ACTION Nr

Research Action Network for Reducing Reactive Nitrogen Losses from Agricultural Ecosystems

Project No. 101079299

ACTION Nr

PhD summer school

“Biological Nitrification Inhibition: Integrating Microbial Functions, Plant Traits, and Technological Innovations for Sustainable Nitrogen Cycling”

University of Thessaly
12-16 May 2025

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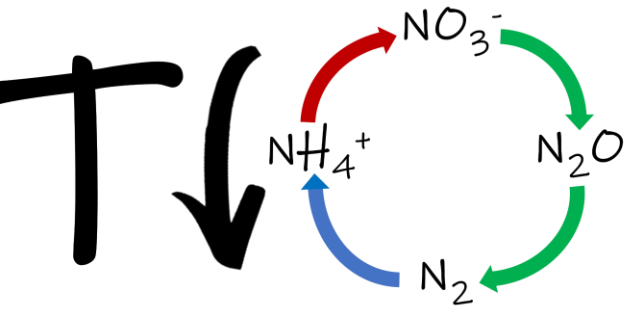

Current methodologies and advancements in screening BNI compounds

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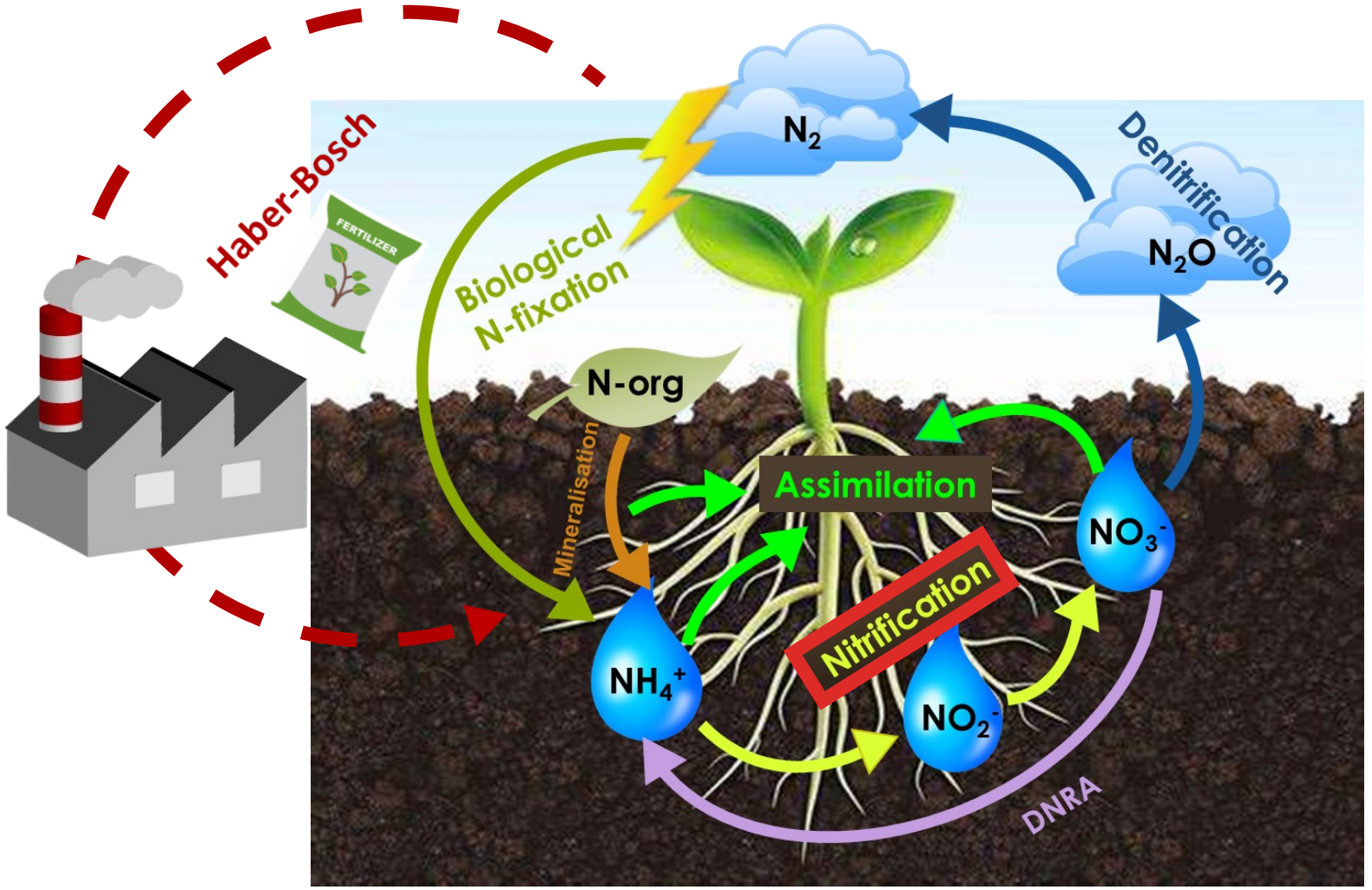
School of Technology
University of Thessaly

May 13, 2025
Larissa, Greece

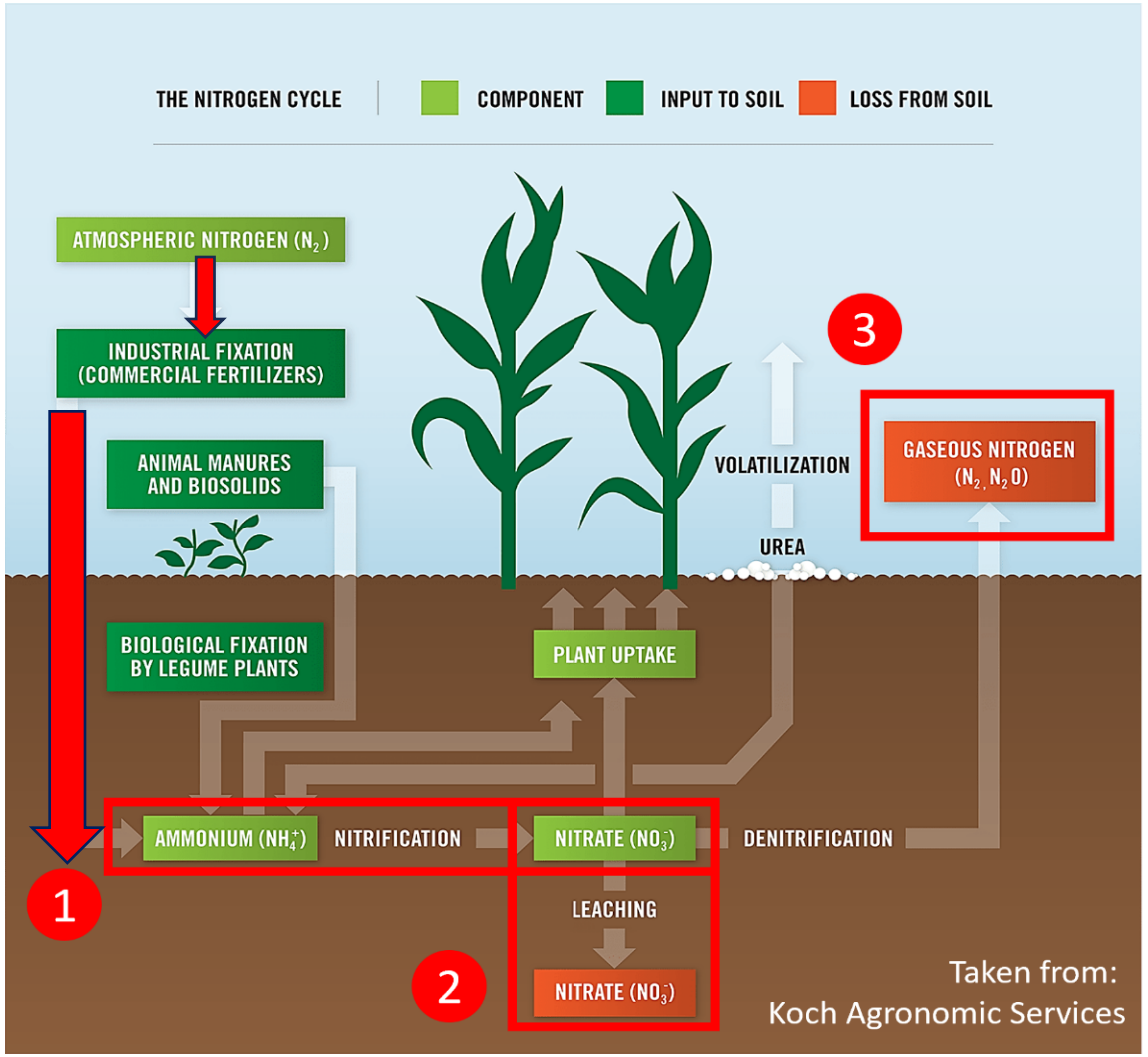
Nitrification: A key process in the global N cycle



