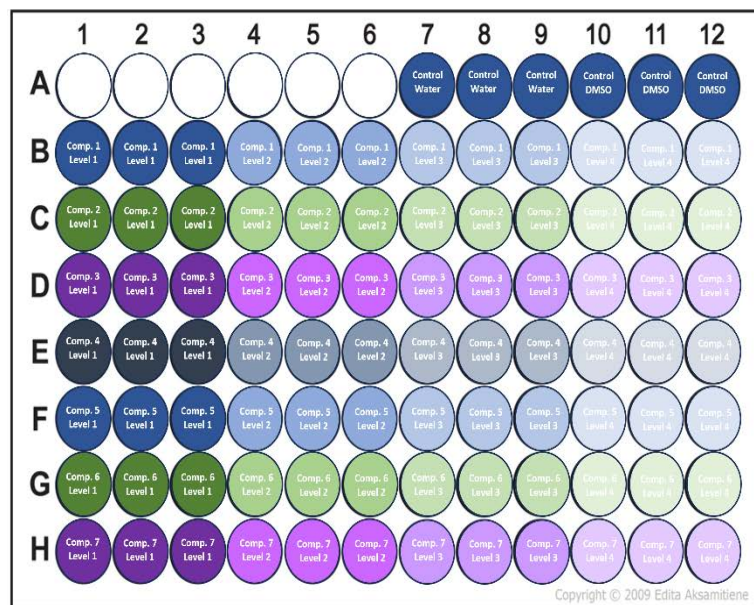


Figure 7. Graphical representation of the fast-track system. The main advantages of this approach are summarized. An overview of the 96-well plate format is shown featuring various test compounds at different concentration levels.

2. Materials and Methods

2.1 Experimental design

Axenic *in vitro* cultures of three AOB were grown, and cells were harvested to create a concentrated inoculum using a filtration-backwash approach. This inoculum was then used to establish concentrated cultures for developing a fast-track bioassay system aimed at screening NIs. The repeatability of the procedure was standardized by measuring both the activity of the AOB in the concentrated cultures and the cell density at specific stages of the process. System performance was validated by testing known NIs at specific concentrations to determine ammonia oxidation inhibition, with results compared to previously reported EC₅₀ values from literature. Additionally, root exudates from wheat and pearl millet genotypes were evaluated for BNI activity at the same concentrations.

2.2 AOM strains and growth conditions

Axenic *in vitro* cultures of *Nitrosospira multiformis* ATCC25196 and *Nitrosomonas ureae* Nm10 were grown in Skinner and Walker (SW) medium (Skinner and Walker 1961) with 1mM of NH_4^+ as the substrate. *Nitrosomonas communis* DSM28436 was grown in 1583 DSMZ medium adopted to a 3mM NH_4^+ substrate. All AOB cultures were incubated statically, in the dark, at 28°C. The AOB cultures were maintained in 30 mL sterile polypropylene bottles (Sterilin™ Polypropylene 30mL Universal Containers, ThermoFischer Scientific) at a working volume of 20 mL, with three or more biological replicates depending on the requirements for downstream applications. The pH of the cultures was adjusted, if deemed necessary, indicated by the decolourisation of the medium. For *Nitrosospira multiformis* and *Nitrosomonas ureae*, pH adjustment was performed using approximately 25 μL of 5% w/v Na_2CO_3 . In contrast, for *Nitrosomonas communis*, 50-100 μL of 10% w/v NaHCO_3 were added, depending on the extent of acidification.

Nitrite production was measured at specific time-points to monitor the growth of the AOB cells. Culture refreshments involved a 1% inoculation into sterile medium, once each culture reached the late logarithmic growth phase (defined as NO_2^- production exceeding 900 μM for *N. multiformis* and *N. ureae* and 2700 μM for *N. communis*). The modelling of the activity of these routine AOB cultures was performed based on nitrite determination data obtained over consecutive generations, employing a single sigmoidal curve fit, for comparison with the fast-track cultures generated later.

2.3 Nitrification inhibitors

The following NIs were used to assess the performance of the fast-track system concerning a range of compounds. For SNIs, 3,4-dimethylpyrazole (phosphate) (DMPP) and nitrapyrin were included as established SNIs of hydrophilic and hydrophobic nature respectively, along with ethoxyquin, a novel, hydrophobic SNI. Methyl-3-(4-hydroxyphenyl) propionate (MHPP), a known, moderately hydrophilic BNI, was selected alongside the hydrophobic compounds sakuranetin and 1,9-decanediol as representative BNIs. DMPP was purchased from Cayman Chemical (1180 East Ellsworth Road, Ann Arbor, Michigan, 48108, USA) with a purity of $\geq 98\%$. Nitrapyrin was obtained from Sigma-Aldrich (Chemie GmbH, Eschenstr. 5, 82024, Taufkirchen, Germany) at $\geq 98\%$ purity. Ethoxyquin was also supplied from Sigma-Aldrich (Chemie GmbH, Eschenstr. 5, 82024, Taufkirchen, Germany) at 96.9% purity. MHPP was purchased from BLDpharm (No.1 Wangdong Middle

Road, Building 5, Songjiang District, Shanghai 201601, China) with purity of 99.21%. Sakuranetin was provided by Syngenta Crop Protection AG (Rosentalstr. 67, 4058 Basel, Switzerland) at 95% purity. 1,9-decanediol was supplied from WuXi AppTec (288 Fute Zhong Road, Shanghai, 200131, China) at 95% purity. Stock solutions of all the compounds were prepared and, the desired concentrations were confirmed via HPLC after the necessary dilutions, except for 1,9-decanediol.

2.3.1 Nitrification inhibitor analysis through High Pressure Liquid Chromatography (HPLC)

Chromatographic analysis was performed using a Shimadzu LC-20ADHPLC system equipped with a UV/VIS PDA detector. NIs were analysed as follows. DMPP was determined using a mobile phase with a ratio of 85:15 H₂O + 0.1% H₃PO₄ to methanol, a flow rate of 1 mL min⁻¹ and a detection wavelength of 225 nm, with the column oven temperature set at 40°C. Nitrapyrin analysis was performed with a mobile phase of acetonitrile and H₂O + 0.1% H₃PO₄ at a 70:30 ratio, a flow rate of 1 mL min⁻¹ and a detection wavelength of 269 nm, with the oven temperature set at 25°C. Ethoxyquin was analysed with a mobile phase of 70:30 acetonitrile to H₂O + 0.25% NH₃⁺, a flow rate of 1 mL min⁻¹ and a detection wavelength of 225 nm, with an oven temperature of 25°C. MHPP analysis utilized a mobile phase of 60:40 acetonitrile to H₂O + 0.1% H₃PO₄, a flow rate of 1 mL min⁻¹ and a detection wavelength of 224 nm, with an oven temperature of 25°C. Sakuranetin determination employed a mobile phase of H₂O + 0.1% H₃PO₄ and methanol in a 30:70 ratio, a flow rate of 0.8 mL min⁻¹ and a detection wavelength of 288 nm. 1,9-decanediol stock solutions were not subjected to HPLC analysis as detection could not be achieved with the described system. Before HPLC analysis, the stock solutions of DMPP, MHPP, and sakuranetin were diluted in methanol, while ethoxyquin and nitrapyrin were diluted in acetonitrile.

2.4 AOM Fast-Track System development

The generation of the fast-track system is described in [Figure 8](#). In total, 180 mL of AOB cultures at the late logarithmic phase were divided into three 60 mL groups and filtered through hydrophilic 0.22 µm polyethersulphone (PES) membrane filters (Frisenette ApS, DK-8420 Knebel, Denmark), using sterile syringes. After filtration, approximately 200 µL of growth medium were added to the membrane each time, and the filter was inverted. The filter was tapped at an approximate frequency of 2-3 taps per second for one minute to detach the cells from the membrane. Then, 2 mL of sterile medium was passed through the filter to resuspend

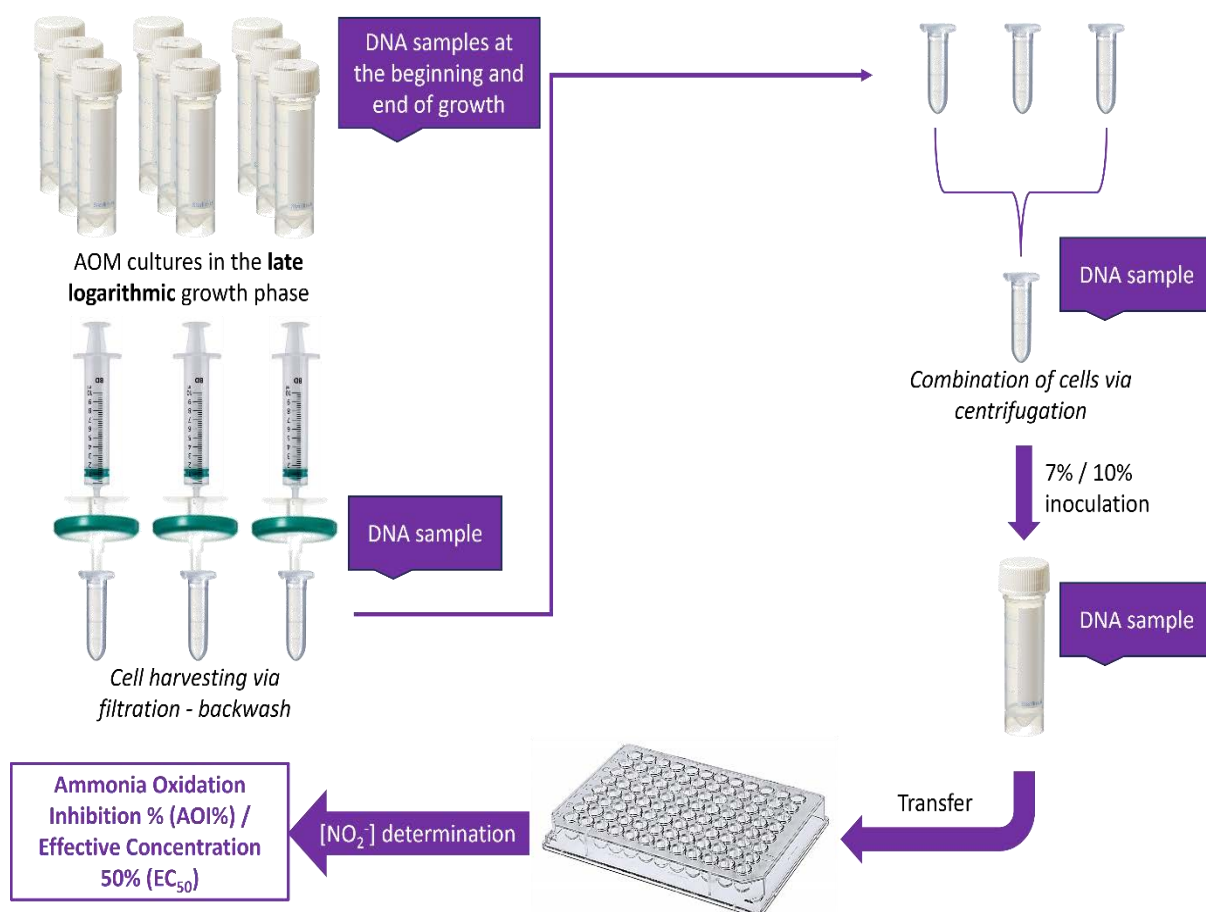


Figure 8. Graphical outline of the cell harvesting procedure. DNA sample collection points shown in the figure refer specifically to the standardization step.

the cells, aiming at a theoretical 30-fold concentration. The first inoculum was then centrifuged at 11.000 g for 10 min. After centrifugation, 1.8 mL of the supernatant was discarded, ensuring that the pellet remained submerged in the liquid. Then, 1.8 mL of the second inoculum were added to the first, and the pellet was resuspended before repeating and the centrifugation process. This procedure was repeated with the third inoculum, combining all three into a final volume of 2 mL. Finally, the combined inoculum was used to generate a concentrated culture. For *N. multiformis* and *N. communis*, 2 mL of the inoculum were added to 18 mL of SW medium and 1583 DSMZ medium respectively, while for *N. ureae* 1.4 mL were added to 18.6 mL of SW medium. The contents of the bottle were gently mixed and the culture was distributed into each well of a cellGrade™ 96-well plate (BRAND GMBH & CO KG, Otto-Schott-Straße 25, 97877 Wertheim, Germany). The plate was incubated in the same conditions as the respective AOB. Cell density and activity were monitored for standardisation.

2.5 Liquid Culture Assays

2.5.1 Liquid Culture Assays: Validation of the Fast-Track system with known SNIs and BNIs

Validation of the performance of the system was conducted through liquid culture assays in a 96-well plate. Various nitrification inhibitors known for their effect on AOB were used, with four or more concentrations employed to range from slight to full inhibition. Different concentration schemes were applied for the different reporter strains due to variations in their sensitivity [Table 3]. Cultures in each well of the plate were prepared as follows. Concentrated culture was added to the plate, followed by an appropriate amount from the stock solutions of each inhibitor to achieve the desired final concentrations. In the case of hydrophobic Nis, a final DMSO concentration of 0.1% v/v was maintained. Control cultures with a final concentration of 0.1% v/v of ddH₂O and DMSO were also prepared. The final DMSO concentration of 0.1% v/v was not expected to impose significant inhibition on AOB (Wright et al. 2020; Zhao et al. 2020).

For the controls (ddH₂O and DMSO), 5 µL were diluted in 495 µL of growth medium. The solutions were vortexed gently, and 20 µL were applied to each well. The same procedure was followed for hydrophilic NIs (i.e., DMPP). For MHPP, the same procedure was applied, with HPLC analysis confirming that initial dilution in growth medium was possible based on the solubility of the compound. For other hydrophobic NIs, where solubility did not allow initial dilution in growth medium, 0.2 µL of the inhibitor was applied directly to the wells, followed by 19.8 µL of growth medium.

Cultures for each treatment (inhibitor and concentration) were grown in triplicates, with each well representing one biological replicate. Nitrite determination at different time points was used to monitor AOB cell activity and determine the Ammonia Oxidation Inhibition percentage (AOI%) and the Effective Concentration 50% (EC₅₀).

2.5.2 Liquid Culture Assays: Proof-of-concept of the fast-track testing system with wheat root exudates

A series of bioassays were set up to screen various wheat genotypes for BNI activity, using the fast-track approach. The wheat root exudates were provided by Prof. Wolfram Weckwerth's group (Molecular Systems Biology, University of Vienna). For each of the 38 wheat genotypes, 2 mL of root exudate were received in pellet form, resuspended in 100 µL of sterile ddH₂O,

and further diluted with appropriate volumes of ddH₂O. This dilution process standardized the concentration of root exudate based on the original dry root weight obtained during the extraction for each genotype. The final concentration of root exudates in the AOB cultures was approximately 10 µg µL⁻¹. The bioassays were conducting using a 96-well plate set-up as previously described, with one concentration level per exudate. 6 µL were applied for each root exudate and a corresponding control treatment was established with addition of 6 µL of ddH₂O in triplicates. The 96-well plate was incubated at 28°C, in the dark, without agitation. Nitrite determination was used to monitor the activity of the AOB cells and determine the AOI% at the optimum time point, when most of the available substrate was consumed.

2.5.3 Liquid Culture Assays: Proof-of-concept of the fast-track testing system with pearl millet root exudates

In addition to root wheat exudates, a series of fast-track bioassays was conducted to examine pearl millet root exudates provided by Prof. Wolfram Weckwerth's group ([Molecular Systems Biology, University of Vienna](#)). Each of the 33 pearl millet genotypes was supplied in 2 mL of root exudate in pellet form, which was resuspended in an appropriate volume of sterile ddH₂O to achieve a standardized concentration of 10 µg µL⁻¹ in culture. The same 96-well plate culture setup used previously was employed, with each sample applied in a 2 µL volumes to triplicate wells containing 200 µL of concentrated AOB culture. Additionally, three replicates of 2 µL ddH₂O were included as a negative control. For selected potent root exudates, a range of concentrations was tested. Four application doses of 20 µg µL⁻¹ (4 µL), 15 µg µL⁻¹ (3 µL), 10 µg µL⁻¹ (2 µL), 5 µg µL⁻¹ (1 µL) were used, along with corresponding negative controls (4 µL, 3 µL, 2 µL and 1 µL of ddH₂O) for *N. multiformis*, by applying different volumes of the root exudate solution. Similarly, root exudate doses of 20 µg µL⁻¹, 10 µg µL⁻¹, 7.5 µg µL⁻¹ and 5 µg µL⁻¹ were established for *N. ureae*. The 96-well plate was incubated at 28 °C, in the dark, without agitation. Nitrite levels were monitored to assess AOB cell activity, and the AOI % was determined at the optimal time point, typically near the end of the culture duration (late logarithmic phase). EC₅₀ values were determined from nitrite measurements at a single time-point, as described below (Section 2.8.2).

2.6 Nitrite determination

Nitrite production measurements were conducted to monitor the activity of the AOM cells in both routine and fast-track cultures. The determination was performed colourimetrically at 540

nm using a 96-well plate format assay by diazotizing and coupling with Griess reagent (Shinn, 1941).

2.7 Cell density determination

DNA from the collected samples was extracted using the NucleoSpin® Tissue kit (Macherey-Nagel GmbH & Co. KG, 52335 Duren, Germany) following the manufacturer's guides. Quantification of the *amoA* gene was used to determine cell density and was performed via qPCR using a Biorad CFX Real-Time PCR system. The bacterial *amoA* gene was quantified over 40 cycles, using the *amoA-1F/amoA-2R* primer set (Rotthauwe et al., 1997), with a final reaction volume of 10 µL per sample. Each reaction contained 5 µL KAPA SYBR R FAST qPCR Master Mix (2x) (KapaBiosystems), 0.1 µL *amoA-1F* primer, 0.1 µL *amoA-2R* primer, 0.2 µL bovine serum albumin (BSA) (New England BioLabs Inc.) (20 mg mL⁻¹), 2.6 µL ddH₂O and 2 µL DNA template. For quantification, an 8 point pre-quantified standard curve, with gene copy concentrations ranging from 10⁸ copies µL⁻¹ to 10¹ copies µL⁻¹, was used in two technical replicates. Annealing temperature was 57°C. The qPCR efficiency was >0.8 and the R² of the external standard curve was >0.98. Cell density determination accounted for the fact that the *amoA* gene is present in 3 copies in *N. multiformis* and *N.s ureae* (as reported by Norton et al. 2008 and Kozlowski et al. 2016a, respectively), and in two copies in *N. communis* (Kozlowski et al. 2016b).

2.8 Data analyses and statistics

Data processing and visualisation were performed using RStudio (R Core Team 2020). Plot generation utilized the “ggplot2” package v3.4.2 (Wickham, 2016) and the “ggpubr” v0.6.0 (Kassambara 2023a) package or the base R functions. Where indicated, figures were finalized using the “patchwork” package v1.1.2 (Pedersen, 2022).

2.8.1 Modelling of the routine AOM cultures growth

The modelling of routine AOB culture activity was performed using data obtained from nitrite determination across different generations, utilizing the “sicegar” package v0.2.4 (Caglar et al. 2018), and employing the equation shown in (1), as described by Caglar et al. (2018).

$$I(t) = f_{sig}(t) = \frac{I_{max}}{1 + e^{-a_1(t-t_{mid})}} \quad (1)$$

The three key estimated parameters for the sigmoidal equation, are: I_{max} -estimated maximum Intensity, t_{mid} -estimated Time at which Intensity is half of the estimated maximum, a_I –slope of the curve at t_{mid} .

The maximum specific growth rate (μ_{max}) of the routine AOB cultures was calculated based on [Shaw et al. \(2006\)](#). Briefly, a semi-logarithmic plot of the natural logarithm of nitrite concentration versus time was generated, using the same data applied in the sigmoidal growth model. The linear phase of the plot was isolated, and the slope coefficient from the linear regression analysis was used at the specific growth rate. Comparison with values reported in the literature was conducted by calculating 95% confidence intervals for each specific maximum growth rate (μ_{max}) using the following formula (2):

$$\mu_{max} - (1.96 \times \text{standard error}) \leq \mu_{max} \leq \mu_{max} + (1.96 \times \text{standard error}) \quad (2)$$

Activity rates of concentrated AOB cultures were calculated using the same approach, incorporating data from the entire incubation period.

2.8.2 EC_{50} calculation

EC_{50} values represent the inhibitor concentrations that reduce ammonia oxidation activity by 50% compared to the respective control treatments. For dose schemes and AOB responses that follow a traditional sigmoidal dose-response curve, normalized nitrite production data were analysed using the “drc” package v3.0.1 ([Ritz et al. 2016](#)) as described in [Papadopoulou et al. \(2020\)](#). In this case, EC_{50} values were generated by the algorithm for each time point, and an average value $\pm$ standard error was calculated using results with satisfactory $R^2 > 0.95$ and statistically significant p-values. For dose schemes that produced linear or polynomial AOB responses, a different approach was applied. This involved the utilization of the slopes of the AOB activity linear regression curves for each dose and the following calculations in (3) to determine the AOI% for each inhibitor concentration, assuming the control had an AOI% of 0%. The linear regression analyses for the activity curves were performed using base R functions.

$$AOI (\%) = \frac{a_{control} - a_i}{a_{control}} \times 100\% \quad (3)$$

In this case, AOI% values were plotted against the inhibitor concentrations. If a satisfactory linear regression (adjusted $R^2 > 0.9$, p-value < 0.05) was achieved using at least 4 out of 5 data

points, the EC₅₀ concentration was calculated from the linear regression equation where $y = 50$. A similar approach was used by [Kaur-Bhambra et al. \(2022\)](#) where $\mu_{\max}$ replaced the AOI%. The standard error of the EC₅₀ was considered equivalent to the standard error produced for the unknown predictor value by the *calibrate ()* function of the “investr” v1.4.2 R package ([Greenwell and Schubert Kabban 2014](#)) at a 95% confidence level, with the observed variable set to 50. If no satisfactory linear relationship was observed, a third-degree polynomial fit was applied. In such cases, the EC₅₀ was calculated from the equation of the polynomial curve where $y = 50$, and the standard error was estimated using the Delta Method. Briefly, this involved calculating the first derivative of the 3rd degree polynomial equation at the root for $y = 50$, followed by computing the sensitivity of the polynomial function with respect to its coefficients at the root to account for variance propagation and derive the standard error.

2.8.3 Ammonia Oxidation Inhibition % (AOI%)

The AOI% refers to the proportional inhibition of AOB activity compared to the negative control and it was calculated for each treatment as shown in equation in (6), following normalisation of all nitrite production values using equations (4) and (5).

$$\text{Normalised } [NO_{2\text{control}}] = \frac{[NO_{2\text{control}}]}{\text{Average } [NO_{2\text{control}}]} \quad (4)$$

$$\text{Normalised } [NO_{2\text{sample}}] = \frac{[NO_{2\text{sample}}]}{\text{Average } [NO_{2\text{control}}]} \quad (5)$$

$$\text{AOI \%} = -(\text{Normalised } [NO_{2\text{sample}}] - \text{Normalised } [NO_{2\text{control}}]) \quad (6)$$

2.8.4 Statistical tests

Data distribution validation was conducted using the Shapiro-Wilk normality test along with Q-Q plots. ANOVA (Analysis of Variance) was performed on nitrite values to compare the means of the linear regression models using the “car” package v3.1.2 ([Fox and Weisberg 2019](#)). Homogeneity of the regression slopes was checked using the linear model given in (7), which tested for interactions between time and protocol repetitions.

$$\begin{aligned} \text{Nitrite} = f_{(\text{Time}, \text{Repetition})} = & \beta_0 + \beta_1 \text{Time} + \beta_2 \text{Repetition}_{(2)} + \beta_3 \text{Repetition}_{(3)} \\ & + \beta_4 \text{Time Repetition}_{(2)} + \beta_5 \text{Time Repetition}_{(3)} \end{aligned} \quad (7)$$

Where applicable, ANCOVA (Analysis of Covariance) was used to assess the effect of protocol repetitions. Independence between time and repetition was tested using one-way ANOVA, linearity was confirmed using univariate linear regression, and homogeneity of residuals was checked via the Levene test from the “car” v3.1.2 package. Tukey’s post-hoc test was performed when necessary, using the “emmeans” v1.10.1 (Lenth 2024) and “multcomp” v1.4.25 (Hothorn et al. 2008) packages, with Bonferroni correction for p-value adjustment. For cell density means obtained via qPCR, the Kruskal-Wallis test and the Dunn’s post-hoc test with Bonferroni correction were performed using the “agricolae” v1.3.7 (de Mendiburu, 2023) and the “rstatix” v0.7.2 (Kassambara 2023b) packages for comparing cell density means obtained from qPCR. Difference in the mean EC₅₀ values of the BNIs and SNIs across the three AOB strains were assessed using the Wilcoxon test facilitated by the “ggplot2” v3.5.0 R package (Wickham 2016) and the “ggpubr” v0.6.0 (Kassambara 2023a) package. The heatmap of the BNIs and SNIs EC₅₀ values were generated with the utilisation of the “ComplexHeatmap” v2.14.0 (Gu et al. 2016) and “dendextend” v1.17.1 (Galili 2015) packages, using hierarchical clustering and Euclidean distances for both rows and columns. EC₅₀ comparisons per NI and AOB strain were made using means and standard errors, and confidence intervals calculated using equation (8).

$$\text{mean EC}_{50} - 1.96 \times \text{standard error} \leq \text{mean EC}_{50} \leq \text{mean EC}_{50} + 1.96 \times \text{standard error} \quad (8)$$

The effect of plant species on root exudates activity was assessed using the Wilcoxon test in “ggplot2” (Wickham 2016) and the “ggpubr” (Kassambara 2023a). The impact of the root exudates on different AOM strains, as well as the effect of wheat genotype origin on the inhibitory capacity of the root exudates was analysed via the Kruskal-Wallis test, followed by Dunn’s post-hoc test with Bonferroni p-value correction using the “agricolae” package v1.3.7 (de Mendiburu, 2023). AOI% heatmaps for the root exudates were generated using the “ComplexHeatmap” v2.14.0 (Gu 2016) and “dendextend” v1.17.1 (Galili 2015) R packages, with hierarchical clustering and Euclidean distances. Principal Component Analysis (PCA) was performed to identify inhibitory patterns among pearl millet and wheat root exudates, as well as the effect of wheat genotype origin on these patterns. AOI% values were scaled using the “vegan” v2.6.4 R package (Oksanen et al. 2022) and the PCA plot was generated using the “factoextra” v1.0.7 R package (Kassambara and Mundt 2020). PERMANOVA was performed on the AOI% values to investigate the effects of plant species and wheat genotype origin on the clustering of the root exudates, using 999 permutations in the “vegan” v2.6.4 R package (Oksanen et al. 2022).