Nitrification **regulates** the availability of **ammonium (NH_4^+)** and **nitrate (NO_3^-)** in soils.

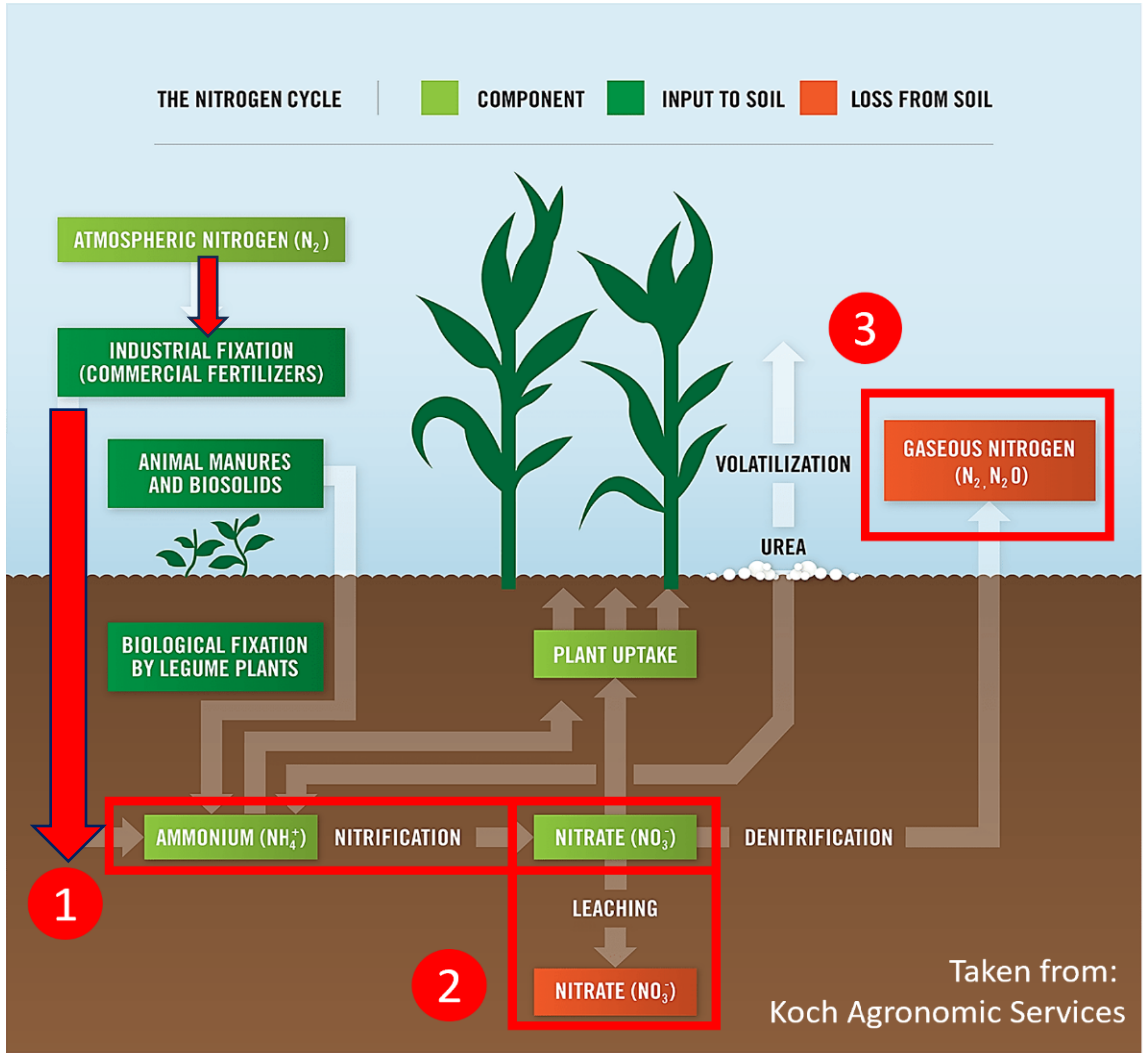
- To sustain crop productivity, these nitrogen forms are often **supplemented through synthetic fertilizers,** industrially fixed via the Haber-Bosch process.

Nitrification: A key factor in agricultural N loss

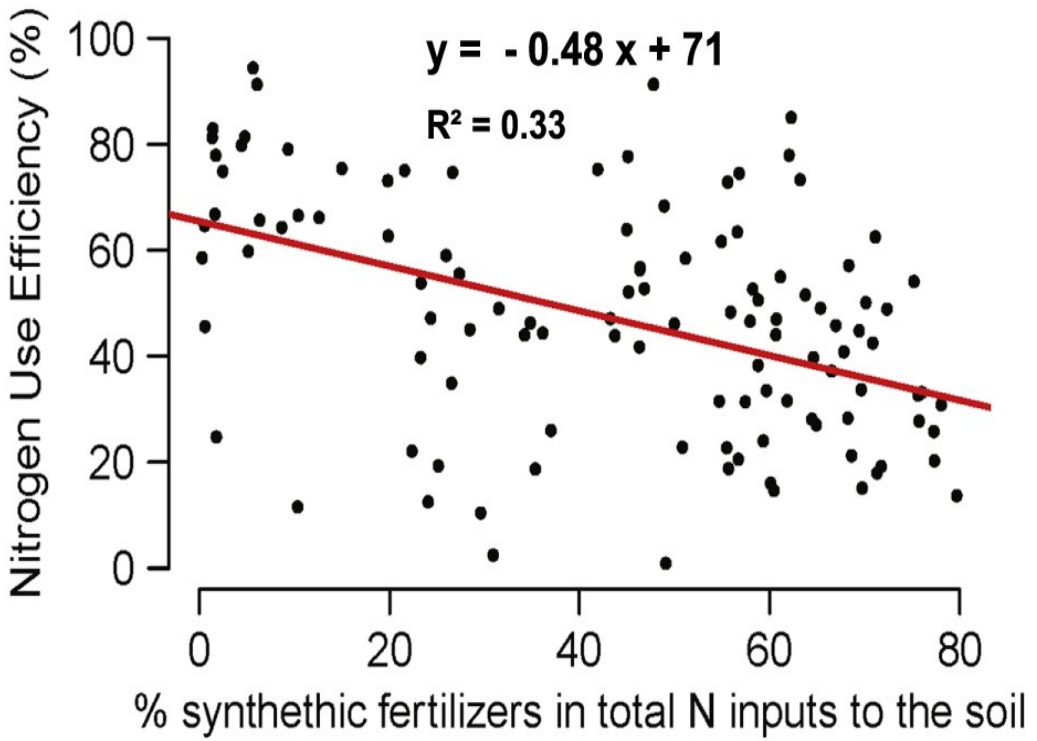


Elevated nitrification rates, driven by excessive NH_4^+ fertilization, pose a significant challenge for both agriculture and the environment.