3. Results

3.1 Fast-track system standardisation

3.1.1 *Nitrosospira multiformis*

Consecutive tests of the cell harvesting protocol were performed, tracking the cell density at various stages, and the subsequent growth of the AOB strains in concentrated cultures within 96-well plates. For *N. multiformis*, the initial cell density in the routine cultures was $2.856 \times 10^5 \pm 1.448 \times 10^5$ cells mL⁻¹ (mean $\pm$ 1 SD). At the end of their growth, these cultures reached a concentration of $3.015 \times 10^7 \pm 9.554 \times 10^6$ cells mL⁻¹, with a mean 105.55-fold increase in cell density. After the cell harvesting procedure, the cell density of the three individual inocula was $1.685 \times 10^8 \pm 7.094 \times 10^7$ cells mL⁻¹, which corresponds to a mean harvesting efficiency of $20.87\% \pm 10.42\%$, when considering the theoretical 30-fold cell density expected from the method. Once the three individual inocula were combined, the cell density increased to $4.350 \times 10^8 \pm 4.166 \times 10^7$ cells mL⁻¹, resulting in a mean efficiency of $86.64\% \pm 12.08\%$ when considering the theoretical cell density expected from this combination. The concentrated culture was initiated with a cell density of $3.571 \times 10^7 \pm 2.104 \times 10^6$ cells mL⁻¹, which is statistically similar to the cell density at the final growth stage of the routine cultures, as confirmed by the Kruskal-Wallis test ($p = 1$) [Figure 9A]. Furthermore, the initial cell density of the concentrated culture was on average 125 times higher than of the initial routine cultures. The cell harvesting procedure successfully produced individual inocula with statistically higher cell densities than those observed at the final growth stage of the routine cultures [Figure 9A] ($p < 0.001$). Additionally, the merged inoculum had a cell density statistically higher than that of the individual inocula [Figure 9A] ($p < 0.05$), as confirmed by the Kruskal-Wallis test ($\chi^2 = 1.59 \times 10^{-5}$). Consistent results were observed in the consecutive tests, with similar cell densities obtained at the different stages of the procedure [Figure 9B].

The activity of the subsequent fast-track cultures appeared to be highly consistent across different tests [Figure 9C]. In both cases, nitrite production followed a clear linear pattern, which was confirmed by linear regression analysis of the pooled data from consecutive tests [Figure 9C] (adjusted $R^2 = 0.9828$, $p < 0.001$). According to an ANOVA test on the linear regression model, nitrite production was significantly influenced by time (F-statistic = 4.23×10^3 , $p < 0.001$) but not by the different repetitions of the protocol (F-statistic = 7.32×10^{-2} , $p = 0.9294$). Homogeneity of the regressions slopes was confirmed, as

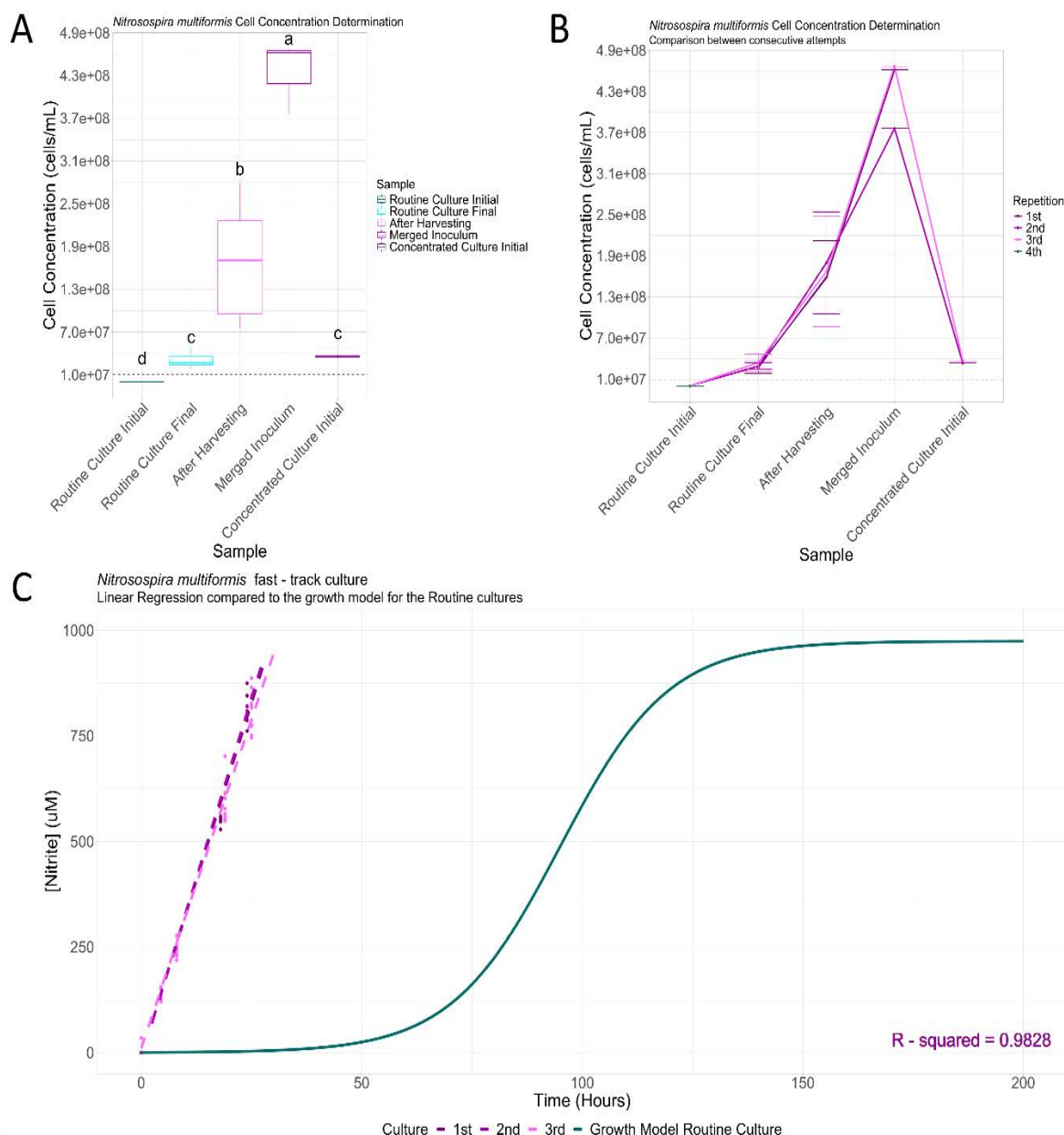


Figure 9. *Nitrosospira multiformis* fast-track culture standardisation. (A) Box plot showing pooled qPCR results at the different stages of the cell harvesting procedure. The y-axis represents cell density, while the x-axis displays the five stages of the procedure. The black dashed line indicates the limit below which the box plot is not scaled. Lowercase letters above the boxes denote groupings based on the Kruskal-Wallis test. (B) Line plots displaying qPCR results across consecutive tests of the cell harvesting procedure. Different tests are distinguished by colour, as indicated in the legend. Coloured error bars represent ± 1 standard deviation from the mean value. The black dashed line indicates the scaling limit for the plot. (C) Activity curve of *N. multiformis* fast-track cultures (magenta lines) compared to 7-days routine culture activity model (blue line). The points show individual nitrite values from biological replicates at each time-point of the fast-track culture. The R^2 value represents the linear regression of pooled fast-track culture data.

there was no significant interaction between the covariate (repetition) with the grouping variable (time) (p-values 0.738 and 0.136 for the 2nd and 3rd repetition respectively). Notably, no exponential activity phase was observed in the activity curve for the fast-track cultures. Compared to the routine cultures, the concentrated *N. multiformis* fast-track cultures exhibited enhanced nitrite production, reaching concentrations of approximately 1000 μM in under 24 h. In contrast, routine cultures required at least 120 h to achieve similar nitrite levels, following a sigmoidal activity pattern where a distinct exponential growth phase was observed [Figure 9C]. The routine cultures demonstrated a μ_{max} of $0.043 \pm 0.002 \text{ h}^{-1}$ (adjusted $R^2 = 0.9434$, $p < 0.001$). This value is statistically similar to the μ_{max} of $0.05 \pm 0.004 \text{ h}^{-1}$ reported by Kaur Bhambra et al. (2021), and comparable to the 0.035 h^{-1} value reported by Shaw et al. (2006). In contrast, the fast-track cultures, exhibited an activity rate of 0.116 ± 0.004 (adjusted $R^2 = 0.9041$, $p < 0.001$), indicating an approximately 3-fold increase in activity compared to the most active point of the routine cultures.

3.1.2 *Nitrosomonas ureae* and *Nitrosomonas communis*

The concentrated cultures of *Nitrosomonas ureae* and *Nitrosomonas communis* exhibited similar behavior to *N. multiformis*. For *N. ureae*, the cell harvesting procedure successfully yielded a concentrated culture with a mean cell density of $9.43 \times 10^7 \pm 1.16 \times 10^7 \text{ cells mL}^{-1}$ (1 SD), which was significantly higher than that of the routine *N. ureae* cultures even at the late logarithmic growth phase (Kruskal-Wallis $p < 0.001$) [Figure 10A]. According to qPCR results, the protocol achieved an average harvesting efficiency of $45.61\% \pm 16.28\%$ (1 SD) and an average centrifugation efficiency of $106.99\% \pm 8.95\%$, both higher than the corresponding values for *N. multiformis*. High repeatability was observed, with cell density trends remaining consistent across consecutive repetitions, particularly for the concentrated cultures [Figure 10B]. In terms of activity, *N. ureae* showed a similar linear nitrite production pattern, with a high quality fit (adjusted $R^2 = 0.9945$, $p < 0.001$) [Figure 10C]. ANOVA revealed that nitrite production was significantly influenced by Time (F-statistic = 25485.89, $p < 0.001$) and repetitions of the protocol (F-statistic = 26.59, $p < 0.001$). Discrepancy in the homogeneity of slopes was identified, as the interactions between different repetitions and time were statistically significant ($p < 0.001$ for both the 2nd and 3rd repetitions respectively). Specifically, the slope of the 1st repetition (49.62 ± 0.50 (1 SE)) was statistically different from the slopes of the 2nd (45.87 ± 0.30 (1 SE)) and 3rd repetitions (45.38 ± 0.28 (1 SE)) at a 95% confidence

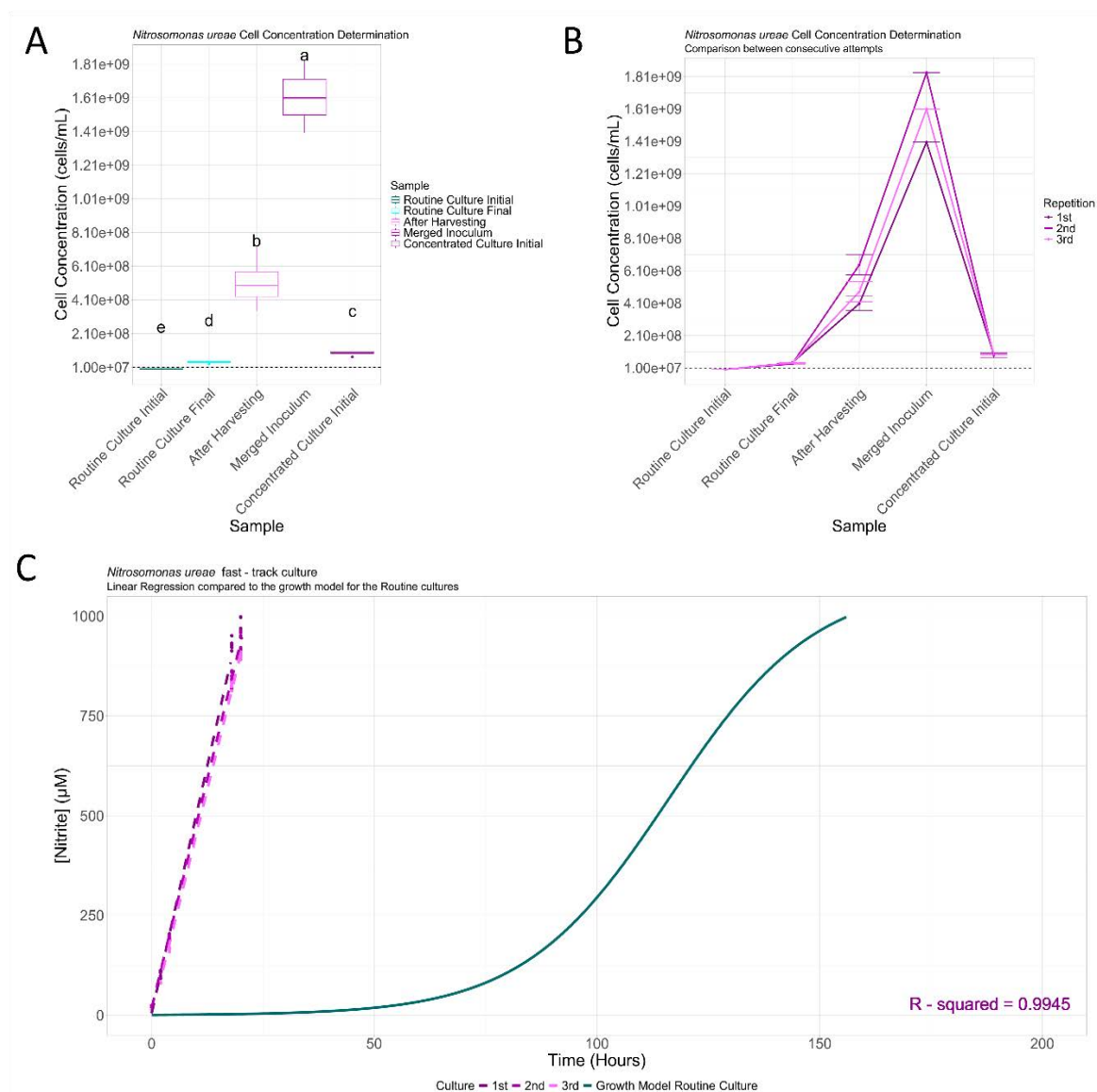


Figure 10. *Nitrosomonas ureae* fast-track culture standardisation. (A) Box plot displaying qPCR pooled results for the various stages of the cell harvesting procedure. The y-axis shows cell density, while the five different stages of the procedure are plotted along the x-axis. The black dashed line indicates the scale limit of the box plot. Lowercase letters above the boxes represent groupings determined by the Kruskal-Wallis test. (B) Line plots showing qPCR results from consecutive tests of the cell harvesting procedure. Each test is depicted in a different colour as indicated in the legend. Coloured error bars represent ± 1 standard deviation from the mean value. The black dashed line marks the scale limit for the line plot. (C) Activity curve comparing *N. ureae* fast-track culture (magenta lines) to the activity model curve of the corresponding 7-days routine cultures (blue line). Points represent nitrite concentrations from biological replicates at each time-point for each fast-track culture. The R^2 value corresponds to the linear regression of pooled fast-track culture data. (D-E) Equations for the activity curves of the routine cultures (blue) and fast-track cultures (magenta) are shown.

interval, although this difference was considered to have no significant biological relevance. Compared to the routine cultures, the concentrated *N. ureae* cultures demonstrated higher activity, reaching nitrite levels of around 1000 μM in less than 24 h, whereas the routine cultures took at least 150h to reach similar levels [Figure 10C]. A logarithmic transformation of the fast-track culture activity resulted in a linear regression curve with a slope of 0.160 ± 0.007 (adjusted $R^2 = 0.844$, $p\text{-value} < 2.2 \times 10^{-16}$, 95% confidence interval), while the μ_{max} of the *N. ureae* routine cultures was estimated at $0.04 \pm 0.001 \text{ h}^{-1}$. This represents an approximate 4-fold increase in activity for the concentrated *N. ureae* cultures compared to the peak activity observed in the routine culture growth.

For *N. communis*, the cell harvesting procedure resulted in a mean harvesting efficiency of $180.57\% \pm 78.20\%$ and a centrifugation efficiency of $51.33\% \pm 24.05\%$. Similar to *N. ureae*, a concentrated culture was produced, with a cell density of $1.94 \times 10^5 \pm 8.79 \times 10^4 \text{ cells mL}^{-1}$ (1 SD) which was significantly higher than the routine cultures at the late logarithmic growth phase ($3.96 \times 10^4 \pm 2.35 \times 10^4 \text{ cells mL}^{-1}$ (1 SD)), confirmed by the Kruskal-Wallis test ($p < 0.05$) [Figure 11A]. Repeatability was lower for *N. communis* compared to other AOB strains, as a deviation was observed in the 3rd repetition for both the individual inocula after harvesting and the merged inoculum [Figure 11B], although the cell density of the final concentrated culture was not significantly affected [Figure 11B]. Crucially, the linear regression curves produced from the three repetitions for *N. communis* were highly consistent, with high quality (adjusted $R^2 = 0.9943$, $p\text{-value} < 2.2 \times 10^{-16}$) [Figure 11C]. Homogeneity was confirmed for the slopes of the 1st and 2nd repetitions ($p < 0.05$ for interaction of 2nd Repetition with Time) but not for the 3rd repetition ($p < 0.05$). The ANCOVA post-hoc test revealed a difference in means between the 2nd and 3rd repetitions ($p < 0.05$), but based on the linear regression curves [Figure 11C] this was not considered biologically significant. According to the linear regression model, the concentrated cultures are expected to completely consume the 3000 μM substrate in approximately 15 h. The *N. communis* concentrated cultures were characterized by an activity rate of 0.132 ± 0.002 . In contrast, the routine cultures required nearly 168 h to consume all the available substrate [Figure 11C] with a μ_{max} of $0.034 \pm 0.002 \text{ h}^{-1}$. Overall, the concentrated cultures exhibited approximately 4-times higher activity compared to the most active stage of the routine culture growth.

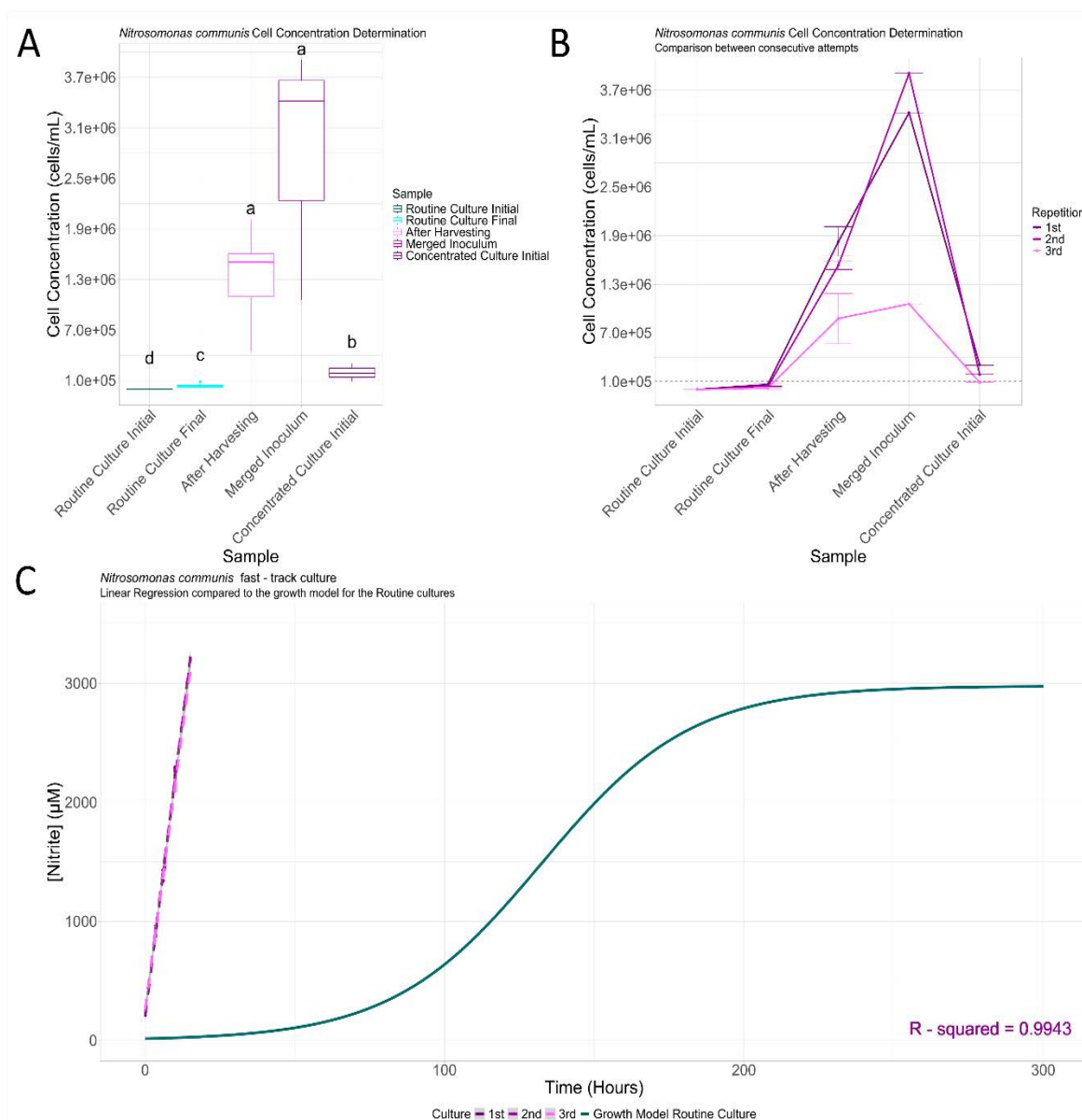


Figure 11. *Nitrosomonas communis* fast-track culture standardisation. (A) Box plot displaying qPCR pooled results for the various stages of the cell harvesting procedure. The y-axis shows cell density, while the five different stages of the procedure are plotted along the x-axis. The black dashed line indicates the scale limit for the box plot. Lowercase letters above the boxes represent groupings determined by the Kruskal-Wallis test. (B) Line plots showing qPCR results from consecutive tests of the cell harvesting procedure. Each test is depicted in a different colours as indicated in the legend. Coloured error bars represent ± 1 standard deviation from the mean value. The black dashed line marks the scale limit for the line plot. (C) Activity curve comparing *N. communis* fast-track culture (magenta lines) to the activity model curve of the corresponding 7-day routine cultures (blue line). Points represent nitrite concentrations from biological replicates at each time-point for each fast-track culture. The R^2 value corresponds to the linear regression of the pooled fast-track culture data.