Excessive nitrification impact on agriculture

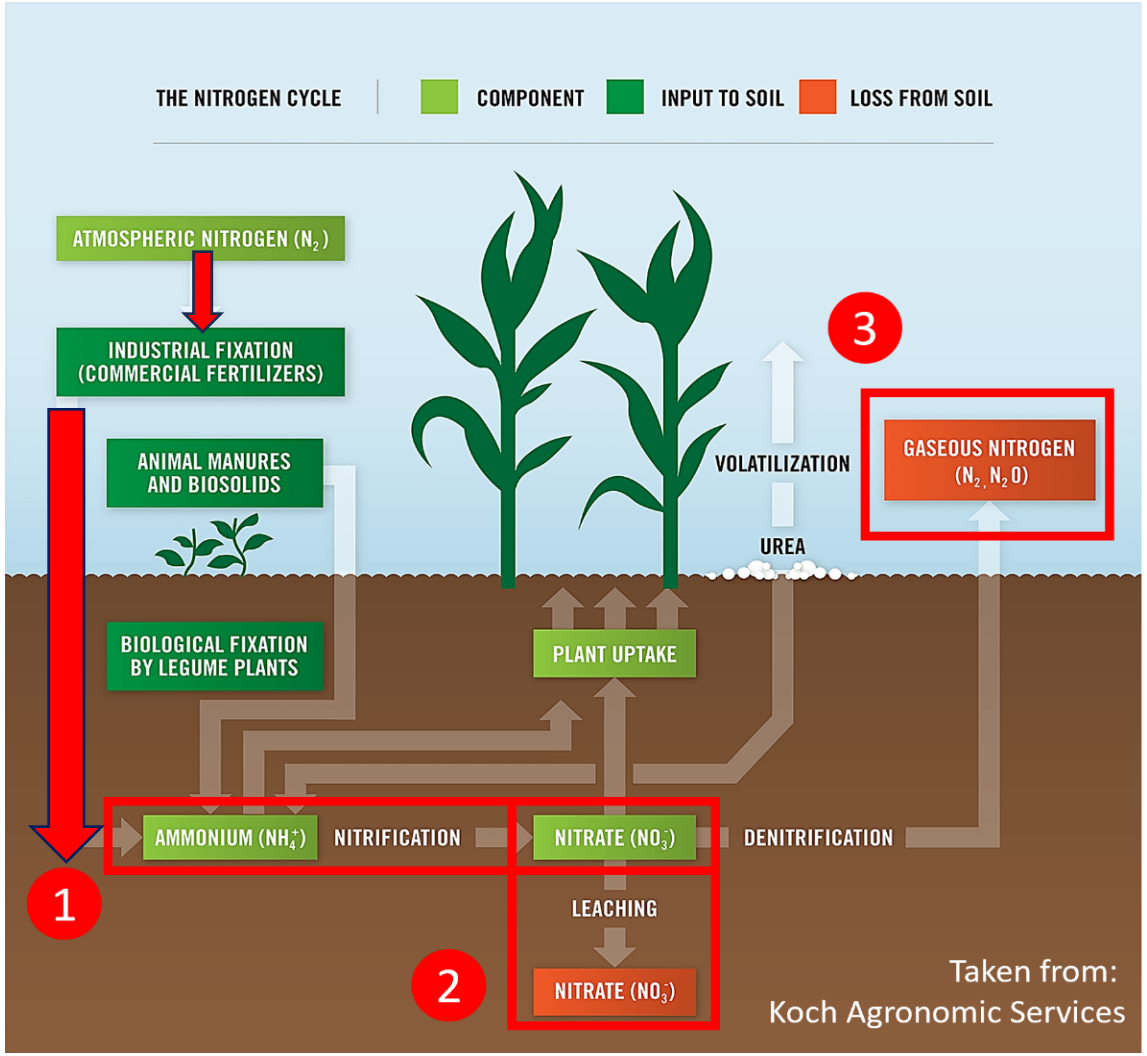


1 **Loss in Nitrogen Use Efficiency**

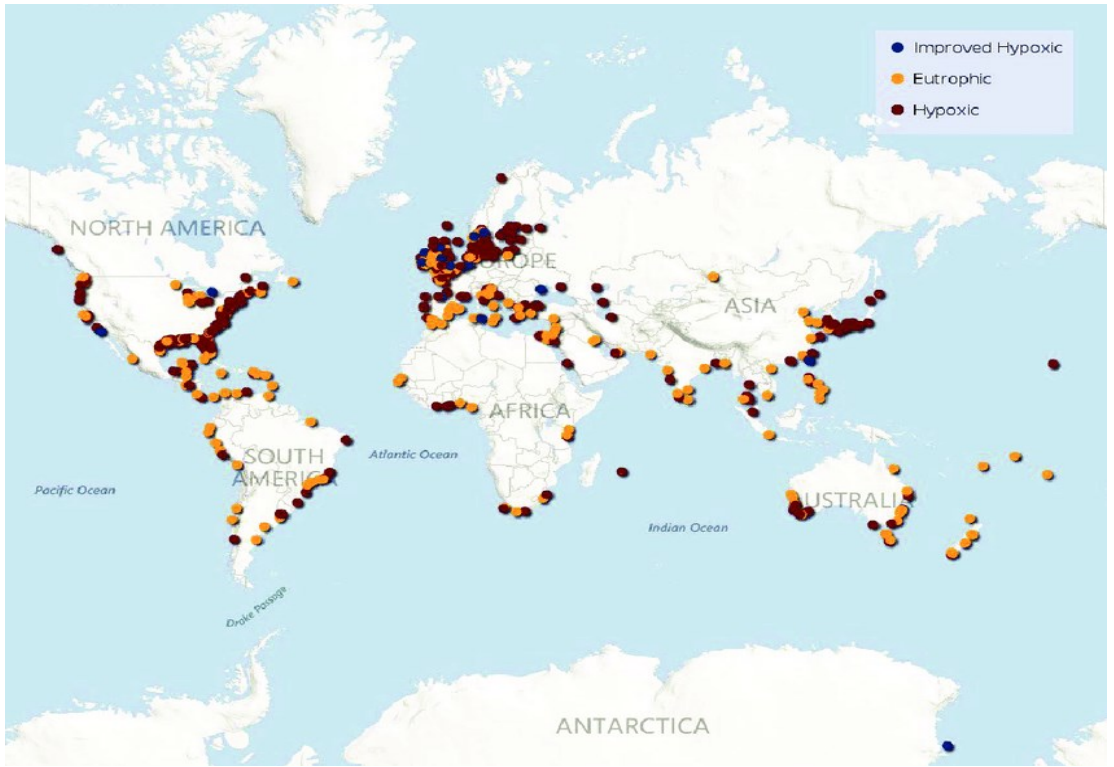


Lassalleta et al. (2014), Environmental Research Letters, 9, 105011

Excessive nitrification impact on the environment

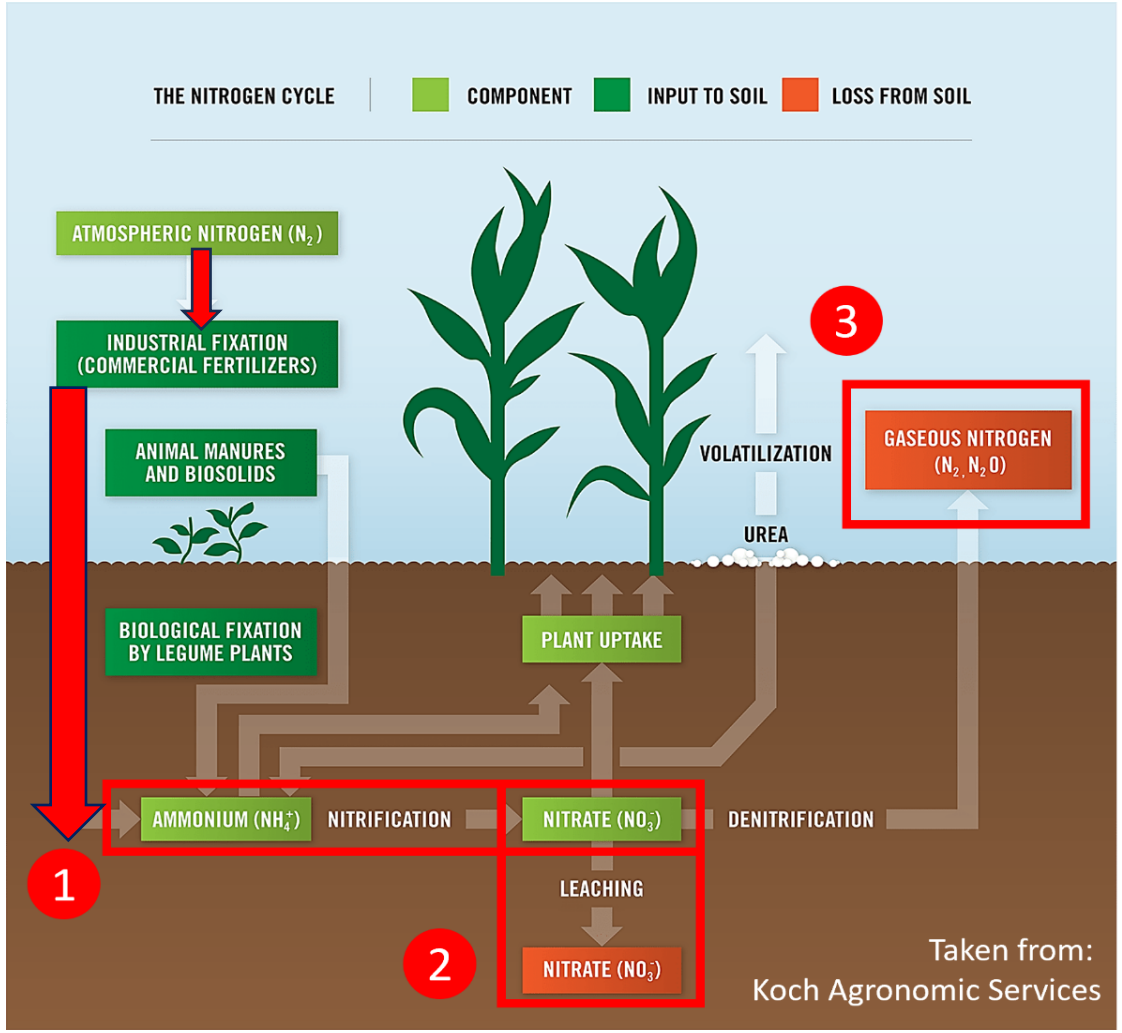


2 Nitrate leaching and eutrophication

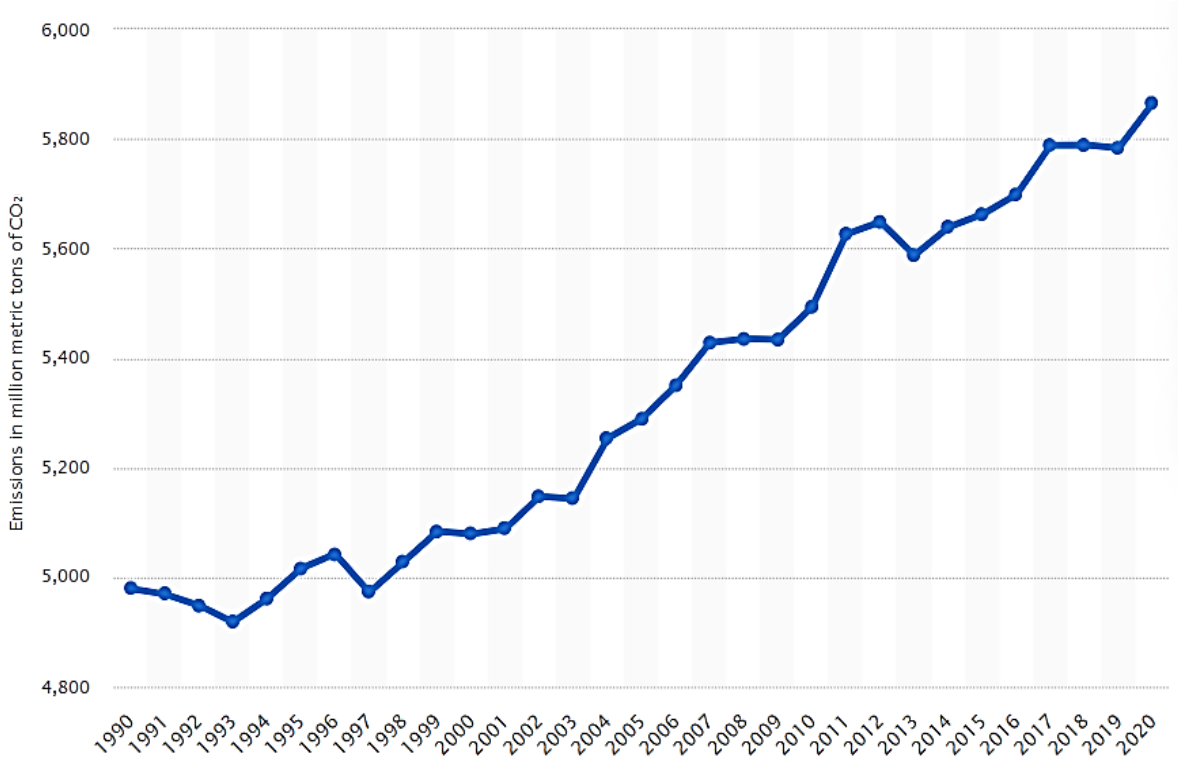


Jessen, C. (2013). PhD Thesis, Leibniz Centre for Tropical Marine Research (ZMT)

Excessive nitrification impact on the environment



3 Emissions of N_2O, a highly potent GHG



Statista Research Department, (2023).
https://www.statista.com/statistics/1351598/agriculture-ghg-emissions-worldwide/

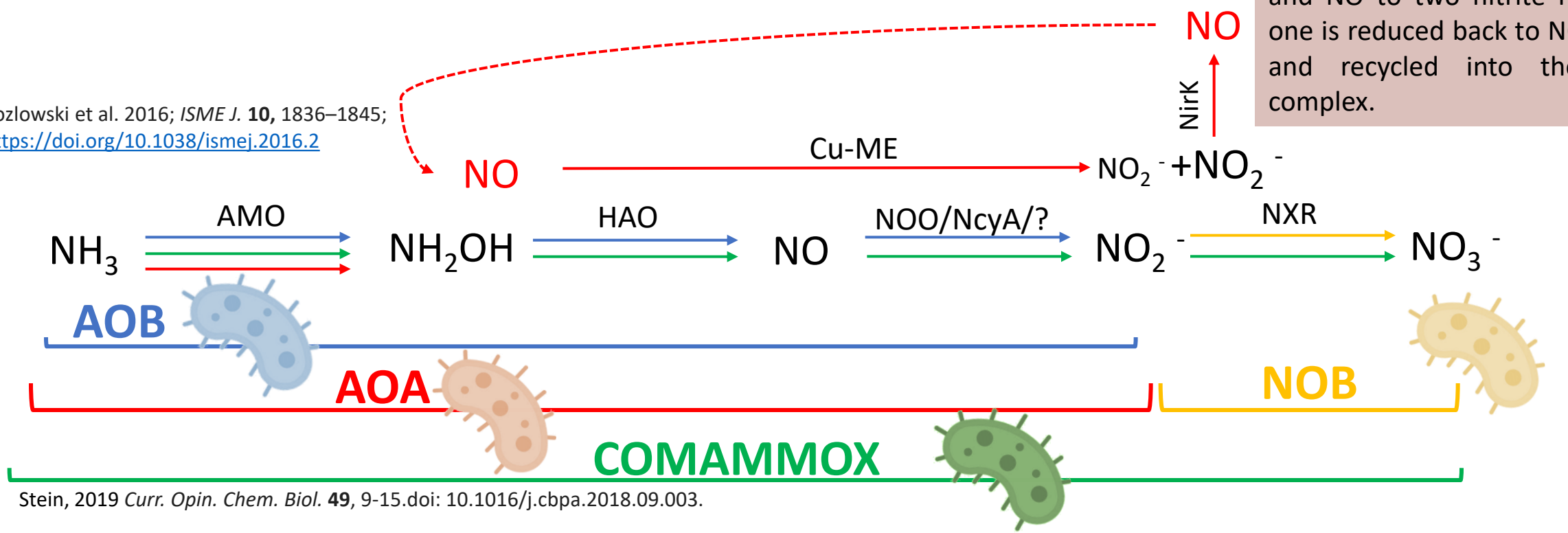
Nitrification is a microbially driven process

❑ Ammonia and nitrite oxidation pathways in chemolithoautotrophs

- Distinct pathways are presented for AOB, AOA, NOB, and comammox bacteria.

Cu-ME co-oxidizes hydroxylamine and NO to two nitrite molecules; one is reduced back to NO via NirK and recycled into the Cu-ME complex.

Kozlowski et al. 2016; *ISME J.* **10,** 1836–1845; https://doi.org/10.1038/ismej.2016.2

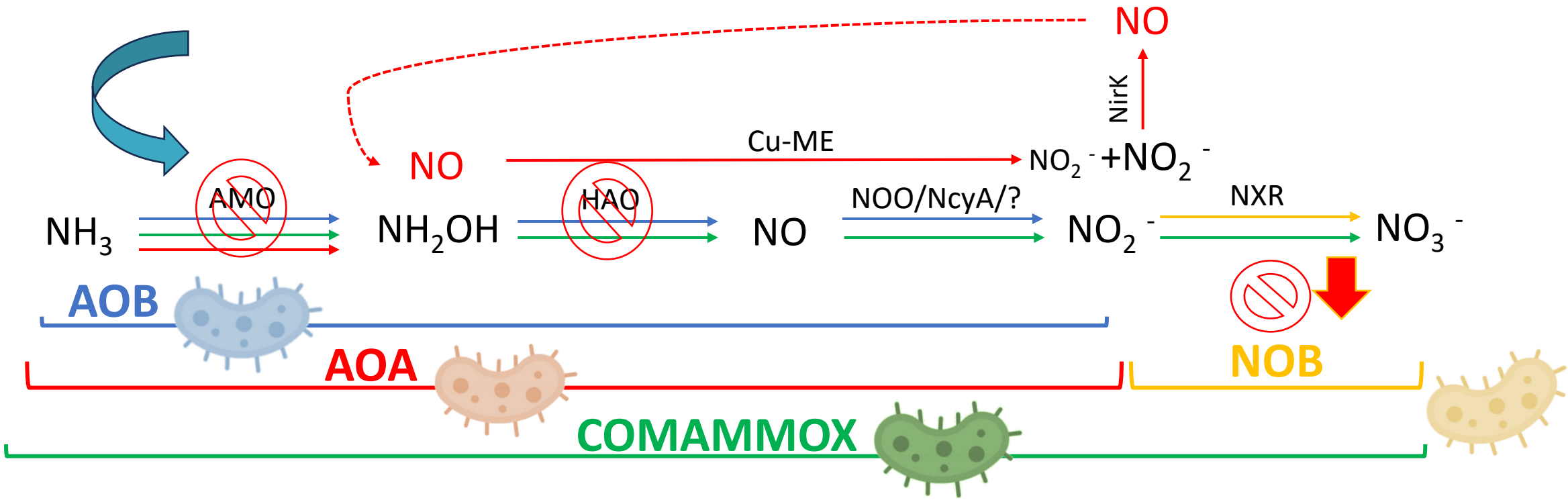


Stein, 2019 *Curr. Opin. Chem. Biol.* **49**, 9-15.doi: 10.1016/j.cbpa.2018.09.003.