3.2 Fast-track system validation with known SNIs and BNIs

Inhibition assays using various SNIs and BNIs were performed on the AOB reporter strains to evaluate the ability of the fast-track system to accurately determine EC₅₀ values. Nitrapyrin proved to be highly efficacious against AOB, with all tested doses showing inhibition across all AOB strains [Figure 12]. EC₅₀ values for all strains were below 1 μM [Figure 13C], with *N. ureae* exhibiting the lowest mean EC₅₀ value of 0.18 μM. *N. multiformis* and *N. communis* had EC₅₀ values of 0.44 ± 0.03 μM and 0.88 ± 0.05 μM, respectively. Comparisons of EC₅₀ values at a 95% confidence interval revealed significant statistical differences among all AOB strains [Figure 13A-C]. DMPP displayed a clear dose-response pattern on the AOB, similar to nitrapyrin [Figure 12]. Among the strains, *N. multiformis* was the most sensitive (EC₅₀ 0.48 ± 0.05 μM), followed by *N. ureae* and *N. communis*, with all values being significantly different [Figure 13A-C]. Ethoxyquin induced a subdued response in AOB. A clear dose-response pattern was observed only for *N. ureae*, which exhibited the highest sensitivity across a broad range of concentrations. The two highest concentrations (150 μM and 250 μM) resulted in higher than 50% inhibition [Figure 12], with an EC₅₀ value of 141.3 ± 43.2 μM. In contrast, EC₅₀ values for *N. multiformis* and *N. communis* were estimated to be higher than the maximum tested dose [Figure 13B-C].

MHPP followed a similar inhibitory pattern to nitrapyrin, but with higher inhibition thresholds [Figure 13B-C]. All AOB strains exhibited a dose-response pattern, with near complete inhibition observed in *N. ureae* (98.3%) and *N. multiformis* (96.5%) at 250 μM [Figure 12]. However, *N. communis*, only showed a 48.6% inhibition at the same MHPP concentration, requiring 500 μM for 90.6% inhibition [Figure 12]. The lowest EC₅₀ value was determined for *N. ureae*, with inhibition thresholds increasing significantly for *N. multiformis* and *N. communis* [Figure 13B-C]. Sakuranetin showed variable inhibitory effects, with *N. ureae* being the most sensitive strain (EC₅₀ 4.6 ± 0.7 μM) [Figure 13C]. In contrast, *N. multiformis* required a sakuranetin concentration of 300 μM to reach 80.9% inhibition [Figure 12], and its EC₅₀ value was estimated at 120.3 ± 17.4 μM, approximately 30 times higher than *N. ureae*. No EC₅₀ value could be determined for *N. communis*, as the highest concentration tested (600 μM) resulted in less than 50% inhibition [Figure 12]. Lastly, 1,9-decanediol exhibited low inhibitory activity, though decisive inhibition thresholds were determined for all AOB strains. *N. ureae* was again the most sensitive strain, as was with MHPP and sakuranetin [Figure 13B]. Interestingly, 1,9-decanediol exhibited statistically higher inhibition of *N. communis* compared to

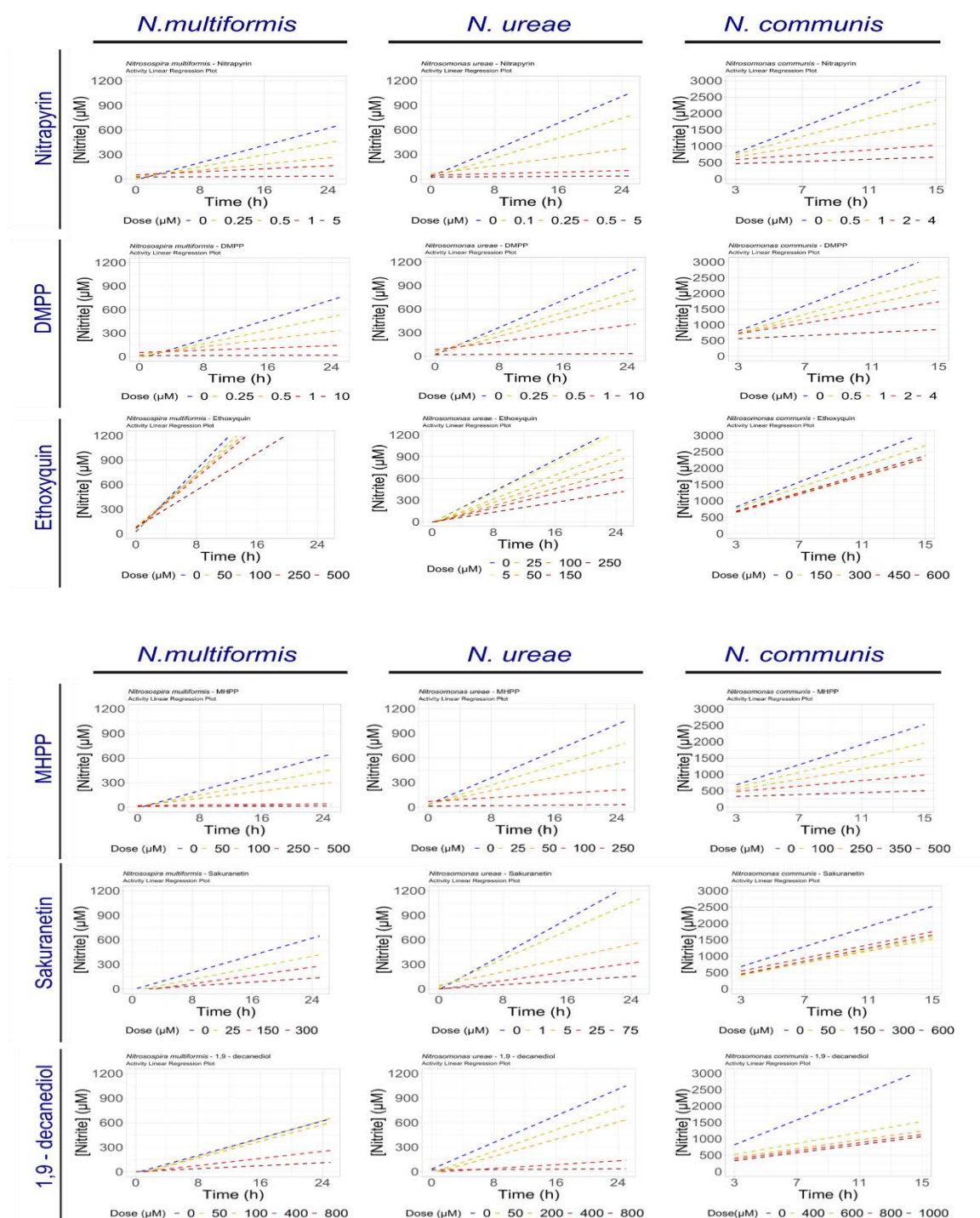


Figure 12. Validation of the AOB fast – track assay with pure SNIs and BNIs. The response of the AOB strains to the tested Biological Nitrification Inhibitors (BNIs) and Synthetic Nitrification Inhibitors (SNIs) is presented. Each of the six panels corresponds to one NI and is divided into three columns, each representing one AOB strain. For each strain and NI combination, the ammonia oxidation activity is illustrated through a linear regression plot, comparing the AOB response to different dose rates, alongside the respective control treatment. The dashed lines in each plot represent the linear regression of nitrite values (data points not shown) at each time point for each treatment.

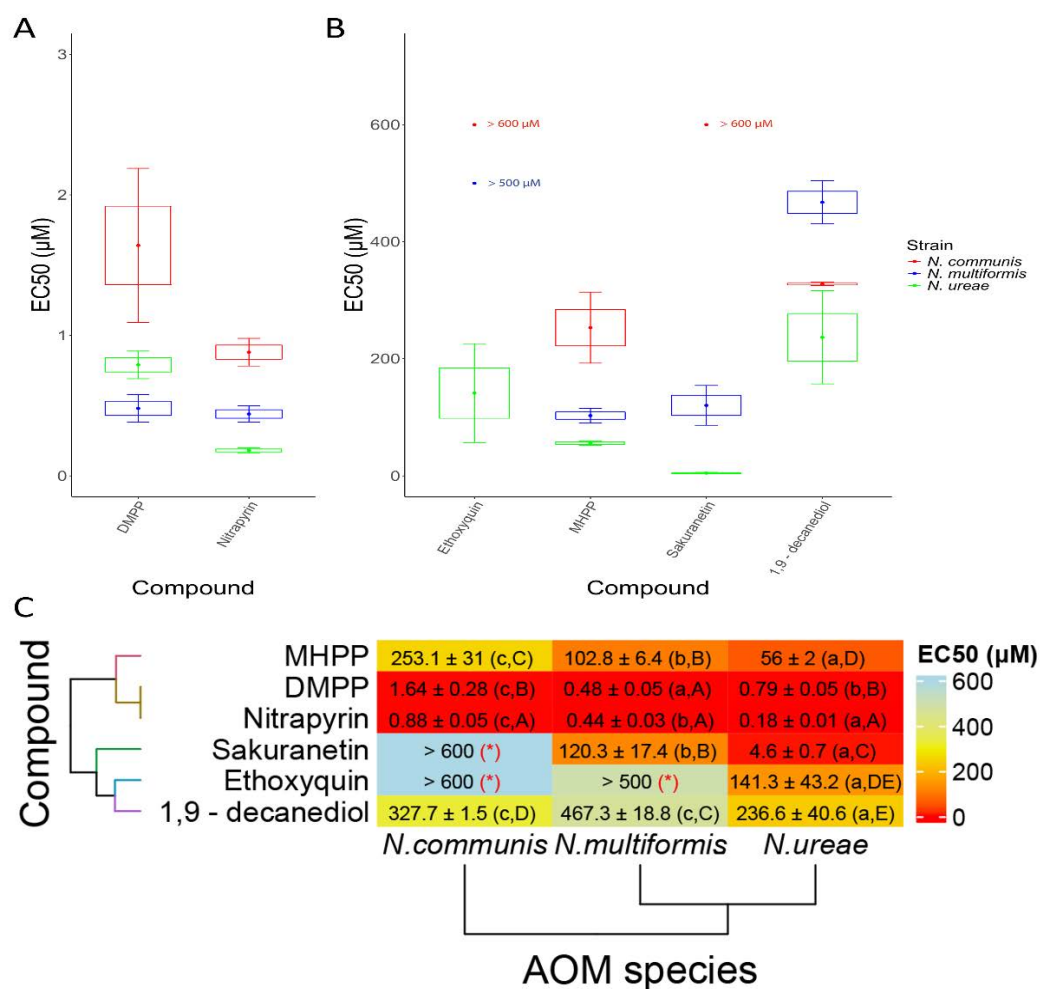


Figure 13. Overview of the inhibition thresholds of the NIs tested on AOB. EC₅₀ values for (A) DMPP and nitrapyrin, and (B) ethoxyquin, MHPP, sakuranetin and 1,9-decanediol are compared across all three AOB strains. Points represent mean EC₅₀ values, while boxes indicate the EC₅₀ ± standard error, and whiskers extend to the EC₅₀ ± 1.96 × standard error, used for comparing mean EC₅₀ values at a 95% confidence interval. Colour-coding for the different AOB strains follows the legend on the right. (C) A heatmap presenting the EC₅₀ values for each nitrification inhibitor (NI) across the reporter AOB strains used in the study, along with their respective standard errors. The colours of the boxes scale logarithmically according to the EC₅₀ values, as indicated by the legend on the right. Dendrograms on the rows and columns were generated using hierarchical clustering of AOB strains and NIs based on Euclidean distances. Values denoted in red are indefinite EC₅₀ values. Lowercase letters in parentheses indicate the grouping of EC₅₀ values for each NI across strains, derived from comparison at a 95% confidence interval. Similarly, uppercase letters signify the grouping of EC₅₀ values of all NIs per AOB strain, obtained from the same comparison. Asterisks (*) accompany indefinite values to indicate that they could not be reasonably compared to other values, while the colour of the respective boxes scales according to the legend based on the lowest limit value presented in each box.

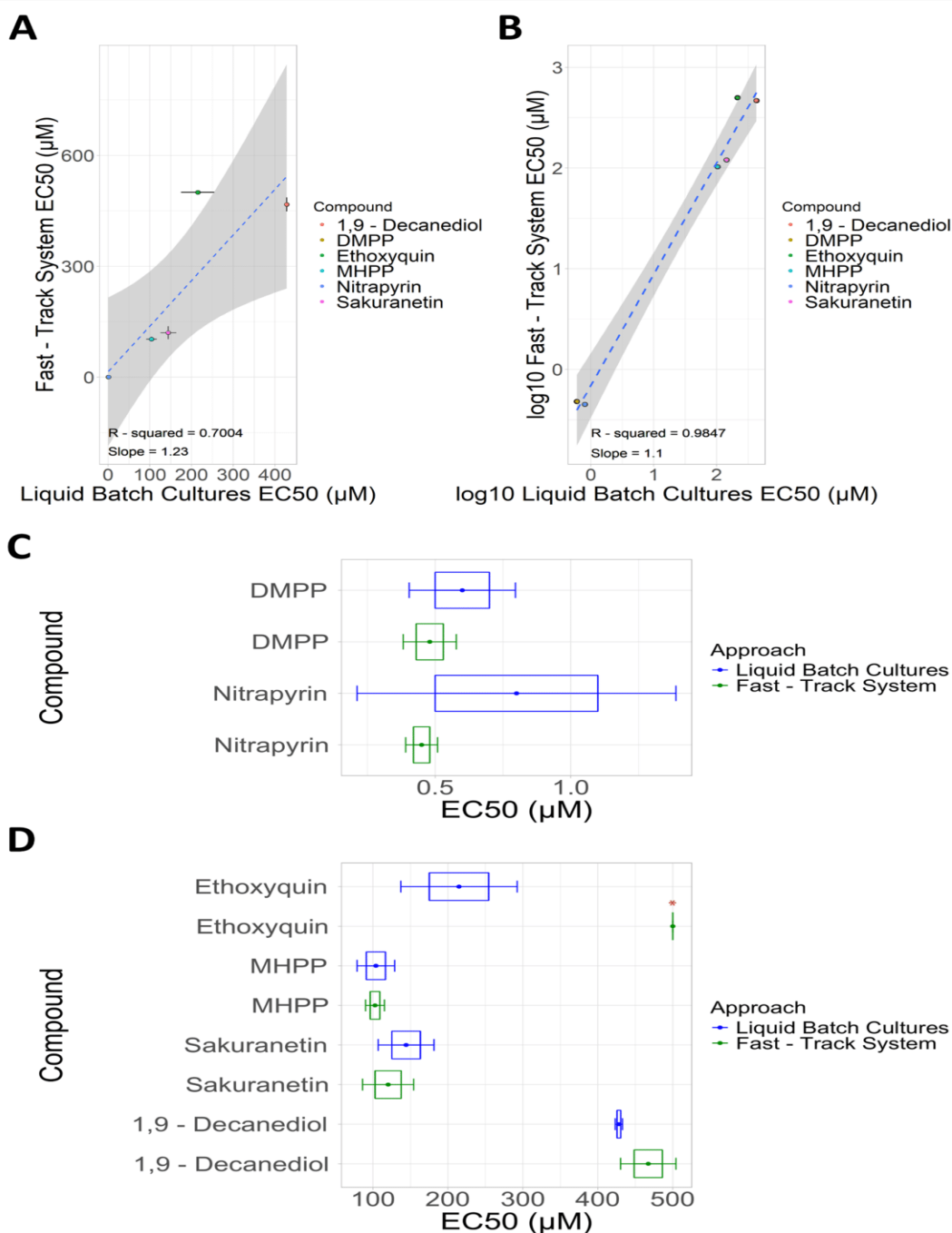


Figure 14. Correlation of EC_{50} values between the fast-track and established liquid batch culture systems for *N. multiformis*. (A) Correlation plots of EC_{50} values for the tested nitrification inhibitors. (B) Transformed plot based on the $\log_{10}$ of the EC_{50} values. The dashed line represents the fitted linear curve, and shaded areas denote the standard error. Error bars illustrate the range of $-1 \text{ SE} < EC_{50} < +1 \text{ SE}$. (C) Comparison of EC_{50} values between the fast-track and established liquid batch culture systems for the tested SNIs and BNIs. White vertical lines represent the EC_{50} values, while blue and green boxes indicate the range $-1 \text{ SE} < EC_{50} < +1 \text{ SE}$. Error bars represent the $-1.96 \text{ SE} < EC_{50} < +1.96 \text{ SE}$, facilitating a 95% confidence interval comparison between the means. [SE: Standard Error].

N. multiformis [Figure 13B]. Overall, higher inhibition thresholds were observed for 1,9-decanediol, with all EC₅₀ values exceeding 200 µM [Figure 13C].

Correlation analysis of EC₅₀ values for *N. multiformis* against literature data yielded a high-quality linear regression (adjusted R² = 0.7004, slope = 1.23) [Figure 14A]. Excluding the ethoxyquin data, which showed a discrepancy with literature, significantly improved the correlation (R² = 0.9907, slope = 1.09; data not shown). Correlation between the log-transformed values was also of higher quality (adjusted R² = 0.9847, slope = 1.10) [Figure 14B]. No significant differences were observed in the EC₅₀ values between the fast-track system and published data for DMPP, nitrapyrin, MHPP, sakuranetin, and 1,9-decanediol [Figure 14C-D].

3.3 Fast-track system proof-of-concept with plant root exudates

3.3.1 Wheat root exudates

After finalizing the standardization and validation processes, the fast-track system was employed to screen wheat root exudates from 38 genotypes collected from three different locations in Austria and India for potential BNI activity. Due to limited material availability, only the two most sensitive AOB reporter strains, *N. multiformis* and *N. ureae*, were tested. In general, the root exudates successfully exerted inhibitory effects on both AOB strains included in the bioassays. The mean AOI% was 18.9% for *N. multiformis* and 17.7% for *N. ureae*, a statistically not significant difference (Wilcoxon test F-statistic = 7020, $p > 0.05$) [Figure 15A]. Regarding the origin of the wheat genotype, the Kruskal-Wallis test indicated that root exudates from at least one of the three regions exhibited significantly different mean AOI % values ($\chi^2 = 7.393$, $p < 0.05$). Pair-wise Wilcoxon tests revealed that the root exudates from genotypes from Vienna-Austria produced root exudates with statistically higher mean AOI% values compared to genotypes from Kashmir-India ($p < 0.01$), but not compared to BHU-India ($p > 0.05$) [Figure 15B].

Hierarchical clustering of the inhibitory effects of all root exudates revealed distinct activity patterns [Figure 16]. Specifically, the bottom cluster includes the two root exudates (RE) that exhibited the highest inhibitory potential, RE32 and RE20. The next three clusters consisted of exudates that were of low activity (AOI% < 20%). Additionally, two other clusters (“turquoise” and “green” clusters) contained 4 and 7 root exudates that exhibited significant inhibition (AOI% > 20%) on either *N. multiformis* or *N. ureae* respectively. Finally, three REs, RE28,

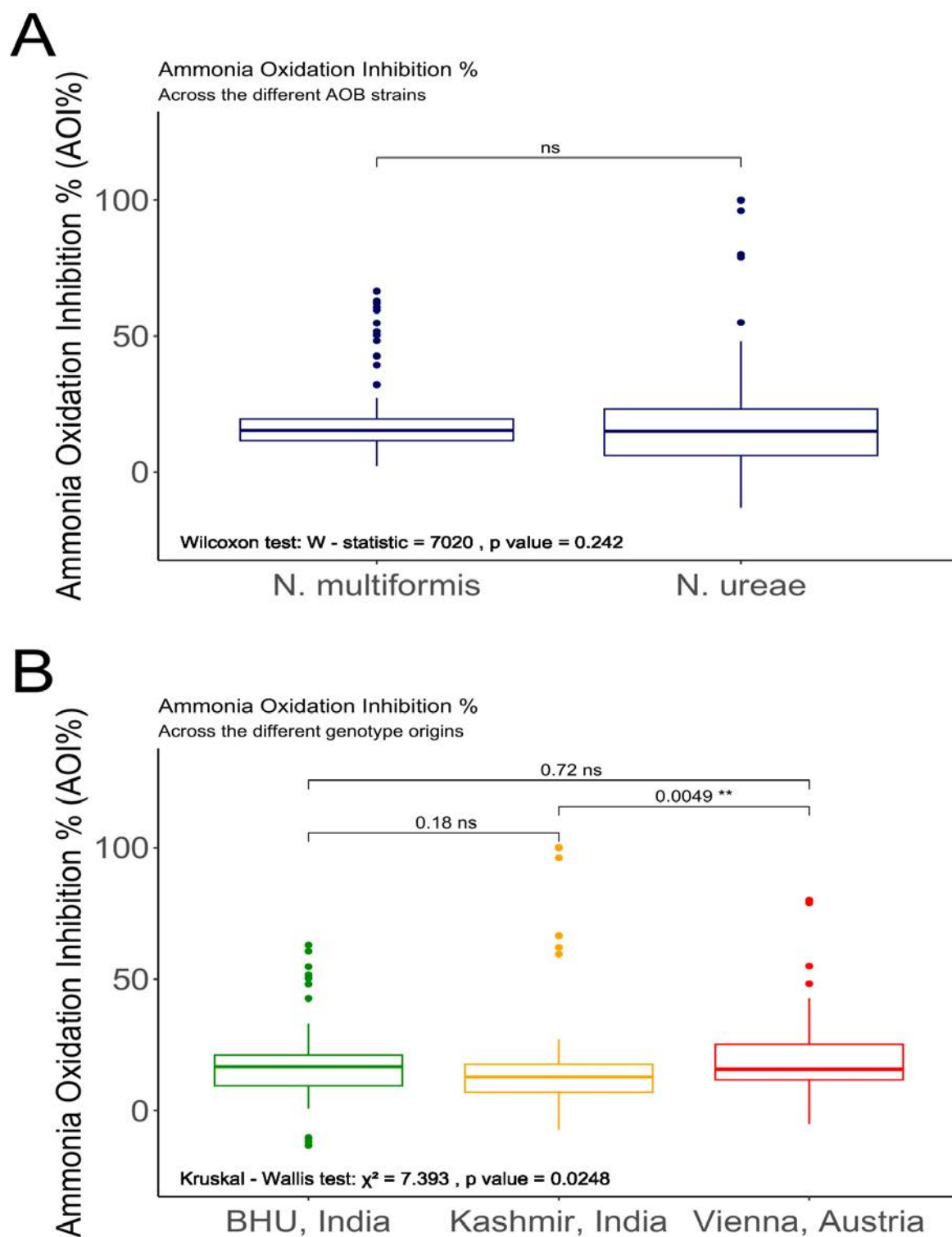


Figure 15. Summary of the wheat root exudate inhibitory patterns on the AOB. Box plot showing (A) the AOI% values for the root exudates on *N. multiformis* and *N. ureae*, and (B) the effect of the wheat genotype origin on the AOI% values. Outliers are represented as points outside the boxes, and whiskers indicate the range of data. Statistical significance from Wilcoxon tests is represented by whiskers, and Wilcoxon and Kruskal-Wallis test results are noted in the bottom left corner of each plot.

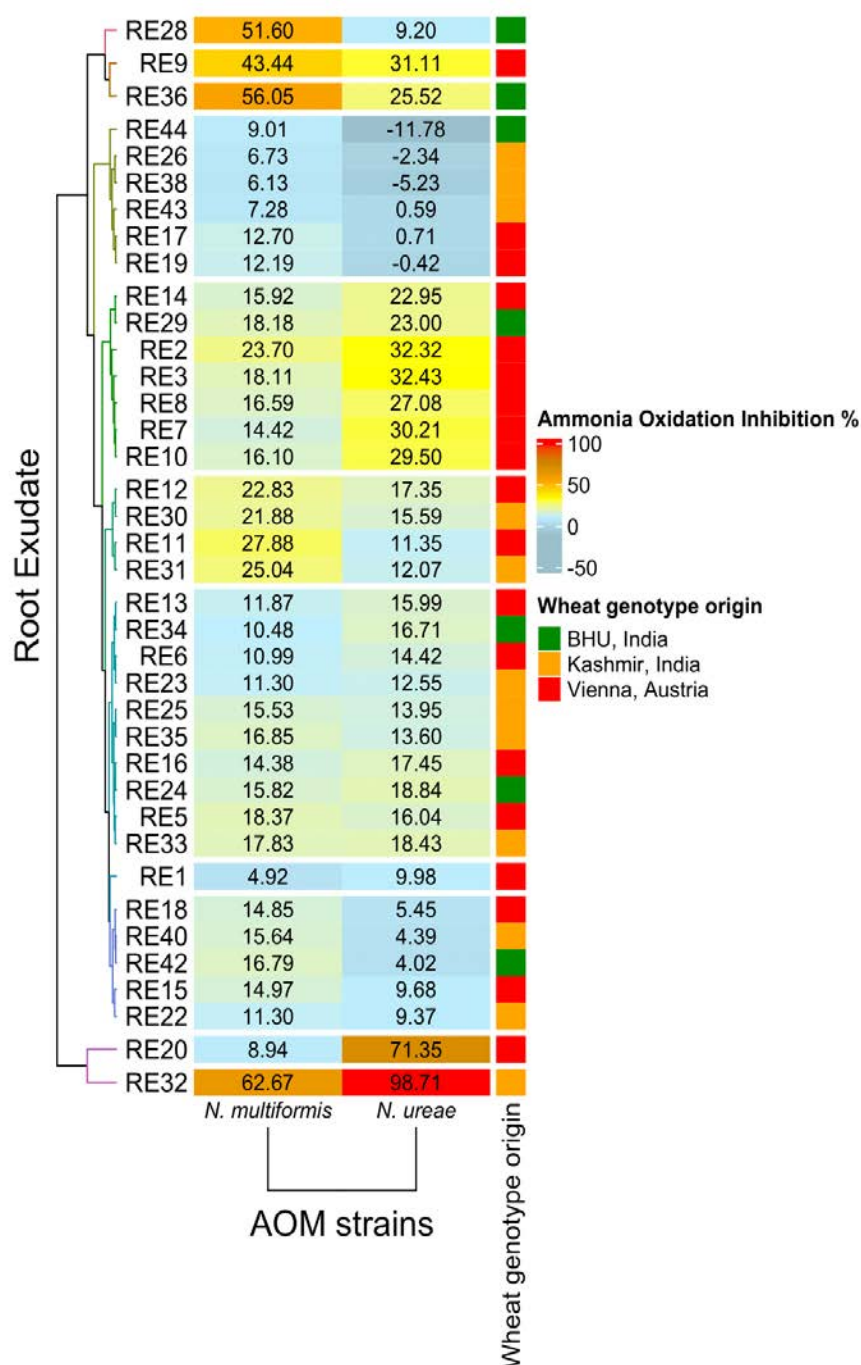


Figure 16. Comparison of root exudate inhibitory activity across the tested ammonia-oxidizing bacteria (AOB) strains. A heatmap showing the mean ammonia oxidation inhibition percentage values (AOI%) for each root exudate (RE) across the two AOB reporter strains. The colours of the boxes scale according to the AOI% values, as indicated by the legend. Dendrograms on the rows and columns were generated using hierarchical clustering of AOB strains and root exudates based on Euclidean distances. The origin of the respective wheat genotypes is annotated on the right, with coloured boxes corresponding to different origin, as indicated in the additional legend. Due to low material availability, the two most sensitive AOB were employed to screen the wheat REs, and *N. communis* was excluded from the analysis.

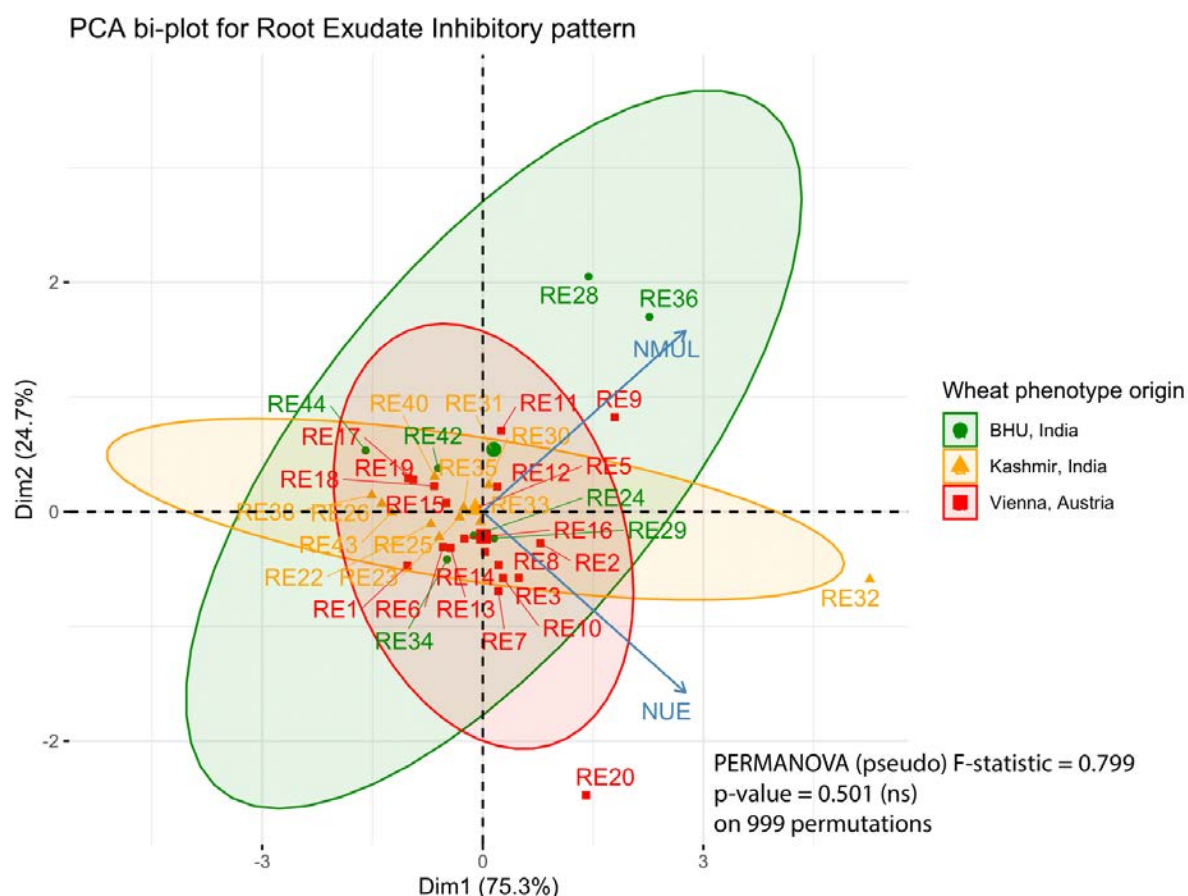


Figure 17. Variation in inhibitory patterns of different root exudates. The plot visualizes the results of PCA, with root exudates shown in two dimensions, represented by different shapes, according to the legend. Colour coding groups exudates based on genotype origin. Ellipses are drawn at a 95% confidence level, and centroids are marked with larger shapes.

RE9 and RE36 are clustered at the top, characterized by increased potential for *N. multiformis* inhibition. Overall, 22 out of the 38 root exudates tested, mostly distributed in the middle of the heatmap, did not exhibit significant nitrification inhibition ($\text{AOI}\% < 20\%$). Twelve root exudates were significantly inhibitory towards only one of the strains, while only 4 yielded an $\text{AOI}\%$ value higher than 20% on both reporter strains [Figure 16]. Genotype origin clustering was partially observed, as root exudates isolated from Vienna-Austria genotypes predominantly formed a cluster with higher activity against *N. ureae* [Figure 16]. In contrast, two of the three root exudates clustered at the top of the heatmap, RE28 and RE36, which showed a strong selective inhibition of *N. multiformis*, were extracted from wheat genotypes originating from BHU-India [Figure 16]. Principal Component Analysis (PCA) was performed to explore the variation in the inhibitory patterns of the tested root exudates. Grouping the exudates based on wheat genotype origin revealed no distinct clustering, as confirmed by PERMANOVA ($F\text{-statistic} = 0.799$, $p > 0.05$ on 999 permutations) [Figure 17]. However, less

variation in inhibitory patterns was observed among exudates from Vienna-Austria genotypes compared to those from the two Indian wheat genotypes. The PCA specifically identified 5 REs as outliers, exhibiting distinct patterns with significant inhibition potential [Figure 17]. RE20 was differentiated due to positive correlation with *N. ureae* inhibition, RE9, RE28 and RE36 were correlated with *N. multiformis* inhibition and RE32 was correlated with inhibition of both AOB reporter strains [Figure 17].

3.3.2 Pearl millet root exudates

In addition to the wheat root exudates, root exudates from 33 different pearl millet genotypes were screened, using all three available AOB reporter strains. The screening revealed a variable impact of the exudates on AOB activity (Kruskal-Wallis $\chi^2 = 87.61$, $p < 0.001$). Distinct inhibitory activity against *N. multiformis* was observed with a low median AOI% value of 4.45% [Figure 18]. Six (6) out of the 33 exudates (18.2%) had AOI% values above the median for *N. multiformis*, though none exceeded the 20% threshold considered significant. Only two exudates, RE27 and RE37, surpassed this threshold with AOI% values of 35.36% and 25.58%, respectively. Similar differences in inhibitory activity were found for *N. ureae* (Kruskal-Wallis $\chi^2 = 90.61$, $p < 0.001$), though with generally higher AOI% values (median = 11.43% compared to 4.45% for *N. multiformis*) [Figure 18]. Eleven (11) (33.3%) out of the 33 exudates produced AOI% values above the 20% threshold [Figure 19]. For *N. communis* a narrower range of AOI% values was observed, with a median value of 13.91% [Figure 18]. Exudates RE27, RE17, RE12 and RE3 were the most potent against *N. communis*, with AOI% values above the 3rd quartile [Figure 19]. Overall, *N. ureae* showed the highest mean AOI% values, but the highest mean AOI% was obtained for *N. communis* [Figure 18], where inhibition was observed in almost all exudates. However, only four exudates surpassed the 20% threshold [Figure 19]. While the difference in mean AOI% values for *N. communis* and *N. ureae* was not statistically significant, both *N. communis* (Kruskal-Wallis $p < 0.001$) and *N. ureae* (Kruskal-Wallis $p < 0.01$) showed higher AOI% values compared to *N. multiformis* [Figure 18].

Hierarchical clustering of the mean AOI% values further supported the distinct inhibitory activity patterns of the root exudates when screened across the AOB reporter strain portfolio. *N. ureae* was again highlighted as the most sensitive strain, clustering separately from *N. communis* and *N. multiformis* [Figure 19]. The exudates exhibited distinct inhibitory patterns, grouped into the following categories from top to bottom on the heatmap: (a) no inhibitory activity against any strain (3 REs), (b) inhibition only against *N. communis* (9 REs), (c)

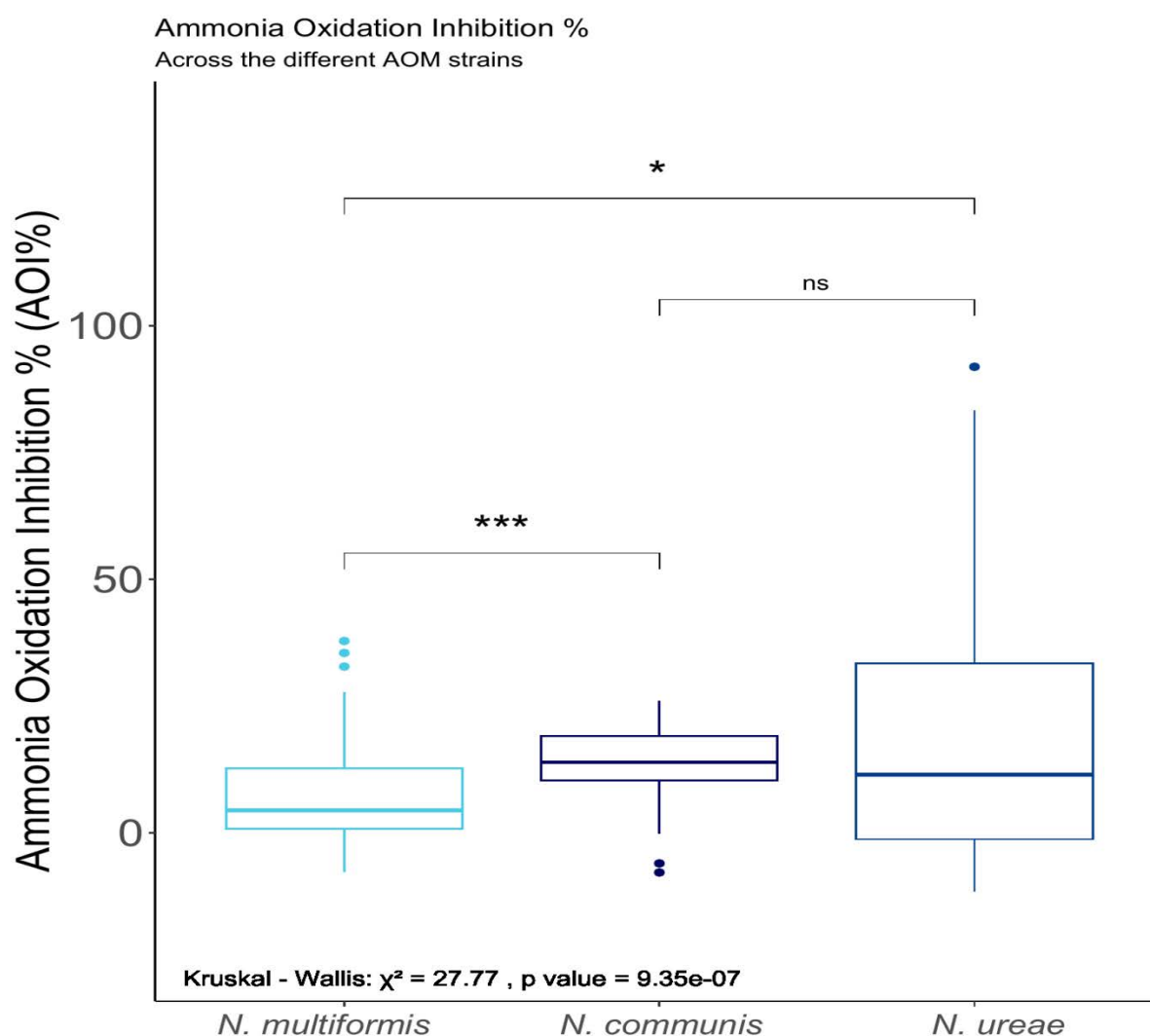


Figure 18. Comparison of pearl millet root exudate inhibitory activity across the AOB reporter strains. Mean AOI% values for the three reporter strains are compared. Asterisks indicate the level of statistical significance (ns - $p > 0.05$, * - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.001$). The Kruskal-Wallis χ^2 and p-value are shown in the bottom left corner. Outliers are represented by coloured points outside of the boxes.

inhibitory activity against all strains but no significant (3 REs), (d) significant inhibition of both *N. ureae* and *N. communis* (8 REs), (e) strong (1RE) and significant (7REs) inhibition across all strains, with selective activity against *N. ureae* and, (f) high inhibition on *N. ureae*, moderate inhibition on *N. multiformis* but no activity against *N. communis* (2 REs) [Figure 19]. A PCA plot was used to visualize these distinct inhibitory patterns, explaining 97.8% of the total variance [Figure 20]. The root exudates that contributed most to the principal components due to their strong inhibitory activity were RE27, RE37 and RE44 [Figure 20]. RE27 was positively correlated with inhibition on *N. ureae*, *N. multiformis* and *N. communis* [Figure 20].

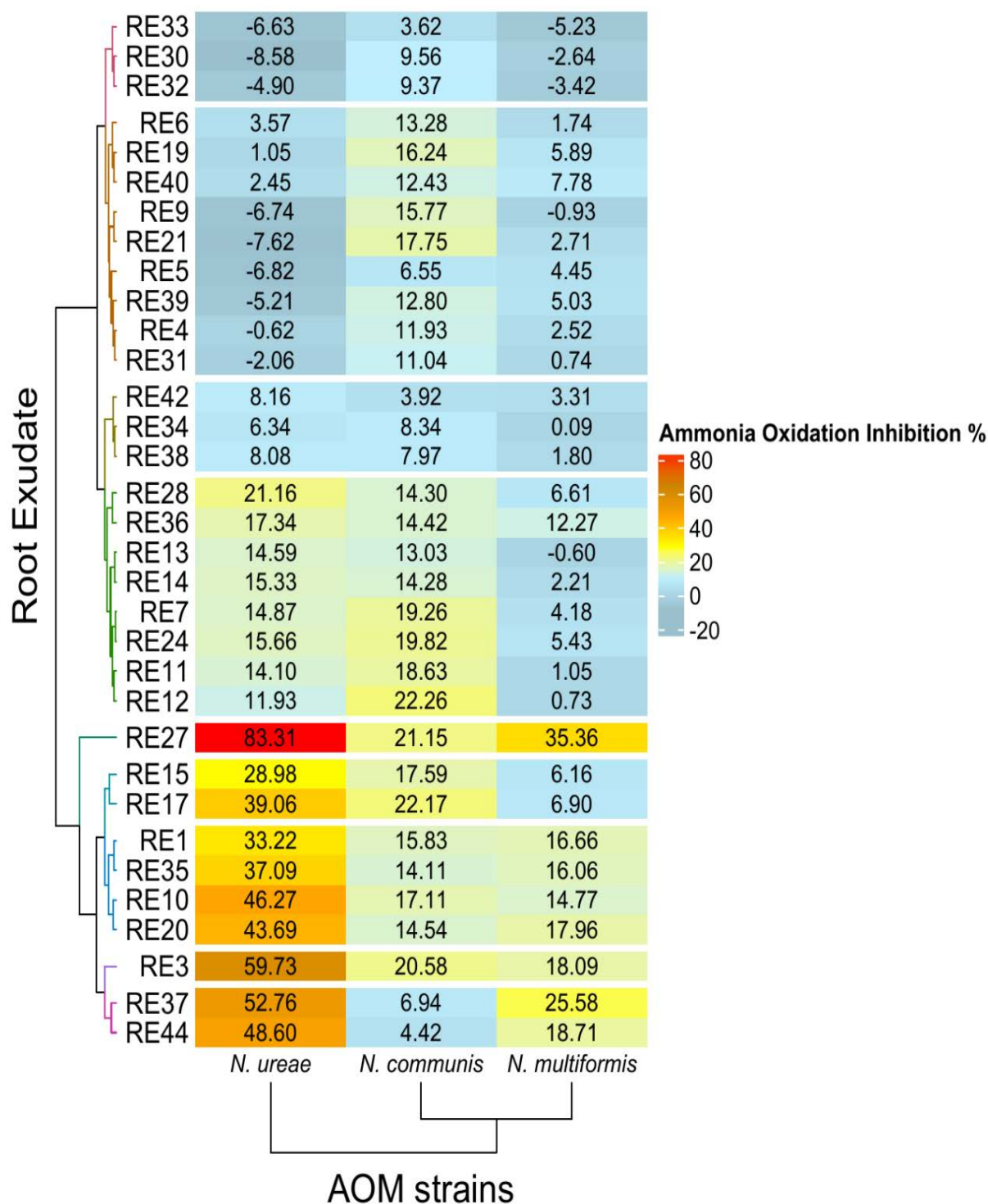


Figure 19. Overview of pearl millet root exudate inhibitory activity across the AOB reporter strains. The heatmap displays the mean AOI% for each root exudate (RE) across the three reporter strains. Box colours are scaled according to AOI% values, as indicated in the legend. Dendrograms for rows (AOB strains) and columns (root exudates) were generated using hierarchical clustering based on Euclidean distances.

Following the initial single-dose screening, and based on the results obtained, a range of concentration tests were performed on some of the most potent root exudates for two AOB strains to identify dose-response patterns. Considering the inhibitory potential and the availability of material, RE27 and RE37 were retested on *N. multiformis*, at in-culture concentrations of 5 $\mu\text{g } \mu\text{L}^{-1}$, 10 $\mu\text{g } \mu\text{L}^{-1}$, 15 $\mu\text{g } \mu\text{L}^{-1}$ and 20 $\mu\text{g } \mu\text{L}^{-1}$. *N. ureae* dose-response patterns were evaluated for RE1, RE3, RE10, RE15, RE20 and RE37, applied in doses of 5 $\mu\text{g } \mu\text{L}^{-1}$, 7.5 $\mu\text{g } \mu\text{L}^{-1}$, 10 $\mu\text{g } \mu\text{L}^{-1}$ and 20 $\mu\text{g } \mu\text{L}^{-1}$. For *N. multiformis*, a clear dose-response pattern was observed for both REs [Figure 21A], with RE27 again proving more potent than RE37 [Figures 19, 21A]. Regarding *N. ureae*, all six REs displayed dose-response patterns, with RE3 and RE37 emerging as the most potent, similar to the single-dose screening [Figures 19, 21B]. Consistent with the single-dose results, *N. multiformis*, was less sensitive compared to *N. ureae*, with neither RE27 nor RE37 achieving AOI% values above 50%, even at the highest concentration tested [Figure 21A]. In contrast, all six REs tested on *N. ureae* successfully inhibited its activity by more than 50% at the highest concentration [Figure 21B], with mean EC₅₀ values ranging from 4.10 $\mu\text{g } \mu\text{L}^{-1}$ (RE3) and 5.53 $\mu\text{g } \mu\text{L}^{-1}$ (RE37) to 9.90 $\mu\text{g } \mu\text{L}^{-1}$ for RE1 and 14.05 $\mu\text{g } \mu\text{L}^{-1}$ for RE10 [Table 3]. The inhibition thresholds estimated for RE3 and RE37, were not significantly different when comparing their 95% confidence intervals, as was also the case for RE1 and RE10.

An overall comparison between the root exudates of the two plant species (wheat and pearl millet) was performed, focusing on *N. multiformis* and *N. ureae*, the two AOB reporter strains used for screening both sets of exudates. No significant difference in the sensitivity of the two reporter strains was observed (Wilcoxon test F-statistic = 20828, $p > 0.05$), although *N. ureae* showed higher AOI% values for certain exudates, which were identified as outliers of higher potency compared to *N. multiformis* [Figure 22A]. However, a significant effect of the plant species was detected, with wheat root exudates showing higher mean AOI% values on the AOB compared to those from pearl millet (Wilcoxon test F-statistic = 15323, $p < 0.001$) [Figure 22B]. Interestingly, but not unexpectedly, the inhibition patterns differed between the two AOB reporter strains [Figure 22C]. For example, wheat root exudate W32 showed a positive correlation with inhibition of both AOB strains, aligning with the high AOI% values obtained for both [Figure 16], while W20 was correlated strongly with *N. ureae* inhibition [Figure 22C].

Table 3. Overview of the inhibition thresholds estimated for the REs on *N. ureae*. Asterisks denote the statistical significance of the applied fit model (ns-nonsignificant, * - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.001$). Mean EC_{50} values $\pm$ standard error are presented.

Root Exudate	EC_{50} value ($\mu\text{g } \mu\text{L}^{-1}$)	Grouping (95% confidence interval comparison)	Dose-Response model	Model R^2	Model p - value
RE1	14.05 ± 2.36	b	Linear	0.8561	< 0.001 ***
RE3	4.10 ± 0.14	a	Log-logistic, Weibull	0.97	< 0.001 ***
RE10	9.90 ± 0.84	b	Polynomial 3 rd degree	0.9566	< 0.001 ***
RE15	Indecisive	-	-	-	-
RE20	Indecisive	-	-	-	-
RE37	5.53 ± 1.38	a	Linear	0.8967	< 0.001 ***

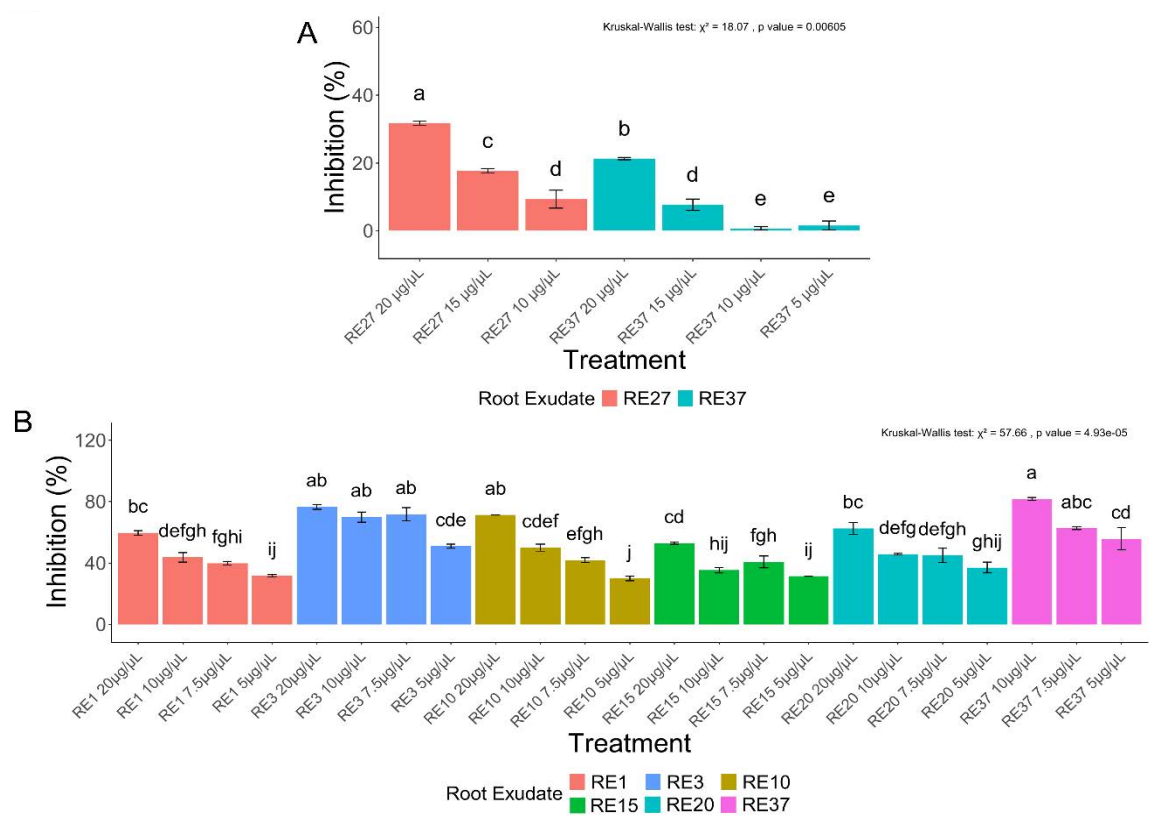


Figure 21. Comparison of the inhibitory effects of the most potent REs. The inhibition effects on (A) *N. multififormis* and (B) *N. ureae* are shown. Bars represent the mean AOI% for each concentration of root exudate, with error bars indicating the standard error of the mean. Different colours correspond to the different REs tested, as indicated in the legend below. Lowercase letters denote treatment groupings based on the Kruskal-Wallis test, with the respective statistics displayed in the upper right corner.