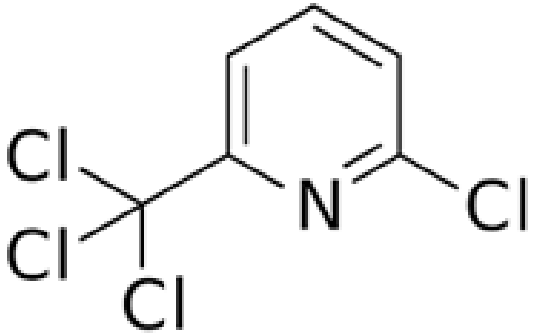
AMO = ammonia monooxygenase, HAO = hydroxylamine dehydrogenase, NOO = nitric oxide oxidoreductase, NXR = nitrite oxidoreductase.
NOO is proposed to be nitrosocyanin (NcyA) or an uncharacterized enzyme

Nitrification inhibitors as a tool to mitigate Nr

- ❑ **Nitrification inhibitors** slow down the biological oxidation of ammonia to nitrate
- ✓ Longer availability of NH_4^+ for plants => Optimized NUE
- ✓ Lower nitrate pollution
- ✓ Reduced greenhouse gas (GHG) emissions



Synthetic Nitrification Inhibitors -SNIs



Instinct® NITROGEN STABILIZER

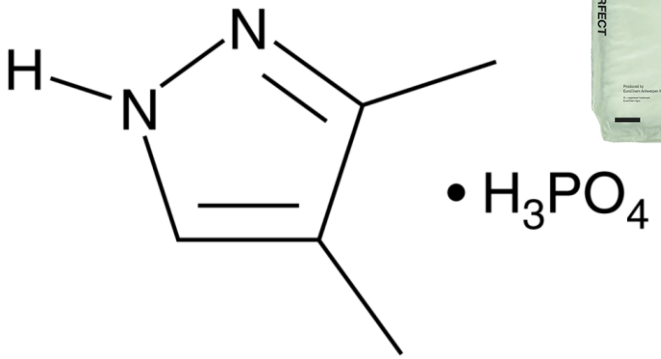
N-Serve® NITROGEN STABILIZER

SNIs currently used in agricultural settings

1962

2-Chloro-6-(trichloromethyl) pyridine
(**Nitrapyrin**)

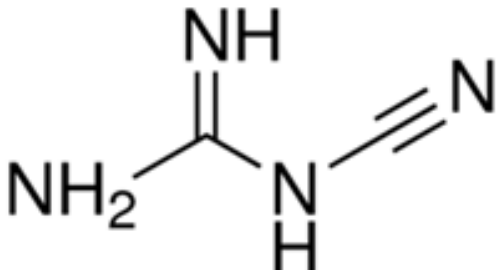
2001



3,4-dimethylpyrazole phosphate
(**DMPP**)

AGROTAIN® ADVANCED 1.0

1982

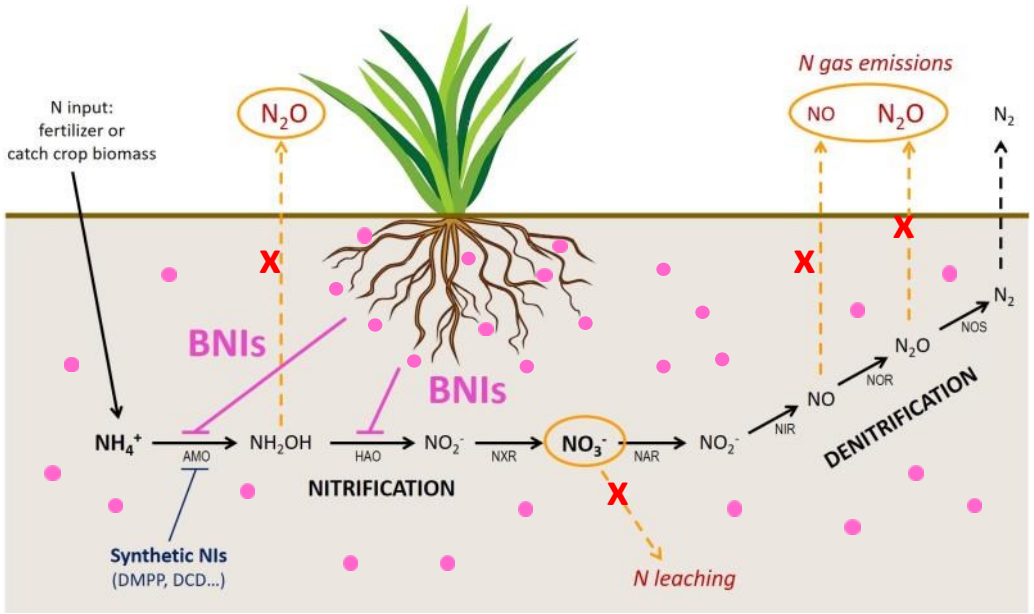


Dicyandiamide
(**DCD**)

ACTIONr

Biological Nitrification Inhibitors - BNIs

Definition: Biological nitrification inhibitors (BNIs) are compounds synthesized or secreted by plants (and microorganisms) with properties that inhibit nitrification.



- BNI proposed as an evolutionary adaptation for N conservation in N-limited soils.

- Occurs via allelopathy, with production and exudation of plant secondary metabolites.

The precise release of BNIs in time and space, gives them an advantage over the commercial SNIs