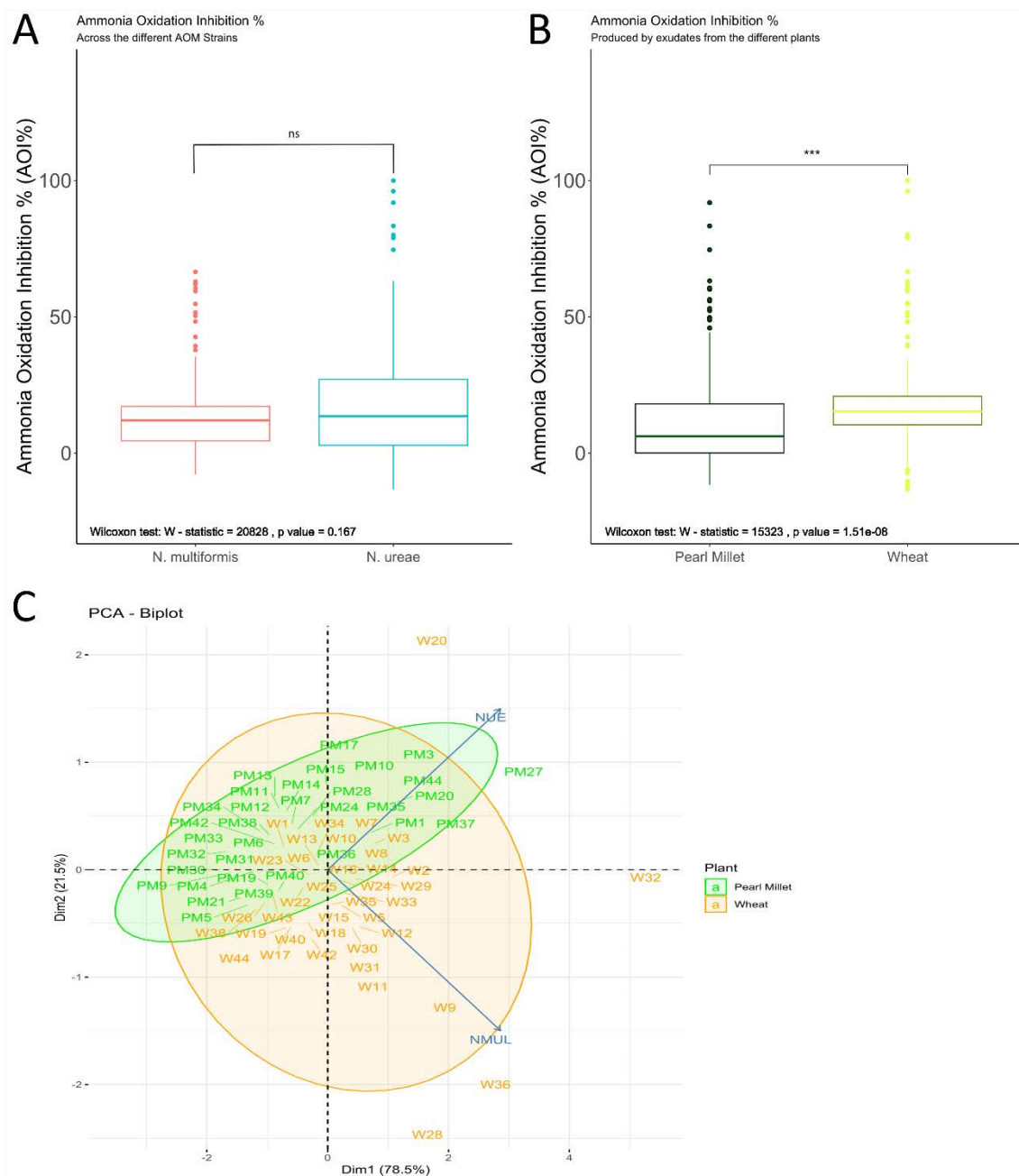


Figure 22. Overview of the comparison of inhibitory activity between wheat and pearl millet root exudates. Box plot showing (A) the AOI% values of the root exudates on *N. multiformis* and *N. ureae*, and (B) the effect of plant species on the inhibitory activity of the exudates. Outliers are represented as points outside the boxes. Whiskers indicate the statistical significance of the comparisons based on Wilcoxon tests. Wilcoxon tests, with the results shown in the bottom left corner of each plot. (C) PCA analysis visualizing the inhibition patterns of the root exudates. Colour coding groups the exudates by plant species with ellipses drawn at a 95% confidence level. Arrow vectors represent the two AOB reporter strains, with positive correlation indicated by vectors and exudates on the same side.

[Figure 22C]. In contrast, wheat exudates, W9, W28 and W36 were identified as outliers more strongly correlated with *N. multiformis* inhibition rather than *N. ureae* [Figure 22C], which is consistent with the lower inhibition of *N. ureae* observed for these exudates [Figure 16]. On the other hand, the most potent pearl millet root exudate, PM27, was mainly correlated with inhibitory activity against *N. ureae*, and not *N. multiformis* [Figure 22C]. This is confirmed by the individual AOI% values of 83.31% for *N. ureae* and 35.36% for *N. multiformis* [Figure 19]. Overall, pearl millet root exudates were more correlated with *N. ureae* inhibition, whereas wheat root exudates were differentiated due to their association with *N. multiformis* inhibition [Figure 22C]. This trend aligns with the lower AOI% values on *N. multiformis* for pearl millet exudates, and the opposite pattern for *N. ureae* when compared to wheat exudates [Figures 16, 19].

4. Discussion

4.1 Evaluation of the proposed fast-track assay

4.1.1 Time frame of the fast-track assay

In this study, the thorough development of a high-throughput, fast-track bioassay for BNI activity screening was described. The resulting pipeline secured high repeatability in both the cell density and activity of the concentrated cultures. The validation and application of the fast-track assay for screening plant root exudates resulted in a turnaround time ranging from 10 h for *N. communis* to 22 h for *N. ureae*, all within a 1-day timespan. This is similar to the timeframes used in studies by O' Sullivan et al. (2016) and Beeckman et al. (2023). Other studies describing fast-track assays report even shorter turnaround times, such as 30 minutes (Subbarao et al. 2006), or 2 hours (Sun et al. 2016). However, it is important to note that use of highly dense AOM cultures, required to achieve these short turnaround times, can potentially reduce the observed effects of tested compounds or exudates, due to high overall metabolic activity, or excess of molecular targets for BNI activity, thus underestimating nitrification inhibition potential. This phenomenon, observed with antibiotics, is referred to as the “inoculum effect” (Brook 1989; Bottery et al. 2020). In case where NI activity arises from direct, irreversible inhibition of AMO or other nitrification-related enzymes, efficacy may be affected not only by target affinity but also by target concentration, which would be significantly increased in a dense culture. This might apply to compounds like nitrpyrin, a suicide inhibitor, oxidized by AMO into 6-chloropicolinic acid (Vannelli and Hooper 1992), where a reduced effect could theoretically occur due to target oversaturation. Another bias

introduced by rapid turnaround times may arise from delayed cell response, leading to higher identified inhibition thresholds. For instance, certain compounds with NI activity, such as 6-chloropicolinic acid, exhibit nitrification inhibition *in vitro* with a lag phase of 8 h, likely due to delayed uptake or transformation into an active compound (Powell and Prosser 1985). Additionally, the ability of putative BNIs to permeate the cell membrane and exert inhibitory activity depends on factors like lipophilicity (indicated by partition coefficient, logP) and molecular polarity (indicated by Topological Polar Surface Area, TPSA) (Nardi et al. 2020). Given that the logP and TPSA of potential BNIs, along with AOA and AOB cell membrane and intracellular space characteristics, can vary significantly (Nardi et al. 2020), impaired or delayed BNI uptake and transfer is a realistic possibility. This could result in the underestimation of BNI potential in assays with very short AOM cultivation times. Overall, while shorter cultivation times are appealing for fast-track screening, slightly longer but still rapid cultivation times, such as those employed in this study, could help mitigate these biases and lead to a more reliable discovery of BNI potential.

4.1.2 Ecological relevance of the fast-track assay

To increase ecological and phylogenetical diversity, three phylogenetically diverse, soil-representative AOB strains were employed in this study. *Nitrosospira* species are considered the most abundant AOB in soil (Lehtovirta-Morley, 2018) and play a key role in ammonia oxidation in fertilized soils (Lin et al. 2018; Cassman et al. 2019). Specifically, *Nitrosospira multiformis* (*Nitrosospira* Cluster 3) is highly adaptive to soil growth (Norton et al. 2008) and abundant in agricultural soils (Norton et al. 2008; Zorz et al. 2018). Therefore *N. multiformis* was chosen as a model (Shen et al. 2013) and ecologically relevant soil-derived AOB reporter strain, aligning with previous studies employing the strain in liquid batch cultures (Shen et al. 2013; Papadopoulou et al. 2020; Kaur-Bhambra et al. 2022; Kolovou et al. 2023) and other fast-track assays (O' Sullivan et al. 2016; Beeckman et al. 2023). *Nitrosomonas* is the second most abundant genus of soil AOB (Lehtovirta-Morley 2018). Two representatives of this lineage, *Nitrosomonas ureae* (*Nitrosomonas* Cluster 6a) (Sedlacek et al. 2019) and *Nitrosomonas communis* (*Nitrosomonas* Cluster 8) (Sedlacek et al. 2019) were included to broaden the AOB reporter strain set. These strains were chosen over *Nitrosomonas europaea*, which has been widely used as a sole AOM reporter strain in other studies (Subbarao et al. 2006; Sun et al. 2016) but is more representative of ammonia-oxidising communities in wastewater treatment plants rather than in soil environments.

While this thesis focuses on the development of the assay using AOB strains, the inclusion of two distinct, soil-representative AOA strains was part of the broader collaborative work conducted by the University of Vienna. “*Candidatus Nitrosocosmicus franklandianus*” (Clade NS-ζ-2) (Alves et al. 2018) and *Nitrososphaera viennensis* (Clade NS-α-3.2.1) (Alves et al. 2018) were selected to further enhance the ecological relevance of the system. AOA are highly active members of the soil AOM community, and their inclusion significantly increases the ecological relevance of the fast-track assay and its ability to assess responses of important soil AOM taxa. *N. viennensis* is one of the best studied soil AOA, regarding its biochemistry (Kerou et al. 2016) and its potential contribution to N₂O emissions, which is comparable to *Nitrosospira multififormis* (Stieglmeier et al. 2014). It has also been used in studies assessing the NI potential of both synthetic (Shen et al. 2013) and biological compounds (Kaur-Bhambra et al. 2022), including fast-track assays (Beeckman et al. 2023). “*Ca. Nitrosocosmicus franklandianus*” has already been employed in liquid batch culture assays to assess NI potential (Papadopoulou et al. 2020; Kaur-Bhambra et al. 2022; Kolovou et al. 2023). It shows high autotrophic growth in environmental samples and is phylogenetically distinct to the *Nitrososphaera* genus (Alves et al. 2018). Additionally, the inclusion of an acidophilic AOA strain of the *Nitrosotaleales* lineage (Lehtovirta-Morley et al. 2014), could further increase the ecological relevance of the fast-track assay. Acidophilic AOA play an important role in global nitrogen cycling, particularly in acidic soils, which cover approximately 30% of the world’s land area (Zhao et al. 2022). Species of the *Nitrosotalea* genus such as *N. sinensis* Nd2 has been found to outcompete AOB in acidic soils (Schleper and Nicol 2010; Lehtovirta-Morley 2018; Zhao et al. 2020) and has been successfully used in bioassays to determine inhibition thresholds for both SNIs (Papadopoulou et al. 2020) and BNIs (Kaur-Bhambra et al. 2022; Kolovou et al. 2023). Its distinct ecophysiology and sensitivity, make it critical candidate for future bioassays aimed at discovering novel BNIs.

In addition to the AOM, potential off-target effects of BNIs could be assessed *in vitro*, with the inclusion of NOB strains, adding another layer to the fast-track screening procedure. Soil - relevant *Nitrobacter* strains, such as the acidophilic “*Candidatus Nitrobacter laanbroekii* NHB1” (Hink et al. 2024), and neutrophilic *Nitrobacter hamburgensis* (Bock et al. 1983), could serve as off-target reporter strains for BNI candidates. Investigating off-target toxicity is crucial to ensure that BNI approaches support sustainability in agricultural intensification. “*Ca. N. laanbroekii*” has already been used in bioassays, identifying significant off-target effects of known BNIs, like MHPP, as well as newer compounds like zeanone and 2-methoxy-1,4-

napthoquinone (Kolovou et al. 2023). Moreover, fast-track co-cultures of either AOM or AOM and NOB, could provide additional insights into how BNIs might influence interactions between different AOM and NOB involved in nitrogen cycling. Evidence for syntrophic relationships between AOM and NOB is growing, with findings that NOB can release ammonia from cyanate, which AOM utilize, while AOM in turn, provide nitrite to NOB (Nardi et al. 2020). Similarly, ureolytic AOM, such as *N. viennensis*, may supply ammonia to non-ureolytic AOM, and ‘*Ca. N. laanbroekii* NHB1’ has been shown to relieve *N. sinensis* from nitrite inhibition (Hink et al. 2024).

In summary, a fast-track assay that includes six AOM reporter strains from both bacterial and archaeal domains, promises to better capture the diversity of soil AOM communities, than previous studies that used less ecologically relevant (Subbarao et al. 2006; Sun et al. 2016) or more limited AOM portfolios (O’Sullivan et al. 2016; Beeckman et al. 2023). Incorporating nitrite oxidisers and synthetic co-cultures would further enhance the assay, offering a multi-level, more holistic fast-track screening process for novel BNIs.

4.2 Determination of inhibition thresholds for pure NIs

The determination of inhibition thresholds for known SNIs and BNIs revealed distinct patterns of ammonia oxidation inhibition, reflecting both the varying efficacy of the NIs, and the differences in sensitivity among specific AOM strains. These findings align with previous studies that advocated using a diverse range of reporter strains (Papadopoulou et al. 2020; Kaur-Bhambra et al. 2022; Kolovou et al. 2023). Notably, this study is the first to report inhibition thresholds for *N. communis* and *N. ureae*, two key representatives of soil AOB communities. Regarding the AOB response to all tested NIs, *N. ureae* appears significantly more sensitive than *N. communis*, and in most cases, it also exhibits lower inhibition thresholds compared to *N. multiformis*. Generally, the tested SNIs, except for ethoxyquin, displayed lower EC₅₀ values for all AOB, compared to the BNIs. Nitrapyrin was highly potent against AOB, with an EC₅₀ of $0.44 \pm 0.03 \mu\text{M}$ determined for *N. multiformis*, consistent with the value of $0.8 \pm 0.3 \mu\text{M}$ reported in previous studies (Papadopoulou et al. 2020). Nitrapyrin exhibited its highest activity on *N. ureae* (EC₅₀ $0.18 \pm 0.01 \mu\text{M}$), while *N. communis* (EC₅₀ $0.88 \pm 0.05 \mu\text{M}$) showed slightly more resistance. Nonetheless, both strains, were more sensitive to nitrapyrin inhibition compared to *N. europaea*, for which the reported EC₅₀ value is $2.1 \pm 0.4 \mu\text{M}$ according to Papadopoulou et al. (2020), with even higher values observed in other studies (Bélser and Schmidt 1981). DMPP exhibited a statistically lower, but highly comparable

inhibitory activity to nitrapyrin on AOB. Impressive specificity towards AOB has been previously reported (Papadopoulou et al. 2020), and the response of AOA strains such as “*Ca. N. franklandia*” to DMPP (Papadopoulou et al. 2020) and *N. viennensis* to DMP (Beeckman et al. 2024) was notably low. The inhibition threshold determined for *N. multiformis* was consistent with previous reports (Papadopoulou et al. 2020), suggesting that similar inhibition patterns might exist among members of the same phylogenetic group (Papadopoulou et al. 2020) due to physiological similarities. Ethoxyquin, which has previously been shown to exhibit specificity towards AOA (Papadopoulou et al. 2020) again demonstrated limited inhibition of AOB. Only *N. ureae* yielded a decisive EC₅₀ value, although it was, still, much higher than those observed for AOA (Papadopoulou et al. 2020), where “*Ca. N. franklandia*” exhibited significant inhibition at doses in the 1-10 µM range (EC₅₀ 1.4 ± 0.3 µM). In contrast, *N. multiformis* (EC₅₀ > 500 µM) and *N. communis* (EC₅₀ > 600 µM) were marginally inhibited by the concentrations used in this study. However, previous studies have reported EC₅₀ values for *N. multiformis* (214.8 ± 39.6 µM) in a liquid batch culture assay (Papadopoulou et al. 2020). This discrepancy could be due to the lower sensitivity of AOB, or the viscous nature of ethoxyquin, which may have impaired its homogenous dissolution in the limited culture volumes involved in the fast-track assay. Additionally, its high lipophilicity and very low TPSA (Kim et al. 2023) could have limited cell uptake and/or intracellular activity. Ethoxyquin is a known antioxidant (Papadopoulou et al. 2020), and NIs with antioxidant activity, such as PTIO (2- phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide) have been observed before (Martens-Habbena et al. 2015). Antioxidants, as electron donors or acceptors (Lü et al. 2010), could disrupt the electron transfer chain, essential for ammonia oxidation, potentially inhibiting AOM viability. Alternatively, by accepting electrons, they could also act as NO scavengers (Sueishi et al. 2011), potentially inhibiting nitrification by removing this obligatory intermediate from the pathway, as is the case with PTIO (Martens-Habbena et al. 2015). The increased efficacy of ethoxyquin against *N. ureae* could be attributed to deviations in the electron transfer chains or nitrite fluxes of different AOB strains.

MHPP, a commonly studied BNI, has previously displayed selective inhibition, based on the AOM strain rather than the domain (Kaur-Bhambra et al. 2022; Kolovou et al. 2023). An EC₅₀ value of 102.8 ± 6.4 µM was obtained for *N. multiformis* consistent with previous reports by Kolovou et al. (2023) (104 ± 12.8 µM). *N. ureae* was the most sensitive AOB, while *N. communis* showed lower inhibition. MHPP has been shown to inhibit AMO but not HAO (Wang et al. 2021), and structural differences in AMO could explain the differing sensitivities

observed. Sakuranetin presented a variable pattern of inhibition across AOB strains. For *N. multiformis*, an EC₅₀ value of $120.3 \pm 17.4 \mu\text{M}$ was similar to thresholds reported in [Kolovou et al. \(2023\)](#) ($144.3 \pm 19 \mu\text{M}$) and derived from the data of [Kaur-Bhambra et al. \(2022\)](#) ($141.1 \pm 0.6 \mu\text{M}$). *N. ureae*, was again the most sensitive strain, while no clear inhibition threshold was determined for *N. communis*. The inability of sakuranetin to produce a clear dose-response pattern for *N. communis* could be due to its reduced sensitivity and potentially impaired solubility or uptake of the compound. Finally, 1,9-decanediol was the weakest NI tested. Its inhibitory activity on AOB followed a unique pattern, with *N. ureae* being the most sensitive strain, followed by *N. communis* rather than *N. multiformis*, which was the least sensitive. An EC₅₀ of $467.3 \pm 18.8 \mu\text{M}$ was determined for *N. multiformis*, closely matching the value derived from [Kaur-Bhambra et al. \(2022\)](#) ($428.1 \pm 2.47 \mu\text{M}$). Similarly to MHPP, 1,9-decanediol is known to inhibit AMO but not HAO enzyme ([Wang et al. 2021](#)), and structural differences in AMO likely contribute to the observed sensitivity differences.

4.3 Determination of BNI activity of plant root exudates

In this study, a robust screening process involving root exudates from various plant genotypes of two different species is presented, aiming to determine their BNI activity and the patterns of nitrification inhibition observed in relevant members of the soil AOM community, as a proof-of-concept. Distinct response patterns were notable among the AOB reporter strains when comparing the two plant species, and even more notable between different genotypes of the same species. Importantly, the sensitivity of the three AOB strains to the root exudates mirrored their response to the pure compounds tested, with *N. ureae* generally being the most sensitive, *N. multiformis* showing intermediate sensitivity, and *N. communis* exhibiting the lowest inhibition. These variations can be attributed not only to the differing sensitivity of AOM species, as discussed earlier, but also to the significant influence of the plant genetic makeup on the composition of root exudates ([Ma et al. 2022](#)). This has been previously described for several accessions of the model plant *Arabidopsis thaliana* grown under similar conditions ([Micallef et al. 2009](#)), and for different wheat varieties from the same geographical region ([Yahya et al. 2021](#)). Additionally, differences in BNI potential have been reported for various maize ([Petroli et al. 2023](#)) and guinea grass varieties ([Villegas et al. 2020](#)), as well as for root exudates from different rice varieties ([Sun et al. 2016](#)). Furthermore, the geographical origin of plant genotypes is a crucial factor shaping the composition of root exudates. Soil types with varying characteristics can impose significant differences in root exudation patterns ([Williams and de Vries 2019](#)). Different soil conditions often lead to variations in root system

architecture, which are believed to regulate both the quantity and quality of root exudation (Badri and Vivanco 2009). Additionally, environmental stressors constantly present in the native environment of the genotype (Williams and de Vries 2019), along with the diversity of native microbial communities (Canarini et al. 2019), can significantly shape the root exudation mechanisms of the plants. Considering these factors, the observation that certain root exudates specifically inhibit one AOB reporter strain more than another could reflect the specific adaptation of plant genotypes to the abiotic and biotic conditions of their original soil environment. Key soil parameters, such as pH and ammonia availability, are known to drive niche differentiation in AOM communities (Prosser and Nicol 2012), and BNI exudation is considered to be an adaptive mechanism (Wang et al. 2021). Therefore, the differential effects of root exudates from different genotypes on various AOM strains should be viewed as a reflection of plants' adaptation to their natural environment including local microbial diversity (Canarini et al. 2019), soil type and pH (Upadhyay et al. 2022).

4.4 Perspectives on the discovery and applicability of BNIs

In recent years, there has been growing interest in utilizing BNIs to reduce nitrification in soils (Nardi et al. 2020), through both chemical- and plant-based strategies (Wang et al. 2021). Various BNIs have been identified that show inhibitory activity in soils alongside their efficacy *in vitro*. For example, MHPP has been observed to inhibit soil nitrification (Subbarao et al. 2013), including reducing the *amoA* gene copy number (Lan et al. 2022), decreasing both gross (Lan et al. 2022) and net (Rojas-Pinzon et al. 2024) nitrification rates, and affecting the composition of soil AOB communities (He et al. 2023). Similarly, 1,9-decanediol has been reported to inhibit potential nitrification, reduce AOB and AOA abundance and induce shifts in the relative abundance of different AOM phylogenetic groups (Lu et al. 2019). However, contrary to these findings, sakuranetin, and despite its demonstrated *in vitro* inhibitory activity (Subbarao et al. 2013; Kaur-Bhambra et al. 2022; Kolovou et al. 2023), including in the present study, has failed to inhibit soil nitrification when applied at concentrations similar to MHPP (Subbarao et al. 2013). Even for MHPP, its inhibitory effect in soil is significantly lower compared to the well-established SNI DMPP (He et al. 2019; Lan et al. 2022). These results, combined with data from *in vitro* tests, suggest that currently known BNIs, although effective, may be less potent in soil environments than traditional SNIs, possibly requiring higher application rates to achieve meaningful effects. Additionally, differences in BNI efficacy have been observed across different soil types, even when the same concentrations are applied (Rojas-Pinzon et al. 2024). BNI activity is heavily influenced by various soil characteristics

rather than solely by the inherent nitrification inhibitory capacity of the BNI compound itself. Soil pH is recognized as a key factor driving variability in BNI performance, with MHPP showing lower activity in neutral soils, like calcareous ones (He et al. 2019), but demonstrating comparable efficacy to DMPP in acidic soils (Lan et al. 2022). Soil pH affects the fate of NIs in soil in multiple ways. For example, higher pH values can accelerate the mineralization of the SNI DCD (Guardia et al. 2018) while MHPP remains more stable in acidic soils (Lan et al. 2022). Moreover, the charge status of NIs, influenced by soil pH, can affect their sorption to metal oxides, soil minerals, or negatively charged soil organic matter (Marsden et al. 2016), reducing their bioavailability and, consequently, their ability to inhibit nitrification.

On the other hand, BNI-producing plants offer an alternative approach to soil nitrification inhibition. Crop rotation models, such as rotating BNI-producing *Brachiaria humidicola* with maize (Karwat et al. 2017), or intercropping BNI-producing *Sorghum bicolor* with maize (Zhang et al. 2023) have shown promise in enhancing NUE efficiency and crop productivity. However, BNI activity via root exudates, is not constant, but rather influenced by environmental factors. For instance, the hydrophilic BNIs of *Sorghum bicolor* are thought to be sensitive to rhizosphere pH levels above 5.0 (Subbarao et al. 2013), and the release of 1,9-decanediol from rice plants is induced at pH values below 5.0, particularly in the presence of high ammonium concentrations and AOB (Zhang et al. 2019). As previously discussed, significant variation in BNI potential exists not only between plant species but also among plant varieties (Janke et al. 2018). This has been demonstrated for *Brachiaria humidicola* (Egenolf et al. 2021), rice (Sun et al. 2016; Illarze et al. 2021) and maize (Petroli et al. 2023), while varying effects were also observed in different soils (Illarze et al. 2021).

Overall, the reduced and variable efficacy of known BNIs in soil, combined with the variability in the BNI potential across plant species and varieties, highlights the importance of discovering and rigorously assessing the activity of pure BNIs, as well as root exudates from novel plant genotypes. This process can be approached in similarity to pesticide risk assessment for soil microorganisms (Karpouzias et al. 2022). A standardised, validated fast-track assay, such as the one described here, could serve as a conservative Tier I assay for systematically screening plant root exudates, their fractions, and pure compounds for BNI activity. By considering the full diversity of soil AOM communities, this high-throughput approach can help identify the most potent candidates for further testing in Tier II and Tier III assays, which, would involve soil microcosm and field studies (Karpouzias et al. 2016). These studies could provide additional insights into the fate, target and off-target effects of BNIs in more complex, real-world

contexts. Finally, incorporating advanced tools that integrate complex ‘-omics’ data with statistical and mathematical approaches (Weckwerth et al. 2020) could further clarify the intricate interactions between root exudates and the soil environment (Ghatak et al. 2023), enhancing the discovery and characterization of novel BNIs.

5. Conclusions

This study described the development of a fast-track assay for discovering BNI activity in plant exudates and pure compounds. This assay, standardized and validated through testing both established and novel SNIs, as well as BNIs, represents a refined approach compared to previous assays. It incorporates a portfolio of AOB reporter strains, which, in combination with the two AOA reporter strains provided by the collaborators from the University of Vienna, provide greater ecological relevance by including a broader range of phylogenetically diverse reporter strains. The inhibition thresholds generated by this assay align closely with the varying sensitivities of AOB to NIs. Additionally, this is the first study to report the inhibition thresholds of SNIs and BNIs for two key soil-representative AOB species, *Nitrosomonas ureae* and *Nitrosomonas communis*. Furthermore, the described assay has demonstrated strong potential for screening plant root exudates for BNI activity, revealing distinct inhibition patterns that reflect the influence of plant genetic makeup and environmental adaptations on BNI potential. This work lays the foundation for more systematic and higher-throughput discovery of candidate BNI-producing plant genotypes and novel BNI compounds, in a more ecologically relevant context than previous methods. When combined with approaches involving more complex and realistic systems, holds significant potential to advance the discovery of novel BNIs and BNI-producing plants, contributing to the mitigation of critical agricultural and environmental challenges, such as reduced NUE, nitrite pollution and greenhouse gas emissions, that drive climate change. In the future, further refinement of the screening procedure could include the addition of acidophilic AOA reporter strains, NOB strains and synthetic co-cultures of ammonia and nitrite oxidizers. This would provide a more holistic assessment of the BNI activity of candidate compounds and root exudates.

6. References

- Abby, S., S., Melcher, M., Kerou, M., Krupovic, M., Stieglmeier, M., Rossel, C., Pfeifer, K., Schleper, C. (2018). Candidatus Nitrosocaldus cavascurensis, an Ammonia Oxidizing, Extremely Thermophilic Archaeon with a Highly Mobile Genome, *Frontiers in Microbiology*, 9. <https://doi.org/10.3389/fmicb.2018.00028>
- Afzal, M. R., Zhang, M., Jin, H., Wang, G., Zhang, M., Ding, M., ... Zhu, Y. (2020). Post-translational regulation of plasma membrane H⁺-ATPase is involved in the release of biological nitrification inhibitors from sorghum roots. *Plant and Soil*. <https://doi.org/10.1007/s11104-020-04511-6>
- Akinnawo, S.O. (2023). Eutrophication: Causes, consequences, physical, chemical and biological techniques for mitigation strategies, *Environmental Challenges*, 12, <https://doi.org/10.1016/j.envc.2023.100733>
- Alves, R. J. E., Minh, B. Q., Urich, T., von Haeseler, A., & Schleper, C. (2018). Unifying the global phylogeny and environmental distribution of ammonia-oxidising archaea based on amoA genes. *Nature Communications*, 9(1). <https://doi.org/10.1038/s41467-018-03861-1>
- Ammar, E. E., Rady, H. A., Khattab, A. M., Amer, M. H., Mohamed, S. A., Elodamy, N. I., Al-Farga, A., & Aioub, A. A. A. (2023). A comprehensive overview of eco-friendly bio-fertilisers extracted from living organisms. *Environmental science and pollution research international*, 30(53), 113119–113137. <https://doi.org/10.1007/s11356-023-30260-x>
- Ayilara, M. S., Adeleke, B. S., Akinola, S. A., Fayose, C. A., Adeyemi, U. T., Gbadegesin, L. A., Omole, R. K., Johnson, R. M., Uthman, Q. O., & Babalola, O. O. (2023). Biopesticides as a promising alternative to synthetic pesticides: A case for microbial pesticides, phytopesticides, and nanobiopesticides. *Frontiers in microbiology*, 14, 1040901. <https://doi.org/10.3389/fmicb.2023.1040901>
- Bachtsevani, E., Papazlatani, C., Rousidou, C., Lampronikou, E., Menkissoglu-Spiroudi, U., Nicol, G., Karpouzas, D., Evangelia, P. (2022). Effects of the nitrification inhibitor 3,4-dimethylpyrazole phosphate (DMPP) on the activity and diversity of the soil microbial community under contrasting soil pH. *Biology and Fertility of Soils*, 57. <https://doi.org/10.1007/s00374-021-01602-z>
- Badri, V., Vivanco, J. M. (2009). Regulation and function of root exudates. *Plant, Cell & Environment*, 32(6), 666–681. <https://doi.org/10.1111/j.1365-3040.2009.01926.x>
- Balcombe, P., Speirs, J. F., Brandon, N. P., & Hawkes, A. D. (2018). Methane emissions: choosing the right climate metric and time horizon. *Environmental science. Processes & impacts*, 20(10), 1323–1339. <https://doi.org/10.1039/c8em00414e>

- Barros-Rodríguez, A., Rangseekaew, P., Lasudee, K., Pathom-Aree, W., & Manzanera, M. (2021). Impacts of Agriculture on the Environment and Soil Microbial Biodiversity. *Plants (Basel, Switzerland)*, 10(11), 2325. <https://doi.org/10.3390/plants10112325>
- Beaulieu, J. J., Del Sontro, T., & Downing, J. A. (2019). Eutrophication will increase methane emissions from lakes and impoundments during the 21st century. *Nature communications*, 10(1), 1375. <https://doi.org/10.1038/s41467-019-09100-5>
- Beeckman, F., Drozdzecki, A., De Knijf, A., Corrochano-Monsalve, M., Bodé, S., Blom, P., Goeminne, G., González-Murua, C., Lückner, S., Boeckx, P., Stevens, C. V., Audenaert, D., Beeckman, T., & Motte, H. (2023). Drug discovery-based approach identifies new nitrification inhibitors. *Journal of environmental management*, 346, 118996. <https://doi.org/10.1016/j.jenvman.2023.118996>
- Beeckman, F., Drozdzecki, A., De Knijf, A., Audenaert, D., Beeckman, T., & Motte, H. (2024). High-throughput assays to identify archaea-targeting nitrification inhibitors. *Frontiers in plant science*, 14, 1283047. <https://doi.org/10.3389/fpls.2023.1283047>
- Belser, L. W., & Schmidt, E. L. (1981). Inhibitory effect of nitrapyrin on three genera of ammonia-oxidizing nitrifiers. *Applied and environmental microbiology*, 41(3), 819–821. <https://doi.org/10.1128/aem.41.3.819-821.1981>
- Bernhard, A. (2010). The Nitrogen Cycle: Processes, Players, and Human Impact. *Nature Education Knowledge* 3(10):25. Retrieved from: <https://www.nature.com/scitable/knowledge/library/the-nitrogen-cycle-processes-players-and-human-15644632/>
- Bock, E., Sundermeyer-Klinger, H., Stackebrandt, E. (1983). New facultative lithoautotrophic nitrite-oxidizing bacteria. *Arch Microbiol* 136:281–284. <https://doi.org/10.1007/BF00425217>
- Bonsdorff, E. (2021). Eutrophication: Early warning signals, ecosystem-level and societal responses, and ways forward: This article belongs to Ambio's 50th Anniversary Collection. Theme: Eutrophication. *Ambio*, 50(4), 753–758. <https://doi.org/10.1007/s13280-020-01432-7>
- Bottery, M. J., Pitchford, J. W., & Friman, V.-P. (2020). Ecology and evolution of antimicrobial resistance in bacterial communities. *The ISME Journal*, 15(4), 939–948. <https://doi.org/10.1038/s41396-020-00832-7>
- Brook, I. (1989). Inoculum Effect. *Clinical Infectious Diseases*, 11(3), 361–368. <https://doi.org/10.1093/clinids/11.3.361>
- Bruhwiller, L., Basu, S., Butler, J. H., Chatterjee, A., Dlugokencky, E., Kenney, M. A., McComiskey, A., Montzka, S. A., & Stanitski, D. (2021). Observations of greenhouse gases as climate indicators. *Climatic change*, 165(1), 12. <https://doi.org/10.1007/s10584-021-03001-7>

- Caglar, M. U., Teufel, A. I., & Wilke, C. O. (2018). Sicegar: R package for sigmoidal and double-sigmoidal curve fitting. *PeerJ*, 6, e4251. <https://doi.org/10.7717/peerj.4251>
- Canarini, A., Kaiser, C., Merchant, A., Richter, A., & Wanek, W. (2019). Root Exudation of Primary Metabolites: Mechanisms and Their Roles in Plant Responses to Environmental Stimuli. *Frontiers in Plant Science*, 10. <https://doi.org/10.3389/fpls.2019.00157>
- Cantarella, H., Otto, R., Soares, J. R., & Silva, A. G. B. (2018). Agronomic efficiency of NBPT as a urease inhibitor: A review. *Journal of advanced research*, 13, 19–27. <https://doi.org/10.1016/j.jare.2018.05.008>
- Capdevila-Cortada, M. (2019). Electrifying the Haber–Bosch. *Nat Catal* 2, 1055. <https://doi.org/10.1038/s41929-019-0414-4>
- Cassman, N. A., Soares, J. R., Pijl, A., Lourenço, K. S., van Veen, J. A., Cantarella, H., & Kuramae, E. E. (2019). Nitrification inhibitors effectively target N₂ O-producing Nitrosospira spp. in tropical soil. *Environmental microbiology*, 21(4), 1241–1254. <https://doi.org/10.1111/1462-2920.14557>
- Castiglione, A. M., Mannino, G., Contartese, V., Berteà, C. M., & Ertani, A. (2021). Microbial Biostimulants as Response to Modern Agriculture Needs: Composition, Role and Application of These Innovative Products. *Plants (Basel, Switzerland)*, 10(8), 1533. <https://doi.org/10.3390/plants10081533>
- Chang, J., Peng, P., DiSpirito, A. A., & Semrau, J. D. (2022). Variable Inhibition of Nitrous Oxide Reduction in Denitrifying Bacteria by Different Forms of Methanobactin. *Applied and environmental microbiology*, 88(7), e0234621. <https://doi.org/10.1128/aem.02346-21>
- Chislock, M. F., Doster, E., Zitomer, R. A. & Wilson, A. E. (2013) Eutrophication: Causes, Consequences, and Controls in Aquatic Ecosystems. *Nature Education Knowledge* 4(4):10. <https://www.nature.com/scitable/knowledge/library/eutrophication-causes-consequences-and-controls-in-aquatic-102364466/>
- Costa, E., Pérez, J., & Kreft, J.-U. (2006). Why is metabolic labour divided in nitrification? *Trends in Microbiology*, 14(5), 213–219. <https://doi.org/10.1016/j.tim.2006.03.006>
- Corrochano-Monsalve, M., González-Murua, C., Bozal-Leorri, A., Lezama, L., & Artetxe, B. (2020). Mechanism of action of nitrification inhibitors based on dimethylpyrazole: A matter of chelation. *Science of The Total Environment*, 141885. <https://doi.org/10.1016/j.scitotenv.2020.141885>
- Corrochano-Monsalve, M., González-Murua, C., Estavillo, J. M., Estonba, A., & Zarraonaindia, I. (2021). Impact of dimethylpyrazole-based nitrification inhibitors on soil-borne bacteria. *The Science of the total environment*, 792, 148374. <https://doi.org/10.1016/j.scitotenv.2021.148374>

Coskun, D., Britto, D. T., Shi, W., & Kronzucker, H. J. (2017). Nitrogen transformations in modern agriculture and the role of biological nitrification inhibition. *Nature Plants*, 3(6), 17074. <https://doi.org/10.1038/nplants.2017.74>

Cui, J., Zhang, M., Chen, L., Zhang, S., Luo, Y., Cao, W., Zhao, J., Wang, L., Jia, Z., & Bao, Z. (2022). Methanotrophs Contribute to Nitrogen Fixation in Emergent Macrophytes. *Frontiers in microbiology*, 13, 851424. <https://doi.org/10.3389/fmicb.2022.851424>

Daims, H., Lebedeva, E., Pjevac, P. et al. (2015). Complete nitrification by *Nitrospira* bacteria. *Nature* 528, 504–509. <https://doi.org/10.1038/nature16461>

Daims, H., Lückner, S., & Wagner, M. (2016). A New Perspective on Microbes Formerly Known as Nitrite-Oxidizing Bacteria. *Trends in microbiology*, 24(9), 699–712. <https://doi.org/10.1016/j.tim.2016.05.004>

da Fonseca, A. B., Santos, C., Nunes, A. P. P., Oliveira, D. P., de Melo, M. E. A., Takayama, T., Mansur, B. L., de Jesus Fernandes, T., do Carmo Alexandrino, G., Dias, M. A. N., & Guelfi, D. (2023). Urease inhibitors technologies as strategy to mitigate agricultural ammonia emissions and enhance the use efficiency of urea-based fertilizers. *Scientific reports*, 13(1), 22739. <https://doi.org/10.1038/s41598-023-50061-z>

De Boer, W., Gunnewiek, P. J., Veenhuis, M., Bock, E., & Laanbroek, H. J. (1991). Nitrification at Low pH by Aggregated Chemolithotrophic Bacteria. *Applied and environmental microbiology*, 57(12), 3600–3604. <https://doi.org/10.1128/aem.57.12.3600-3604.1991>

de Mendiburu F (2023). *_agricolae: Statistical Procedures for Agricultural Research_*. R package version 1.3-7, <https://CRAN.R-project.org/package=agricolae>

Dos Santos, P.C., Fang, Z., Mason, S.W. *et al.* (2012). Distribution of nitrogen fixation and nitrogenase-like sequences amongst microbial genomes. *BMC Genomics* 13, 162. <https://doi.org/10.1186/1471-2164-13-162>

Egenolf, K., Schad, P., Arévalo, A., Villegas, D., Arango, J., Hannes Karwat, Georg Cadisch, & Rasche, F. (2021). Inter-microbial competition for N and plant NO₃[–] uptake rather than BNI determines soil net nitrification under intensively managed *Brachiaria humidicola*. *Biology and Fertility of Soils*, 58(3), 307–319. <https://doi.org/10.1007/s00374-021-01606-9>

European Commission. 2030 Climate Targets. Climate Action. https://climate.ec.europa.eu/eu-action/climate-strategies-targets/2030-climate-targets_en

EEA. European Environment Agency. (2023). Nitrate in Groundwater: 8th Environment Action Programme. European Environment Agency. Retrieved from: <https://www.eea.europa.eu/en/analysis/indicators/nitrate-in-groundwater-8th-eap>

FAO. Food and Agriculture Organization of the United Nations. (2009). Hunger. <http://www.fao.org/hunger/en/>

FAOSTAT. Food and Agriculture Organization of the United Nations. (2020). FAOSTAT statistical database. <https://www.fao.org/faostat/en/>

FAOSTAT. Food and Agriculture Organization of the United Nations. (2022). FAOSTAT statistical database. <https://www.fao.org/faostat/en/#data/QCL>

FAOSTAT. Food and Agriculture Organization of the United Nations. (2023a). FAOSTAT statistical database <http://www.fao.org/faostat/en/#data/FS>

FAOSTAT. Food and Agriculture Organization of the United Nations. (2023b). FAOSTAT statistical database. <https://www.fao.org/faostat/en/#data/ESB/visualize>

Fenibo, E. O., Ijoma, G. N., Matambo, T. (2021). Biopesticides in Sustainable Agriculture: A Critical Sustainable Development Driver Governed by Green Chemistry Principles. *Front. Sustain. Food Syst.*, 5. <https://doi.org/10.3389/fsufs.2021.619058>

Fowler, D., Coyle, M., Skiba, U., Sutton, M. A., Cape, J. N., Reis, S., Sheppard, L. J., Jenkins, A., Grizzetti, B., Galloway, J. N., Vitousek, P., Leach, A., Bouwman, A. F., Butterbach-Bahl, K., Dentener, F., Stevenson, D., Amann, M., & Voss, M. (2013). The global nitrogen cycle in the twenty-first century. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, 368(1621), 20130164. <https://doi.org/10.1098/rstb.2013.0164>

Fox, J., Weisberg, S. (2019). An R Companion to Applied Regression, Third edition. Sage, Thousand Oaks CA. <https://socialsciences.mcmaster.ca/jfox/Books/Companion/>

Francis, C. A., Roberts, K. J., Beman, J. M., Santoro, A. E., and Oakley, B. B. (2005). Ubiquity and diversity of ammonia-oxidizing archaea in water column sand sediments of the ocean. *Proc. Natl. Acad. Sci. U.S.A.* 102, 14683–14688. <https://doi.org/10.1073/pnas.0506625102>

Frankland, P.F., Frankland G.C. (1890). The nitrifying process and its specific ferment. Part I. *Philos Trans Roy Soc B* 181:107–28.

Galili T. (2015). dendextend: an R package for visualizing, adjusting and comparing trees of hierarchical clustering. *Bioinformatics (Oxford, England)*, 31(22), 3718–3720. <https://doi.org/10.1093/bioinformatics/btv428>

Ghafoor, I., Rahman, M. H. U., Hasnain, M. U., Ikram, R. M., Khan, M. A., Iqbal, R., Hussain, M. I., & Sabagh, A. E. (2022). Effect of slow-release nitrogenous fertilizers on dry matter

accumulation, grain nutritional quality, water productivity and wheat yield under an arid environment. *Scientific reports*, 12(1), 14783. <https://doi.org/10.1038/s41598-022-18867-5>

Ghatak, A., Chaturvedi, P., Waldherr, S., Subbarao, G. V., & Weckwerth, W. (2023). PANOMICS at the interface of root–soil microbiome and BNI. *Trends in Plant Science*, 28(1), 106–122. <https://doi.org/10.1016/j.tplants.2022.08.016>

Ghimire-Kafle, S., Weaver, M. E., Jr, & Bollmann, A. (2023). Ecophysiological and Genomic Characterization of the Freshwater Complete Ammonia Oxidizer *Nitrospira* sp. Strain BO4. *Applied and environmental microbiology*, 89(2), e0196522. <https://doi.org/10.1128/aem.01965-22>

Goring, C. A. I. (1962). Control of nitrification by 2-chloro-6-(trichloromethyl) pyridine, *Soil Sci.*, 93, pp. 211-218. <https://doi.org/10.1097/00010694-196203000-00010>

Greenwell, B. M., Kabban, C. M. S. (2014). “**Investr**: An R Package for Inverse Estimation.” *The R Journal*, 6(1), 90–100. <https://doi.org/10.32614/RJ-2014-009>

Gu, Z., Eils, R., & Schlesner, M. (2016). Complex heatmaps reveal patterns and correlations in multidimensional genomic data. *Bioinformatics (Oxford, England)*, 32(18), 2847–2849. <https://doi.org/10.1093/bioinformatics/btw313>

Guardia, G., Marsden, K. A., Vallejo, A., Jones, D. L., & Chadwick, D. R. (2018). Determining the influence of environmental and edaphic factors on the fate of the nitrification inhibitors DCD and DMPP in soil. *Science of the Total Environment*, 624, 1202–1212. <https://doi.org/10.1016/j.scitotenv.2017.12.250>

Harris, E., Diaz-Pines, E., Stoll, E., Schlöter, M., Schulz, S., Duffner, C., Li, K., Moore, K. L., Ingrisch, J., Reinthaler, D., Zechmeister-Boltenstern, S., Glatzel, S., Brüggemann, N., & Bahn, M. (2021). Denitrifying pathways dominate nitrous oxide emissions from managed grassland during drought and rewetting. *Science advances*, 7(6), eabb7118. <https://doi.org/10.1126/sciadv.abb7118>

Hautier, Y., Zhang, P., Loreau, M. *et al.* (2020). General destabilizing effects of eutrophication on grassland productivity at multiple spatial scales. *Nat Commun* 11, 5375. <https://doi.org/10.1038/s41467-020-19252-4>

Hayatsu, M., Tago, K., Uchiyama, I. *et al.* (2017). An acid-tolerant ammonia-oxidizing γ -proteobacterium from soil. *ISME J* 11, 1130–1141. <https://doi.org/10.1038/ismej.2016.191>

He, X., Li, M., Zhou, M., Zhou, M., Chen, G., Zhang, Y., Li, Y., Zeng, M., Gao, X., Lan, T. (2023). Gross nitrogen transformations and ammonia oxidizers affected by nitrification inhibitors and/or organic amendments in a calcareous soil: A ^{15}N tracing study, *Applied Soil Ecology*, vol 188, <https://doi.org/10.1016/j.apsoil.2023.104926>

Hink, L., Gubry-Rangin, C., Nicol, G.W. *et al.* (2018). The consequences of niche and physiological differentiation of archaeal and bacterial ammonia oxidisers for nitrous oxide emissions. *ISME J* 12, 1084–1093. <https://doi.org/10.1038/s41396-017-0025-5>

Hink, L., Bachtsevani, E., Meng, Y., Sedlacek, C. J., Lee, S., Daims, H., Wagner, M., Gubry-Rangin, C., de Boer, W., Hazard, C., Prosser, J. I., Nicol, G. W. (2024). Acidotolerant soil nitrite oxidiser ‘*Candidatus Nitrobacter laanbroekii*’ NHB1 alleviates constraints on growth of acidophilic soil ammonia oxidisers. *bioRxiv*, <https://doi.org/10.1101/2024.07.06.601931>

Hothorn, T., Bretz, F., Westfall, P. (2008). Simultaneous Inference in General Parametric Models. *Biometrical Journal*, 50 (3), 346-363.