environmental microbiology

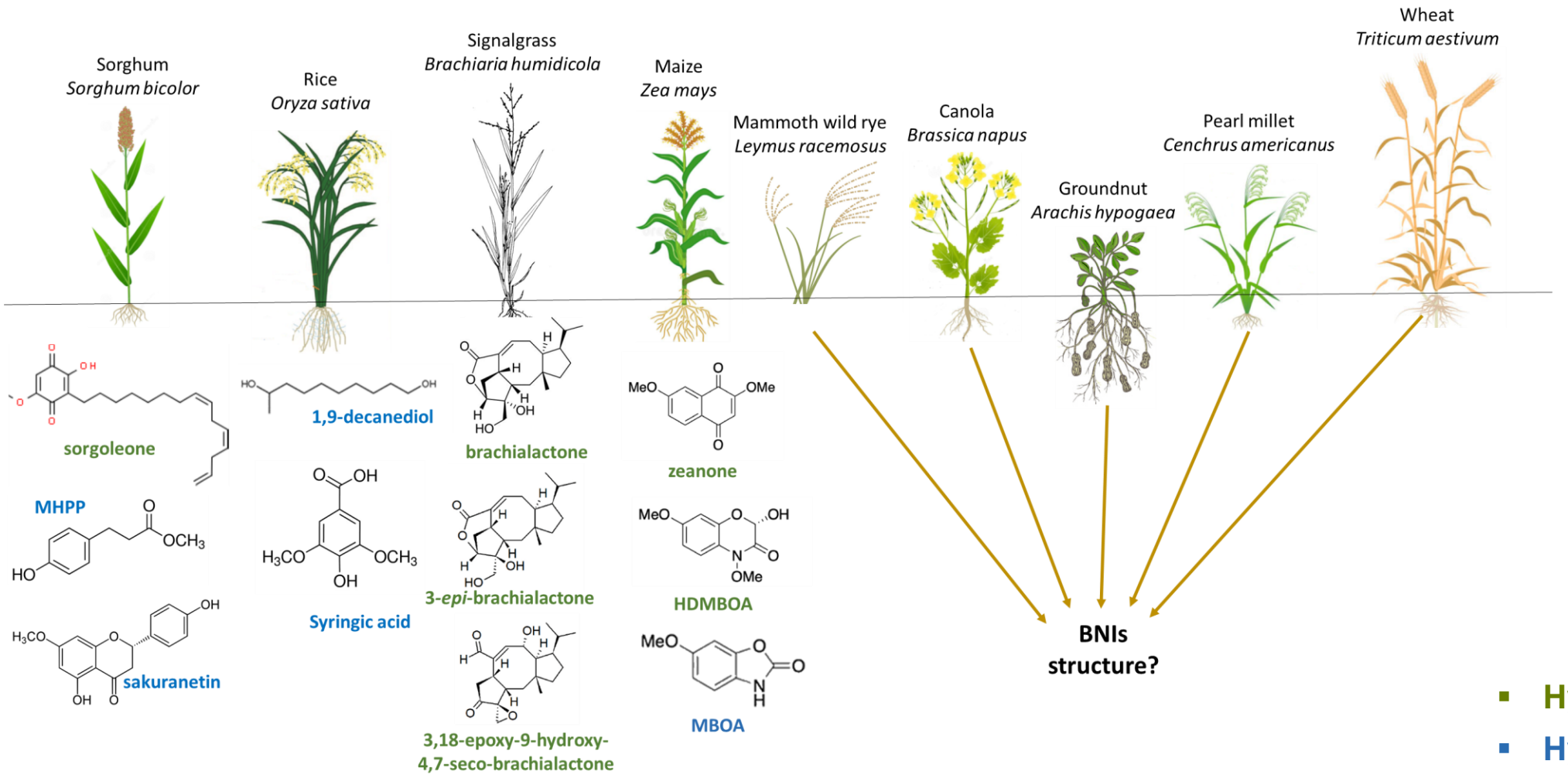
Environmental Microbiology (2020) **22**(3), 1141–1153 doi:10.1111/1462-2920.14905

Biological inhibition of soil nitrification by forest tree species affects *Nitrobacter* populations

Amandine Laffite,[1] Alessandro Florio,[1] Kasaina Sitraka Andrianarisoa,[2] Charline Creuze des Chatelliers,[1] Brigitte Schloter-Hai,[3] Sidy M. Ndaw,[1] Charlotte Periot,[1] Michael Schloter,[3] Bernd Zeller,[2] Franck Poly[1†] and Xavier Le Roux[1*†]

- Represses AOM and NOB, reducing nitrification, NO_3^- leaching, and N_2O emissions.

Biological Nitrification Inhibitors -BNIs



- **Hydrophobic BNIs**
- **Hydrophilic BNIs**

SNIs vs. BNIs

Synthetic Nitrification Inhibitors (SNIs)

- ✓ High efficacy
- ✓ Established use

- ○ Erratic performance
- ○ High cost of synthesis
- ○ Difficulties in application
- ○ Degradation in soil
- ○ Off – target effects
- ○ Potential groundwater pollution
- ○ Entry into the food system

Biological Nitrification Inhibitors (BNIs)

- ✓ Environmentally friendly
- ✓ Great potential of BNIs and BNI - producing plants in agricultural settings

- ○ Degradation in soil
- ○ Variable efficacy in soil
- ○ Variability in BNI potential of currently known BNI – producing plant species and varieties

➢ **Identifying pure BNIs and root exudates from new plant types is becoming more important!**

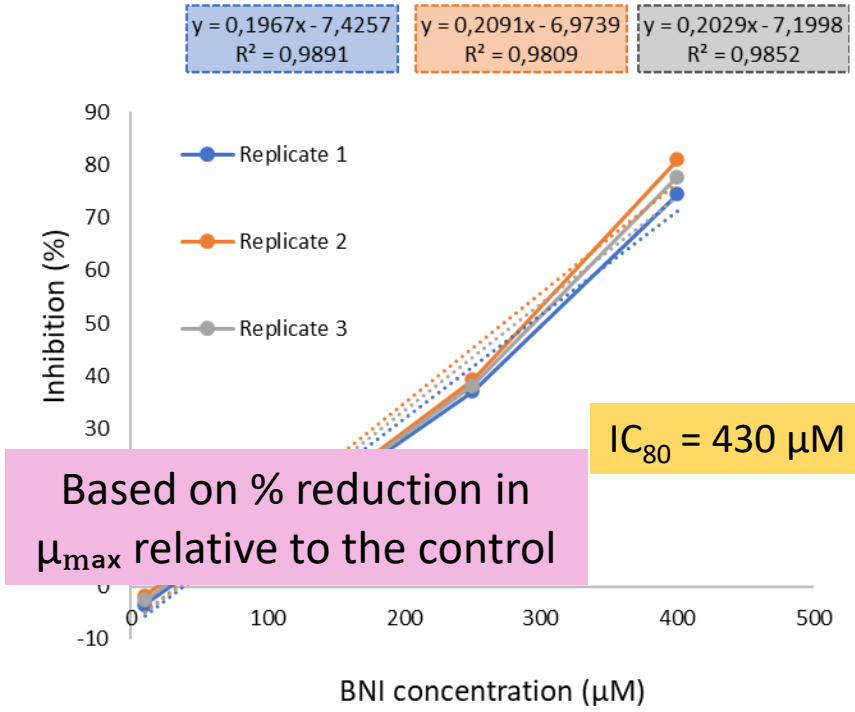
Current methodologies in BNIs screening

1 Liquid batch culture bioassays

✓ MHPP against *Nitrosospira multiformis*

- Triplicate BNI assays (5–14 days, dose series) determine nitrite-inhibition thresholds.
- Daily nitrite measurements with DMSO/H_2O controls confirm inhibitor effects.

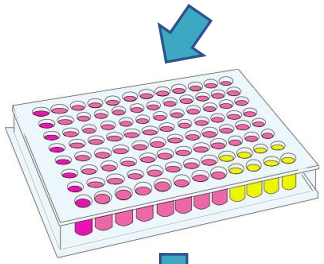
Powell & Prosser, 1985; FEMS Microbiol Lett. 28: 51-54