Hu, L., Dong, Z., Wang, Z., Xiao, L., & Zhu, B. (2022). The contributions of ammonia oxidizing bacteria and archaea to nitrification-dependent N₂O emission in alkaline and neutral purple soils. *Scientific reports*, 12(1), 19928. <https://doi.org/10.1038/s41598-022-23084-1>

Huang, T., Ju, X. & Yang, H. (2017). Nitrate leaching in a winter wheat-summer maize rotation on a calcareous soil as affected by nitrogen and straw management. *Sci Rep* 7, 42247. <https://doi.org/10.1038/srep42247>

Illarze, G., Arango, J., Nuñez, J., & Irisarri, P. (2021). Biological nitrification inhibition by rice root exudates in two different soils of Uruguay. *Acta Agriculturae Scandinavica, Section B — Soil & Plant Science*, 1–11. <https://doi.org/10.1080/09064710.2021.1948602>

Iizumi, T., Mizumoto, M., & Nakamura, K. (1998). A bioluminescence assay using *Nitrosomonas europaea* for rapid and sensitive detection of nitrification inhibitors. *Applied and environmental microbiology*, 64(10), 3656–3662. <https://doi.org/10.1128/AEM.64.10.3656-3662.1998>

IndustryARC. (2021). Nitrification and Urease Inhibitors Market. IndustryARC. <https://www.industryarc.com/Report/18613/nitrification-and-urease-inhibitors-market.html>

Janke, C. K., Wendling, L. A., & Fujinuma, R. (2018). Biological nitrification inhibition by root exudates of native species, *Hibiscus splendens* and *Solanum echinatum*. *PeerJ*, 6, e4960. <https://doi.org/10.7717/peerj.4960>

Jessen, C. (2013). Effects of Simulated Eutrophication and Overfishing on Coral Reef Invertebrates, Algae and Microbes in the Red Sea, *PhD Thesis*, Leibniz Centre for Tropical Marine Research (ZMT). https://www.researchgate.net/publication/279400700_Effects_of_Simulated_Eutrophication_and_Overfishing_on_Coral_Reef_Invertebrates_Algae_and_Microbes_in_the_Red_Sea

Kafle, A., Garcia, K., Wang, X., Pfeffer, P. E., Strahan, G. D., & Bücking, H. (2019). Nutrient demand and fungal access to resources control the carbon allocation to the symbiotic partners

in tripartite interactions of *Medicago truncatula*. *Plant, cell & environment*, 42(1), 270–284. <https://doi.org/10.1111/pce.13359>

Karpouzas, D. G., Tsiamis, G., Trevisan, M., Ferrari, F., Malandain, C., Sibourg, O., & Martin-Laurent, F. (2016). “LOVE TO HATE” pesticides: felicity or curse for the soil microbial community? An FP7 IAPP Marie Curie project aiming to establish tools for the assessment of the mechanisms controlling the interactions of pesticides with soil microorganisms. *Environmental Science and Pollution Research*, 23(18), 18947–18951. <https://doi.org/10.1007/s11356-016-7319-4>

Karpouzas, D., Vryzas, Z. & Martin-Laurent, F. (2022). Pesticide soil microbial toxicity: setting the scene for a new pesticide risk assessment for soil microorganisms (IUPAC Technical Report). *Pure and Applied Chemistry*, 94(10), 1161-1194. <https://doi.org/10.1515/pac-2022-0201>

Karwat, H., Moreta, D., Arango, J., Núñez, J., Rao, I., Rincón, Á., Rasche, F., & Cadisch, G. (2017). Residual effect of BNI by *Brachiaria humidicola* pasture on nitrogen recovery and grain yield of subsequent maize. *Plant and Soil*, 420(1-2), 389–406. <https://doi.org/10.1007/s11104-017-3381-z>

Kassambara, A., Mundt, F. (2020). *_factoextra: Extract and Visualize the Results of Multivariate Data Analyses_*. R package version 1.0.7. <https://CRAN.R-project.org/package=factoextra>

Kassambara, A. (2023a). *ggpubr: 'ggplot2' Based Publication Ready Plots_*. R package version 0.6.0, <https://CRAN.R-project.org/package=ggpubr>

Kassambara, A. (2023b). *rstatix: Pipe-Friendly Framework for Basic Statistical Tests*. R package version 0.7.2

Kaur-Bhambra, J., Wardak, D. L. R., Prosser, J. I., & Gubry-Rangin, C. (2022). Revisiting plant biological nitrification inhibition efficiency using multiple archaeal and bacterial ammonia-oxidising cultures. *Biology and Fertility of Soils*, 58(3), 241–249. <https://doi.org/10.1007/s00374-020-01533-1>

Kerou, M., Offre, P., Valledor, L., Abby, S. S., Melcher, M., Nagler, M., Weckwerth, W., & Schleper, C. (2016). Proteomics and comparative genomics of *Nitrososphaera viennensis* reveal the core genome and adaptations of archaeal ammonia oxidizers. *Proceedings of the National Academy of Sciences of the United States of America*, 113(49), E7937–E7946. <https://doi.org/10.1073/pnas.1601212113>

- Kim, S., Chen, J., Cheng, T., Gindulyte, A., He, J., He, S., Li, Q., Shoemaker, B. A., Thiessen, P. A., Yu, B., Zaslavsky, L., Zhang, J., & Bolton, E. E. (2023). PubChem 2023 update. *Nucleic Acids Res.*, 51(D1), D1373–D1380. <https://doi.org/10.1093/nar/gkac956>
- Knowles R. (1982). Denitrification. *Microbiological reviews*, 46(1), 43–70. <https://doi.org/10.1128/mr.46.1.43-70.1982>
- Kolovou, M., Panagiotou, D., Süße, L., Loiseleur, O., Williams, S., Karpouzas, D.G., Papadopoulou, E. (2023). Assessing the activity of different plant-derived molecules and potential biological nitrification inhibitors on a range of soil ammonia- and nitrite- oxidizing strains. *Appl Environ Microbiol* 89: e01380-23. <https://doi.org/10.1128/aem.01380-23>
- Kong, X., Duan, Y., Schramm, A., Eriksen, J., & Petersen, S. O. (2016). 3,4-Dimethylpyrazole phosphate (DMPP) reduces activity of ammonia oxidizers without adverse effects on non-target soil microorganisms and functions. *Applied Soil Ecology*, 105, 67–75. <https://doi.org/10.1016/j.apsoil.2016.03.018>
- Könneke, M., Bernhard, A. E., de la Torre, J. R., Walker, C. B., Waterbury, J. B., & Stahl, D. A. (2005). Isolation of an autotrophic ammonia-oxidizing marine archaeon. *Nature*, 437(7058), 543–546. <https://doi.org/10.1038/nature03911>
- Kop, L. F. M., Koch, H., Jetten, M. S. M., Daims, H., & Lüscher, S. (2024). Metabolic and phylogenetic diversity in the phylum *Nitrospinota* revealed by comparative genome analyses. *ISME communications*, 4(1), ycad017. <https://doi.org/10.1093/ismeco/ycad017>
- Kozlowski, J. A., Kits, K. D., & Stein, L. Y. (2016a). Complete Genome Sequence of Nitrosomonas ureae Strain Nm10, an Oligotrophic Group 6a Nitrosomonad. *Genome announcements*, 4(2), e00094-16. <https://doi.org/10.1128/genomeA.00094-16>
- Kozlowski, J. A., Kits, K. D., & Stein, L. Y. (2016b). Genome Sequence of Nitrosomonas communis Strain Nm2, a Mesophilic Ammonia-Oxidizing Bacterium Isolated from Mediterranean Soil. *Genome announcements*, 4(1), e01541-15. <https://doi.org/10.1128/genomeA.01541-15>
- Kremen C. (2020). Ecological intensification and diversification approach to maintain biodiversity, ecosystem services and food production in a changing world. *Emerging topics in life sciences*, 4(2), 229–240. <https://doi.org/10.1042/ETLS20190205>
- Krysenko S. (2023). Impact of Nitrogen-Containing Compounds on Secondary Metabolism in *Streptomyces* spp.—A Source of Metabolic Engineering Strategies. *SynBio* 1(3):204-225. <https://doi.org/10.3390/synbio1030015>

- Kumar, S., Diksha, Sindhu, S. S., & Kumar, R. (2021). Biofertilizers: An ecofriendly technology for nutrient recycling and environmental sustainability. *Current research in microbial sciences*, 3, 100094. <https://doi.org/10.1016/j.crmicr.2021.100094>
- Kuypers, M., Marchant, H., Kartal, B. (2018). The microbial nitrogen-cycling network. *Nat Rev Microbiol* 16, 263–276. <https://doi.org/10.1038/nrmicro.2018.9>
- Lan, T., Li, M., He, X. *et al.* (2022). Effects of synthetic nitrification inhibitor (3,4-dimethylpyrazole phosphate; DMPP) and biological nitrification inhibitor (methyl 3-(4-hydroxyphenyl) propionate; MHPP) on the gross N nitrification rate and ammonia oxidizers in two contrasting soils. *Biol Fertil Soils* 58, 333–344. <https://doi.org/10.1007/s00374-022-01628-x>
- Lassaletta, L., Billen, G., Grizzetti, B., Anglade, J., & Garnier, J. (2014). 50-year trends in nitrogen use efficiency of world cropping systems: the relationship between yield and nitrogen input to cropland. *Environmental Research Letters*, 9(10), 105011. <https://doi.org/10.1088/1748-9326/9/10/105011>
- Lawrence, N. C., Tenesaca, C. G., VanLoocke, A., & Hall, S. J. (2021). Nitrous oxide emissions from agricultural soils challenge climate sustainability in the US Corn Belt. *Proceedings of the National Academy of Sciences of the United States of America*, 118(46), e2112108118. <https://doi.org/10.1073/pnas.2112108118>
- Lawrencja, D., Wong, S. K., Low, D. Y. S., Goh, B. H., Goh, J. K., Ruktanonchai, U. R., Soottitantawat, A., Lee, L. H., & Tang, S. Y. (2021). Controlled Release Fertilizers: A Review on Coating Materials and Mechanism of Release. *Plants (Basel, Switzerland)*, 10(2), 238. <https://doi.org/10.3390/plants10020238>
- Lehtovirta-Morley, L. E., Stoecker, K., Vilcinskis, A., Prosser, J. I., & Nicol, G. W. (2011). Cultivation of an obligate acidophilic ammonia oxidizer from a nitrifying acid soil. *Proceedings of the National Academy of Sciences*, 108(38), 15892–15897. <https://doi.org/10.1073/pnas.1107196108>
- Lehtovirta-Morley, L. E., Ge, C., Ross, J., Yao, H., Nicol, G. W., & Prosser, J. I. (2014). Characterisation of terrestrial acidophilic archaeal ammonia oxidisers and their inhibition and stimulation by organic compounds. *FEMS microbiology ecology*, 89(3), 542–552. <https://doi.org/10.1111/1574-6941.12353>
- Lehtovirta-Morley, L. E., Ross, J., Hink, L., Weber, E. B., Gubry-Rangin, C., Thion, C., Prosser, J. I., & Nicol, G. W. (2016). Isolation of 'Candidatus Nitrosocosmicus franklandus', a novel ureolytic soil archaeal ammonia oxidiser with tolerance to high ammonia concentration. *FEMS microbiology ecology*, 92(5), fiw057. <https://doi.org/10.1093/femsec/fiw057>

Lehtovirta-Morley, L.E. (2018). Ammonia oxidation: Ecology, physiology, biochemistry and why they must all come together, *FEMS Microbiology Letters*, Volume 365, Issue 9, fny058, <https://doi.org/10.1093/femsle/fny058>

Lei, J., Fan, Q., Yu, J., Ma, Y., Yin, J. and Liu, R. (2022). A meta-analysis to examine whether nitrification inhibitors work through selectively inhibiting ammonia-oxidizing bacteria. *Front. Microbiol.* 13:962146. <https://doi.org/10.3389/fmicb.2022.962146>

Lenth, R. (2024). emmeans: Estimated Marginal Means, aka Least-Squares Means. R package version 1.10.1. <https://CRAN.R-project.org/package=emmeans>

Lewe, N., Hermans, S., Lear, G., Kelly, L. T., Thomson-Laing, G., Weisbrod, B., ... Deslippe, J. R. (2021). Phospholipid fatty acid (PLFA) analysis as a tool to estimate absolute abundances from compositional 16S rRNA bacterial metabarcoding data. *Journal of Microbiological Methods*, 188, 106271. <https://doi.org/10.1016/j.mimet.2021.106271>

Li, C., He, Z.-Y., Hu, H.-W., He, J.-Z., et al. (2023). Niche specialization of comammox *Nitrospira* in terrestrial ecosystems: Oligotrophic or copiotrophic? *Crit. Rev. Environ.* 53, 161–176. <https://doi.org/10.1080/10643389.2022.2049578>.

Li, J., Jin, Q., Liang, Y., Geng, J., Xia, J., Chen, H., & Yun, M. (2022). Highly Efficient Removal of Nitrate and Phosphate to Control Eutrophication by the Dielectrophoresis-Assisted Adsorption Method. *International journal of environmental research and public health*, 19(3), 1890. <https://doi.org/10.3390/ijerph19031890>

Lin, L., St Clair, S., Gamble, G.D. *et al* (2023). Nitrate contamination in drinking water and adverse reproductive and birth outcomes: a systematic review and meta-analysis. *Sci Rep* 13, 563. <https://doi.org/10.1038/s41598-022-27345-x>

Lin, Y., Ye, G., Luo, J., Di, H. J., Liu, D., Fan, J., & Ding, W. (2018). Nitrospira Cluster 8a Plays a Predominant Role in the Nitrification Process of a Subtropical Ultisol under Long-Term Inorganic and Organic Fertilization. *Applied and environmental microbiology*, 84(18), e01031-18. <https://doi.org/10.1128/AEM.01031-18>

Lin, Y., Hu, H.-W., Ye, G., Fan, J., Ding, W., He, Z.-Y., ... He, J.-Z. (2021). Ammonia-oxidizing bacteria play an important role in nitrification of acidic soils: A meta-analysis. *Geoderma*, 404, 115395. <https://doi.org/10.1016/j.geoderma.2021.115395>

Lu, L., Han, W., Zhang, J. *et al.* (2012). Nitrification of archaeal ammonia oxidizers in acid soils is supported by hydrolysis of urea. *ISME J* 6, 1978–1984. <https://doi.org/10.1038/ismej.2012.45>

Lu, Y., Zhang, X., Jiang, J., Kronzucker, H. J., Shen, W., & Shi, W. (2018). Effects of the biological nitrification inhibitor 1,9-decanediol on nitrification and ammonia oxidizers in three

Lu, Y., Zhang, X., Ma, M., Zu, W., Kronzucker, H. J., Shi, W. (2022). Syringic acid from rice as a biological nitrification and urease inhibitor and its synergism with 1,9-decanediol. *Biol Fertil Soils* 58:277–289. <https://doi.org/10.1007/s00374-021-01584-y>

Lü, J. M., Lin, P. H., Yao, Q., & Chen, C. (2010). Chemical and molecular mechanisms of antioxidants: experimental approaches and model systems. *Journal of cellular and molecular medicine*, 14(4), 840–860. <https://doi.org/10.1111/j.1582-4934.2009.00897.x>

Ma, W., Tang, S., Dengzeng, Z., Zhang, D., Zhang, T., Ma, X. (2022). Root exudates contribute to belowground ecosystem hotspots: A review, *Frontiers in Microbiology*, vol 13, <https://doi.org/10.3389/fmicb.2022.937940>

Mahmud, K., Makaju, S., Ibrahim, R., & Missaoui, A. (2020). Current Progress in Nitrogen Fixing Plants and Microbiome Research. *Plants (Basel, Switzerland)*, 9(1), 97. <https://doi.org/10.3390/plants9010097>

Marsden, K. A., Marín-Martínez, A. J., Vallejo, A., Hill, P. W., Jones, D. L., & Chadwick, D. R. (2016). The mobility of nitrification inhibitors under simulated ruminant urine deposition and rainfall: a comparison between DCD and DMPP. *Biology and Fertility of Soils*, 52(4), 491–503. <https://doi.org/10.1007/s00374-016-1092-x>

Martens-Habbena, W., Qin, W., Horak, R. E. A., Urakawa, H., Schauer, A. J., Moffett, J. W., ... Stahl, D. A. (2015). The production of nitric oxide by marine ammonia-oxidizing archaea and inhibition of archaeal ammonia oxidation by a nitric oxide scavenger. *Environmental Microbiology*, <https://doi.org/10.1111/1462-2920.12677>

Massey, M.S., Davis, J.G. (2023). Beyond Soil Inoculation: Cyanobacteria as a Fertiliser Replacement. *Nitrogen*, 4, 253-262. <https://doi.org/10.3390/nitrogen4030018>

Mateo-Marín, N., Quílez, D., Guillén, M., & Isla, R. (2020). Feasibility of stabilised nitrogen fertilisers decreasing greenhouse gas emissions under optimal management in sprinkler irrigated conditions. *Agriculture, Ecosystems & Environment*, 290, 106725. <https://doi.org/10.1016/j.agee.2019.106725>

Mefferd, C. C., Zhou, E., Seymour, C. O., Bernardo, N. A., Srivastava, S., Bengtson, A. J., Jiao, J. Y., Dong, H., Li, W. J., & Hedlund, B. P. (2022). Incomplete denitrification phenotypes in diverse *Thermus* species from diverse geothermal spring sediments and adjacent soils in southwest China. *Extremophiles: life under extreme conditions*, 26(2), 23. <https://doi.org/10.1007/s00792-022-01272-1>

Melillo, J. M. (2021). Disruption of the global nitrogen cycle: A grand challenge for the twenty-first century. *Ambio*, 50(4), 759–763. <https://doi.org/10.1007/s13280-020-01429-2>

Mercurio, P., Eaglesham, G., Parks, S., Kenway, M., Beltran, V., Flores, F., Mueller, J. F., & Negri, A. P. (2018). Contribution of transformation products towards the total herbicide toxicity to tropical marine organisms. *Scientific reports*, 8(1), 4808. <https://doi.org/10.1038/s41598-018-23153-4>

Micallef, S. A., Shiari, M. P., & Colón-Carmona, A. (2009). Influence of *Arabidopsis thaliana* accessions on rhizobacterial communities and natural variation in root exudates. *Journal of experimental botany*, 60(6), 1729–1742. <https://doi.org/10.1093/jxb/erp053>

Moeller, F.U., Webster, N.S., Herbold, C.W., Behnam, F., Domman, D., Albertsen, M., Mooshammer, M., Markert, S., Turaev, D., Becher, D., Rattei, T., Schweder, T., Richter, A., Watzka, M., Nielsen, P.H. and Wagner, M. (2019), Characterization of a thaumarchaeal symbiont that drives incomplete nitrification in the tropical sponge *Ianthella basta*. *Environ Microbiol*, 21: 3831-3854. <https://doi.org/10.1111/1462-2920.14732>

Montzka, S., Dlugokencky, E. & Butler, J. (2011). Non-CO₂ greenhouse gases and climate change. *Nature* 476, 43–50. <https://doi.org/10.1038/nature10322>

Nardi, P., Laanbroek, H. J., Nicol, G. W., Renella, G., Cardinale, M., Pietramellara, G., Weckwerth, W., Trinchera, A., Ghatak, A., & Nannipieri, P. (2020). Biological nitrification inhibition in the rhizosphere: determining interactions and impact on microbially mediated processes and potential applications. *FEMS microbiology reviews*, 44(6), 874–908. <https://doi.org/10.1093/femsre/fuaa037>

Nature Synthesis Editorial (2023). Green ammonia synthesis. *Nat. Synth* 2, 581–582. <https://doi.org/10.1038/s44160-023-00362-y>

Norton, J. M., Klotz, M. G., Stein, L. Y., Arp, D. J., Bottomley, P. J., Chain, P. S., Hauser, L. J., Land, M. L., Larimer, F. W., Shin, M. W., & Starkenburg, S. R. (2008). Complete genome sequence of *Nitrosospira multififormis*, an ammonia-oxidizing bacterium from the soil environment. *Applied and environmental microbiology*, 74(11), 3559–3572. <https://doi.org/10.1128/AEM.02722-07>

Nosherwani, S. A., & Neto, R. C. (2021). Techno-economic assessment of commercial ammonia synthesis methods in coastal areas of Germany. *Journal of Energy Storage*, 34, 102201. <https://doi.org/10.1016/j.est.2020.102201>

Núñez, J., Arevalo, A., Karwat, H., Egenolf, K., Miles, J., Chirinda, N., ... Arango, J. (2018). Biological nitrification inhibition activity in a soil-grown biparental population of the forage grass, *Brachiaria humidicola*. *Plant and Soil*, 426(1-2), 401–411. <https://doi.org/10.1007/s11104-018-3626-5>

O'Sullivan, C. A., Fillery, I. R. P., Roper, M. M., & Richards, R. A. (2016). Identification of several wheat landraces with biological nitrification inhibition capacity. *Plant and Soil*, 404(1/2), 61–74. <http://www.jstor.org/stable/43872665>

Ofori-Bah, C.O., & Amanor-Boadu, V. (2023). Directing the wind: Techno-economic feasibility of green ammonia for farmers and community economic viability. *Front. Environ. Sci.* <https://doi.org/10.3389/fenvs.2022.1070212>

Oksanen, J., Simpson, G., Blanchet, F., Kindt, R., Legendre, P., Minchin, P., O'Hara, R., Solymos, P., Stevens, M., Szoecs, E., Wagner, H., Barbour, M., Bedward, M., Bolker, B., Borcard, D., Carvalho, G., Chirico, M., De Caceres, M., Durand, S., Evangelista, H., FitzJohn, R., Friendly, M., Furneaux, B., Hannigan, G., Hill, M., Lahti, L., McGlinn, D., Ouellette, M., Ribeiro Cunha, E., Smith, T., Stier, A., Ter Braak, C., Weedon, J. (2022). *_vegan: Community Ecology Package_*. R package version 2.6-4, <https://CRAN.R-project.org/package=vegan>

Orr, C. H., James, A., Leifert, C., Cooper, J. M., & Cummings, S. P. (2011). Diversity and activity of free-living nitrogen-fixing bacteria and total bacteria in organic and conventionally managed soils. *Applied and environmental microbiology*, 77(3), 911–919. <https://doi.org/10.1128/AEM.01250-10>

Otaka, J., Subbarao, G. V., Ono, H., Yoshihashi, T. (2022). Biological nitrification inhibition in maize—isolation and identification of hydrophobic inhibitors from root exudates. *Biol Fertil Soils* 58:251–264. <https://doi.org/10.1007/s00374-021-01577-x>

Otaka, J., Subbarao, G. V., MingLi, J., Ono, H., Yoshihashi, T. (2023). Isolation and characterization of the hydrophilic BNI compound, 6-methoxy-2(3h)-benzoxazolone (MBOA), from maize roots. *Plant Soil* 489:341–359. <https://doi.org/10.1007/s11104-023-06021-7>

Ouyang, Y., & Norton, J. M. (2020). Short-Term Nitrogen Fertilisation Affects Microbial Community Composition and Nitrogen Mineralization Functions in an Agricultural Soil. *Applied and environmental microbiology*, 86(5), e02278–19. <https://doi.org/10.1128/AEM.02278-19>

Pang, Y., & Wang, J. (2021). Various electron donors for biological nitrate removal: A review. *Science of The Total Environment*, 794, 148699. <https://doi.org/10.1016/j.scitotenv.2021.1486>

Papadopoulou, E. S., Tsachidou, B., Sułowicz, S., Menkissoglu-Spiroudi, U., Karpouzas, D. G. (2016). Land Spreading of Wastewaters from the Fruit-Packaging Industry and Potential Effects on Soil Microbes: Effects of the Antioxidant Ethoxyquin and Its Metabolites on Ammonia Oxidizers. *Appl Environ Microbiol* 82 <https://doi.org/10.1128/AEM.03437-15>

Papadopoulou, E. S., Bachtsevani, E., Lampronikou, E., Adamou, E., Katsaouni, A.,

Vasileiadis, S., ... Karpouzas, D. G. (2020). Comparison of Novel and Established Nitrification Inhibitors Relevant to Agriculture on Soil Ammonia- and Nitrite-Oxidizing Isolates. *Frontiers in Microbiology*, 11. <https://doi.org/10.3389/fmicb.2020.581283>

Papadopoulou, E. S., Bachtsevani, E., Papazlatani, C. V., Rousidou, C., Brouziotis, A., Lampronikou, E., Tsiknia, M., Vasileiadis, S., Ipsilantis, I., Menkissoglu-Spiroudi, U., Ehaliotis, C., Philippot, L., Nicol, G. W., & Karpouzas, D. G. (2022). The Effects of Quinone Imine, a New Potent Nitrification Inhibitor, Dicyandiamide, and Nitrapyrin on Target and Off-Target Soil Microbiota. *Microbiology spectrum*, 10(4), e0240321. <https://doi.org/10.1128/spectrum.02403-21>

Pedersen, T. (2022). patchwork: The Composer of Plots. R package version 1.1.2

Pedrinho, A., Karas, P. A., Kanellopoulos, A., Feray, E., Korman, I., Wittenberg, G., Ramot, O., Karpouzas, D. G. (2024). The effect of natural products used as pesticides on the soil microbiota: OECD 216 nitrogen transformation test fails to identify effects that were detected via q-PCR microbial abundance measurement. *Pest Management Science*. <https://doi.org/10.1002/ps.7961>

Pester, M., Maixner, F., Berry, D., Rattei, T., Koch, H., Lückner, S., Nowka, B., Richter, A., Spieck, E., Lebedeva, E., Loy, A., Wagner, M., & Daims, H. (2014). NxrB encoding the beta subunit of nitrite oxidoreductase as functional and phylogenetic marker for nitrite-oxidizing Nitrospira. *Environmental microbiology*, 16(10), 3055–3071. <https://doi.org/10.1111/1462-2920.12300>

Petroli, C. D., Subbarao, G. V., Burgueño, J., Tadashi Yoshihashi, Li, H., Jorge Franco Durán, & Pixley, K. V. (2023). Genetic variation among elite inbred lines suggests potential to breed for BNI-capacity in maize. *Scientific Reports*, 13(1). <https://doi.org/10.1038/s41598-023-39720-3>

Pi, H. W., Lin, J. J., Chen, C. A., Wang, P. H., Chiang, Y. R., Huang, C. C., Young, C. C., & Li, W. H. (2022). Origin and Evolution of Nitrogen Fixation in Prokaryotes. *Molecular biology and evolution*, 39(9), msac181. <https://doi.org/10.1093/molbev/msac181>

Powell, S. J., & Prosser, J. I. (1986). Effect of copper on inhibition by nitrapyrin of growth of *Nitrosomonas europaea*. *Current Microbiology*, 14(3), 177–179. <https://doi.org/10.1007/bf01568371>

Prosser, J. I., & Nicol, G. W. (2012). Archaeal and bacterial ammonia-oxidisers in soil: the quest for niche specialisation and differentiation. *Trends in microbiology*, 20(11), 523–531. <https://doi.org/10.1016/j.tim.2012.08.001>

R Core Team (2020). *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing

Ritz, C., Baty, F., Streibig, J. C., and Gerhard, D. (2016). Dose-response analysis using R. *PLoS One* 10: e0146021. <https://doi.org/10.1371/journal.pone.0146021>

Rojas-Pinzon, P. A., Prommer, J., Sedlacek, C. J., Sandén, T., Spiegel, H., Pjevac, P., Fuchslueger, L., & Giguere, A. T. (2024). Inhibition profile of three biological nitrification inhibitors and their response to soil pH modification in two contrasting soils. *FEMS microbiology ecology*, 100(6), fae072. <https://doi.org/10.1093/femsec/fae072>

Rotthauwe, J. H., Witzel, K. P., and Liesack, W. (1997). The ammonia monooxygenase structural gene amoA as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. *Appl. Environ. Microbiol.* 63, 4704–4712. <https://doi.org/10.1128/aem.63.12.4704-4712.1997>

Rowan, A. K., Snape, J. R., Fearnside, D., Barer, M. R., Curtis, T. P., & Head, I. M. (2003). Composition and diversity of ammonia-oxidising bacterial communities in wastewater treatment reactors of different design treating identical wastewater. *FEMS microbiology ecology*, 43(2), 195–206. <https://doi.org/10.1111/j.1574-6941.2003.tb01059.x>

Sanders, T., Fiencke, C., Hüpeden, J., Pfeiffer, E. M., & Spieck, E. (2019). Cold Adapted *Nitrosospora* sp.: A Potential Crucial Contributor of Ammonia Oxidation in Cryosols of Permafrost-Affected Landscapes in Northeast Siberia. *Microorganisms*, 7(12), 699. <https://doi.org/10.3390/microorganisms7120699>

Schleper, C., & Nicol, G. W. (2010). Ammonia-oxidising archaea--physiology, ecology and evolution. *Advances in microbial physiology*, 57, 1–41. <https://doi.org/10.1016/B978-0-12-381045-8.00001-1>

Sedlacek, C. J., McGowan, B., Suwa, Y., Sayavedra-Soto, L., Laanbroek, H. J., Stein, L. Y., ... Bollmann, A. (2019). A Physiological and Genomic Comparison of Nitrosomonas Cluster 6a and 7 Ammonia-Oxidizing Bacteria. *Microbial Ecology*, 78(4), 985–994. <https://doi.org/10.1007/s00248-019-01378-8>

Seppelt, R., Klotz, S., Peiter, E., & Volk, M. (2022). Agriculture and food security under a changing climate: An underestimated challenge. *iScience*, 25(12), 105551. <https://doi.org/10.1016/j.isci.2022.105551>

Shakoor, A., Ashraf, F., Shakoor, S. *et al.* (2020). Biogeochemical transformation of greenhouse gas emissions from terrestrial to atmospheric environment and potential feedback

to climate forcing. *Environ Sci Pollut Res* **27**, 38513–38536. <https://doi.org/10.1007/s11356-020-10151-1>

Shaw, L. J., Nicol, G. W., Smith, Z., Fear, J., Prosser, J. I., & Baggs, E. M. (2006). Nitrosospira spp. can produce nitrous oxide via a nitrifier denitrification pathway. *Environmental Microbiology*, **8**(2), 214–222. <https://doi.org/10.1111/j.1462-2920.2005.00882.x>

Shen, T., Stieglmeier, M., Dai, J., Urich, T., & Schleper, C. (2013). Responses of the terrestrial ammonia-oxidizing archaeon *Ca. Nitrososphaera viennensis* and the ammonia-oxidizing bacterium *Nitrosospira multiformis* to nitrification inhibitors. *FEMS microbiology letters*, **344**(2), 121–129. <https://doi.org/10.1111/1574-6968.12164>

Shinn, M. B. (1941). Colorimetric method for determination of nitrite. *Ind. Eng. Chem.* **13**, 33–35.

Sieradzki, E. T., Nuccio, E. E., Pett-Ridge, J. *et al.* (2023). Expression of macromolecular organic nitrogen degrading enzymes identifies potential mediators of soil organic N availability to an annual grass. *ISME J* **17**, 967–975. <https://doi.org/10.1038/s41396-023-01402-3>

Singh, S., Anil, A. G., Kumar, V., Kapoor, D., Subramanian, S., Singh, J., & Ramamurthy, P. C. (2022). *Nitrates in the environment: A critical review of their distribution, sensing techniques, ecological effects and remediation*. *Chemosphere*, **287**, 131996. <https://doi.org/10.1016/j.chemosphere.2021.13>

Singh, S.K. (2018). Explorations of Plant's Chemodiversity: Role of Nitrogen-Containing Secondary Metabolites in Plant Defense. In: Singh, A., Singh, I. (eds) *Molecular Aspects of Plant-Pathogen Interaction*. Springer, Singapore. https://doi.org/10.1007/978-981-10-7371-7_14

Skinner, F. A., and Walker, N. (1961). Growth of *Nitrosomonas europaea* in batch and continuous culture. *Arch.Mikrobiol.* **38**, 339–349. <https://doi.org/10.1007/bf00408008>

Solansky, S. (1982). N-stabilisator SKW-DIDIN verbessert die stickstoffwirkung der Gülle. *Blickfeld* **61**, 1–4. <https://doi.org/10.1159/000404405>

Soumare, A., Diedhiou, A. G., Thuita, M., Hafidi, M., Ouhdouch, Y., Gopalakrishnan, S., & Kouisni, L. (2020). Exploiting Biological Nitrogen Fixation: A Route Towards a Sustainable Agriculture. *Plants (Basel, Switzerland)*, **9**(8), 1011. <https://doi.org/10.3390/plants9081011>

Statista Research Department, (2023a). Global fertiliser consumption 1965-2020, by nutrient. [infographic], Statista, <https://www.statista.com/statistics/438967/fertiliser-consumption-globally-by-nutrient/>

Statista Research Department, (2023b). Annual agriculture greenhouse gas (GHG) emissions worldwide from 1990 to 2020. [infographic], Statista, <https://www.statista.com/statistics/1351598/agriculture-ghg-emissions-worldwide/>

Stein L. Y. (2023). Agritech to Tame the Nitrogen Cycle. *Cold Spring Harbor perspectives in biology*, a041668. Advance online publication. <https://doi.org/10.1101/cshperspect.a041668>

Stieglmeier, M., Mooshammer, M., Kitzler, B., Wanek, W., Zechmeister-Boltenstern, S., Richter, A., & Schleper, C. (2014). Aerobic nitrous oxide production through N-nitrosating hybrid formation in ammonia-oxidizing archaea. *The ISME journal*, 8(5), 1135–1146. <https://doi.org/10.1038/ismej.2013.220>

Strock, J. S. (2008). Ammonification. *Encyclopedia of Ecology*, 162–165. <https://doi.org/10.1016/b978-008045405-4.00256-1>

Subbarao, G. V., Ishikawa, T., Ito, O., Nakahara, K., Wang, H. Y., & Berry, W. L. (2006). A bioluminescence assay to detect nitrification inhibitors released from plant roots: a case study with *Brachiaria humidicola*. *Plant and Soil*, 288(1-2), 101–112. <https://doi.org/10.1007/s11104-006-9094-3>

Subbarao, G. V., Tomohiro, B., Masahiro, K., Osamu, I., Samejima, H., Wang, H. Y., ... Berry, W. L. (2007). Can biological nitrification inhibition (BNI) genes from perennial *Leymus racemosus* (Triticeae) combat nitrification in wheat farming? *Plant and Soil*, 299(1-2), 55–64. <https://doi.org/10.1007/s11104-007-9360-z>

Subbarao, G. V., Nakahara, K., Hurtado, M. P., Ono, H., Moreta, D. E., Salcedo, A. F., Yoshihashi, A. T., Ishikawa, T., Ishitani, M., Ohnishi-Kameyama, M., Yoshida, M., Rondon, M., Rao, I. M., Lascano, C. E., Berry, W. L., & Ito, O. (2009). Evidence for biological nitrification inhibition in *Brachiaria* pastures. *Proceedings of the National Academy of Sciences of the United States of America*, 106(41), 17302–17307. <https://doi.org/10.1073/pnas.0903694106>

Subbarao, G. V., Sahrawat, K. L., Nakahara, K., Ishikawa, T., Kishii, M., Rao, I. M., ... Lata, J. C. (2012). Biological Nitrification Inhibition—A Novel Strategy to Regulate Nitrification in Agricultural Systems. *Advances in Agronomy*, 249–302. <https://doi.org/10.1016/b978-0-12-394275-3.00001-8>

Subbarao, G. V., Nakahara, K., Ishikawa, T., Ono, H., Yoshida, M., Yoshihashi, T., Zhu, Y., Zakir, H. A. K. M., Deshpande, S. P. et al. (2013) Biological nitrification inhibition (BNI) activity in sorghum and its characterization. *Plant Soil* 366:243–259. <https://doi.org/10.1007/s11104-012-1419-9>

Sueishi, Y., Hori, M., Kita, M., & Kotake, Y. (2011). Nitric oxide (NO) scavenging capacity of natural antioxidants. *Food Chemistry*, 129(3), 866–870. <https://doi.org/10.1016/j.foodchem.2011.05.0>

- Sun, L., Lu, Y., Yu, F., Kronzucker, H. J., Shi, W. (2016). Biological nitrification inhibition by rice root exudates and its relationship with nitrogen-use efficiency. *New Phytol* 212:646–656. <https://doi.org/10.1111/nph.14057>
- Suri, N., Zhang, Y., Gieg, L. M., & Ryan, M. C. (2021). Denitrification Biokinetics: Towards Optimization for Industrial Applications. *Frontiers in microbiology*, 12, 610389. <https://doi.org/10.3389/fmicb.2021.610389>
- Svane, S., Sigurdarson, J. J., Finkenwirth, F., Eitinger, T., & Karring, H. (2020). Inhibition of urease activity by different compounds provides insight into the modulation and association of bacterial nickel import and ureolysis. *Scientific reports*, 10(1), 8503. <https://doi.org/10.1038/s41598-020-65107-9>
- Taggert, B. I., Walker, C., Chen, D., & Wille, U. (2021). Substituted 1,2,3-triazoles: a new class of nitrification inhibitors. *Scientific reports*, 11(1), 14980. <https://doi.org/10.1038/s41598-021-94306-1>
- Tourna, M., Stieglmeier, M., Spang, A., Könneke, M., Schintlmeister, A., Urich, T., et al. (2011). *Nitrososphaera viennensis*, an ammonia oxidizing archaeon from soil. *Proc. Natl. Acad. Sci. U.S.A.* 108, 8420–8425. <https://doi.org/10.1073/pnas.1013488108>
- United Nations. (2023). Food Security and Nutrition and Sustainable Agriculture. Sustainable Development Goals. <https://sdgs.un.org/topics/food-security-and-nutrition-and-sustainable-agriculture>
- Upadhyay, S. K., Srivastava, A. K., Rajput, V. D., Chauhan, P. K., Bhojiya, A. A., Jain, D., Chaubey, G., Dwivedi, P., Sharma, B., & Minkina, T. (2022). Root Exudates: Mechanistic Insight of Plant Growth Promoting Rhizobacteria for Sustainable Crop Production. *Frontiers in microbiology*, 13, 916488. <https://doi.org/10.3389/fmicb.2022.916488>
- Van Groenigen, J. W., Huygens, D., Boeckx, P., Kuyper, T. W., Lubbers, I. M., Rütting, T., & Groffman, P. M. (2015). The soil N cycle: new insights and key challenges. *SOIL*, 1(1), 235–256. <https://doi.org/10.5194/soil-1-235-2015>
- Vannelli, T., and Hooper, A. B. (1992). Oxidation of nitrapyrin to 6- chloropicolinic acid by the ammonia-oxidizing bacterium *Nitrosomonas europaea*. *Appl. Environ. Microbiol.* 58, 2321–2325. <https://doi.org/10.1128/aem.58.7.2321-2325>
- Vanparys, B., Spieck, E., Heylen, K., Wittebolle, L., Geets, J., Boon, N., & De Vos, P. (2007). The phylogeny of the genus *Nitrobacter* based on comparative rep-PCR, 16S rRNA and nitrite oxidoreductase gene sequence analysis. *Systematic and Applied Microbiology*, 30(4), 297–308. <https://doi.org/10.1016/j.syapm.2006.11.006>

Villegas, D., Arevalo, A., Nuñez, J., Mazabel, J., Subbarao, G., Rao, I., De Vega, J., & Arango, J. (2020). Biological Nitrification Inhibition (BNI): Phenotyping of a Core Germplasm Collection of the Tropical Forage Grass *Megathyrsus maximus* Under Greenhouse Conditions. *Frontiers in plant science*, 11, 820. <https://doi.org/10.3389/fpls.2020.00820>

Wang, J., Sharaf, F. & Kanwal, A. (2023) Nitrate pollution and its solutions with special emphasis on electrochemical reduction removal. *Environ Sci Pollut Res* 30, 9290–9310 (2023). <https://doi.org/10.1007/s11356-022-24450-2>

Wang, Q., Zhao, Z., Yuan, M., Zhang, Z., Chen, S., Ruan, Y., & Huang, Q. (2022). Impacts of urea and 3,4-dimethylpyrazole phosphate on nitrification, targeted ammonia oxidizers, non-targeted nitrite oxidizers, and bacteria in two contrasting soils. *Frontiers in microbiology*, 13, 952967. <https://doi.org/10.3389/fmicb.2022.952967>

Wang, X., Bai, J., Xie, T., Wang, W., Zhang, G., Yin, S., & Wang, D. (2021). Effects of biological nitrification inhibitors on nitrogen use efficiency and greenhouse gas emissions in agricultural soils: A review. *Ecotoxicology and Environmental Safety*, 220, 112338. <https://doi.org/10.1016/j.ecoenv.2021.112338>

Watson, S. W., Graham, L.B., Remsen, C.C., Valois, F.W. (1971). A lobular, ammonia-oxidizing bacterium, *Nitrosolobus multiiformis* nov. gen. nov. sp. *Arch. Microbiol.* 76:183-203. <https://doi.org/10.1007/BF00409115>

Weckwerth, W., Ghatak, A., Bellaire, A., Chaturvedi, P., & Varshney, R. K. (2020). PANOMICS meets germplasm. *Plant biotechnology journal*, 18(7), 1507–1525. <https://doi.org/10.1111/pbi.13372>

Webb, P., Benton, T.G., Beddington, J. et al. (2020). The urgency of food system transformation is now irrefutable. *Nat Food* 1, 584–585. <https://doi.org/10.1038/s43016-020-00161-0>

Wickham, H. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York, 2016.

Williams, A., & de Vries, F. T. (2019). Plant root exudation under drought: implications for ecosystem functioning. *New Phytologist*. <https://doi.org/10.1111/nph.16223>

Winogradsky S (1890). Recherches sur les organismes de la nitrification. *Ann Inst Pasteur*; 4: 213–331.

Woodward, E. E., Edwards, T. M., Givens, C. E., Kolpin, D. W., & Hladik, M. L. (2021). Widespread Use of the Nitrification Inhibitor Nitrapyrin: Assessing Benefits and Costs to Agriculture, Ecosystems, and Environmental Health. *Environmental Science & Technology*, 55(3), 1345–1353. <https://doi.org/10.1021/acs.est.0c05732>

Wright, C. L., Schatteman, A., Crombie, A. T., Murrell, J. C., & Lehtovirta-Morley, L. E.

(2020). Inhibition of Ammonia Monooxygenase from Ammonia-Oxidizing Archaea by Linear and Aromatic Alkynes. *Applied and environmental microbiology*, 86(9), e02388-19. <https://doi.org/10.1128/AEM.02388-19>

Wright, C.L., Lehtovirta-Morley, L.E. (2023). Nitrification and beyond: metabolic versatility of ammonia oxidising archaea. *ISME J* 17, 1358–1368. <https://doi.org/10.1038/s41396-023-01467-0>

Xu, S., Wang, B., Li, Y., Jiang, D., Zhou, Y., Ding, A., Zong, Y., Ling, X., Zhang, S., & Lu, H. (2020). Ubiquity, diversity, and activity of comammox Nitrospira in agricultural soils. *The Science of the total environment*, 706, 135684. <https://doi.org/10.1016/j.scitotenv.2019.135684>

Yahya, M., Islam, E. U., Rasul, M., Farooq, I., Mahreen, N., Tawab, A., Irfan, M., Rajput, L., Amin, I., Yasmin, S. (2021). Differential Root Exudation and Architecture for Improved Growth of Wheat Mediated by Phosphate Solubilizing Bacteria. *Frontiers in Microbiology*, vol 12, <https://doi.org/10.3389/fmicb.2021.744094>

Zakir, H. A. K. M., Subbarao, G. V., Pearse, S. J., Gopalakrishnan, S., Ito, O., Ishikawa, T., Kawano, N., Nakahara, K., Yoshihashi, T., Ono, H., & Yoshida, M. (2008). Detection, isolation and characterization of a root-exuded compound, methyl 3-(4-hydroxyphenyl) propionate, responsible for biological nitrification inhibition by sorghum (*Sorghum bicolor*). *The New phytologist*, 180(2), 442–451. <https://doi.org/10.1111/j.1469-8137.2008.02576.x>

Zayed, O., Hewedy, O. A., Abdelmoteleb, A., Ali, M., Youssef, M. S., Roumia, A. F., Seymour, D., & Yuan, Z. C. (2023). Nitrogen Journey in Plants: From Uptake to Metabolism, Stress Response, and Microbe Interaction. *Biomolecules*, 13(10), 1443. <https://doi.org/10.3390/biom13101443>

Zerulla, W., Barth, T., Dressel, J., Erhardt, K., von Locquenghien, K. H., Pasda, G., et al. (2001). 3,4-Dimethylpyrazole phosphate (DMPP)-a new nitrification inhibitor for agriculture and horticulture-an introduction. *Biol. Fertil. Soils* 34, 79–84. <https://doi.org/10.1007/s003740100380>

Zhang, H., Zhang, L., Tao, R., Hu, J., & Chu, G. (2022). Nitrapyrin Addition Mitigated CO₂ Emission from a Calcareous Soil Was Closely Associated with Its Effect on Decreasing Cellulolytic Fungal Community Diversity. *Journal of agricultural and food chemistry*, 70(17), 5299–5309. <https://doi.org/10.1021/acs.jafc.1c08020>

Zhang, M., Wang, W., Zhang, Y., Teng, Y., & Xu, Z. (2017). Effects of fungicide iprodione and nitrification inhibitor 3, 4-dimethylpyrazole phosphate on soil enzyme and bacterial properties. *The Science of the total environment*, 599-600, 254–263. <https://doi.org/10.1016/j.scitotenv.2017.05.011>

Zhang, M., Gao, X., Chen, G., Afzal, M. R., Wei, T., Zeng, H., Subbarao, G. V., Wei, Z., Zhu, Y. (2023). Intercropping with BNI-sorghum benefits neighbouring maize productivity and

mitigates soil nitrification and N₂O emission, *Agriculture, Ecosystems & Environment*, vol 352, <https://doi.org/10.1016/j.agee.2023.108510>

Zhang, X., Lu, Y., Yang, T. *et al.* Factors influencing the release of the biological nitrification inhibitor 1,9-decanediol from rice (*Oryza sativa* L.) roots. *Plant Soil* **436**, 253–265 (2019). <https://doi.org/10.1007/s11104-019-03933-1>

Zhao, J., Bello, M. O., Meng, Y., Prosser, J. I., Gubry-Rangin, C. (2020). Selective inhibition of ammonia oxidising archaea by simvastatin stimulates growth of ammonia oxidising bacteria, *Soil Biology and Biochemistry*, Volume 141, 107673, <https://doi.org/10.1016/j.soilbio.2019.107673>

Zhao, J., Wang, B., Zhou, X., Alam, M. S., Fan, J., Guo, Z., Zhang, H., Gubry-Rangin, C., & Zhongjun, J. (2022). Long-Term Adaptation of Acidophilic Archaeal Ammonia Oxidisers Following Different Soil Fertilisation Histories. *Microbial ecology*, 83(2), 424–435. <https://doi.org/10.1007/s00248-021-01763-2>

Zhao, Y., Hu, J., Zhou, Z., Li, L., Zhang, X., He, Y., Zhang, C., Wang, J., & Hong, G. (2024). Biofortified Rice Provides Rich Sakuranetin in Endosperm. *Rice (New York, N.Y.)*, 17(1), 19. <https://doi.org/10.1186/s12284-024-00697-w>

Zhou, X., Wang, S., Ma, S., Zheng, X., Wang, Z., & Lu, C. (2020). Effects of commonly used nitrification inhibitors—dicyandiamide (DCD), 3,4-dimethylpyrazole phosphate (DMPP), and nitrapyrin—on soil nitrogen dynamics and nitrifiers in three typical paddy soils. *Geoderma*, 380, 114637. <https://doi.org/10.1016/j.geoderma.2020.114637>

Zhu, Y., Zeng, H., Shen, Q., Ishikawa, T., & Subbarao, G. V. (2012). Interplay among NH₄⁺ uptake, rhizosphere pH and plasma membrane H⁺-ATPase determine the release of BNIs in sorghum roots-possible mechanisms and underlying hypothesis. *Plant and Soil*, 358(1-2), 131–141. <https://doi.org/10.1007/s11104-012-1151-5>

Zorz, J. K., Kozlowski, J. A., Stein, L. Y., Strous, M., & Kleiner, M. (2018). Comparative Proteomics of Three Species of Ammonia-Oxidizing Bacteria. *Frontiers in microbiology*, 9, 938. <https://doi.org/10.3389/fmicb.2018.00938>

Zumft W. G. (1997). Cell biology and molecular basis of denitrification. *Microbiology and molecular biology reviews: MMBR*, 61(4), 533–616. <https://doi.org/10.1128/mmbr.61.4.533-616.1997>