IC_{80} = 430 µM

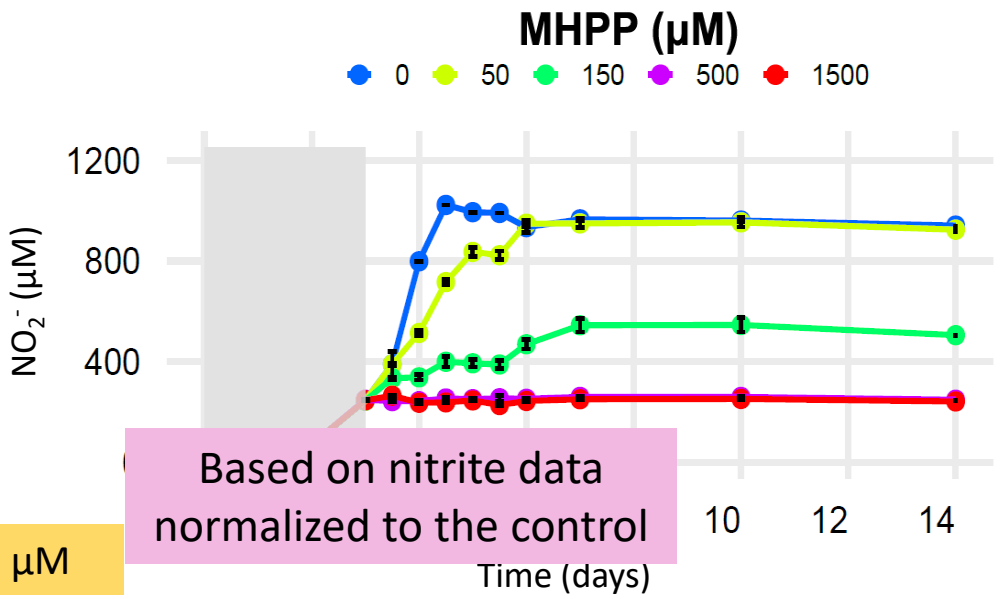
Based on % reduction in μ_{max} relative to the control

Kaur-Bhambra et al. 2021, Biol. Fertil. Soils



Estimation of BNIs inhibition thresholds

EC_{50} = 104 µM



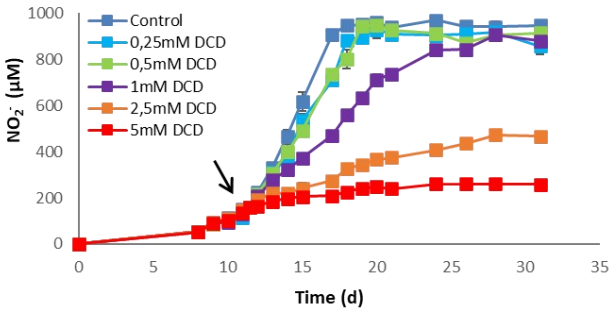
Based on nitrite data normalized to the control

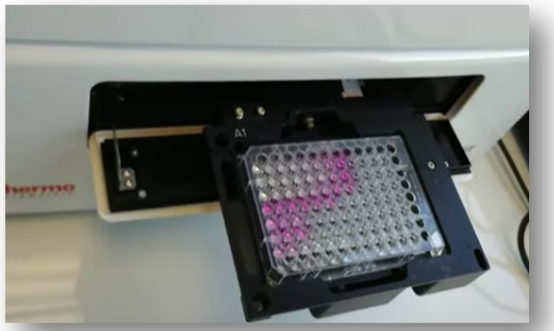
Kolovou et al. 2023 Appl Environ Microbiol 89:e01380-23

Current methodologies in BNIs screening

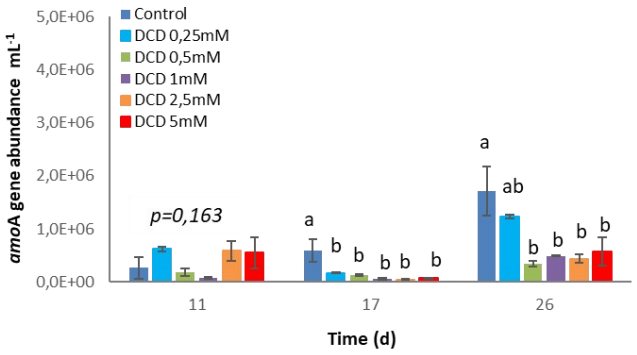
1 Liquid batch culture bioassays

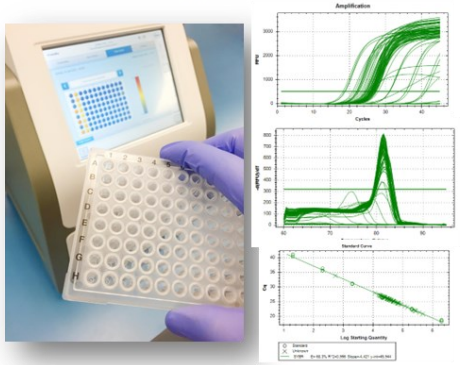
ACTIVITY



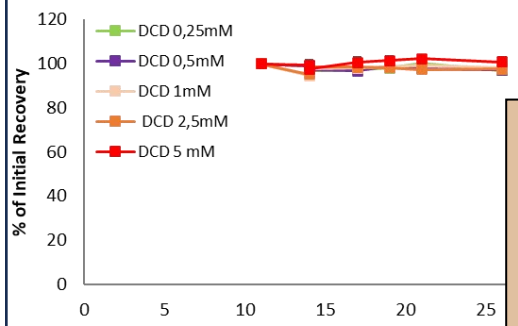


GROWTH





STABILITY

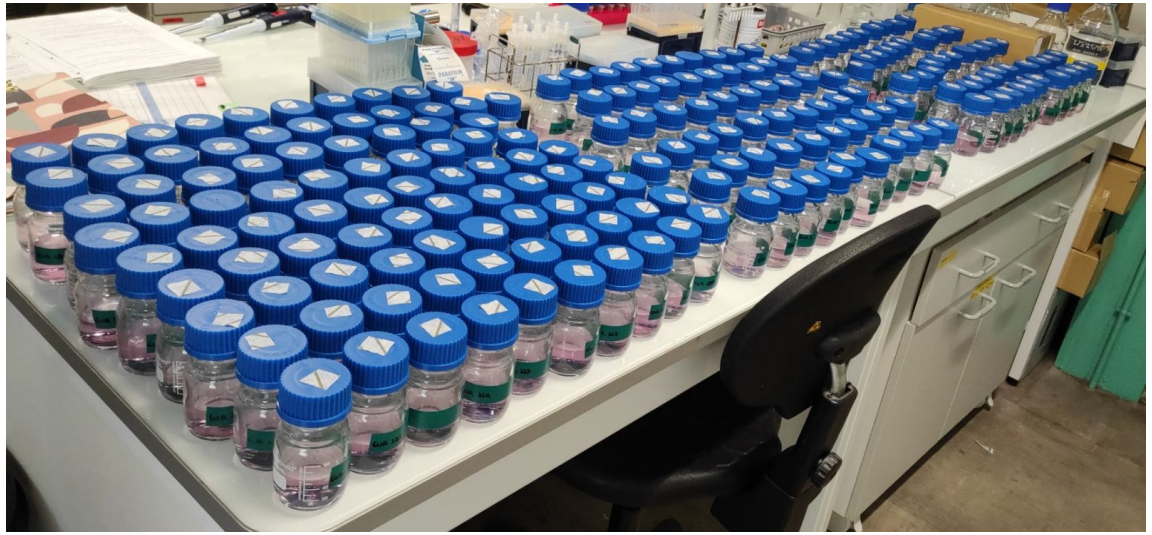


Ability to monitor NI **compound stability & persistence** → crucial for **interpreting inhibition (exposure-dependent)**

Papadopoulou et al. 2020, Front. Microbiol., https://doi.org/10.3389/fmicb.2020.581283

Current methodologies in BNIs screening

1 Liquid batch culture bioassays



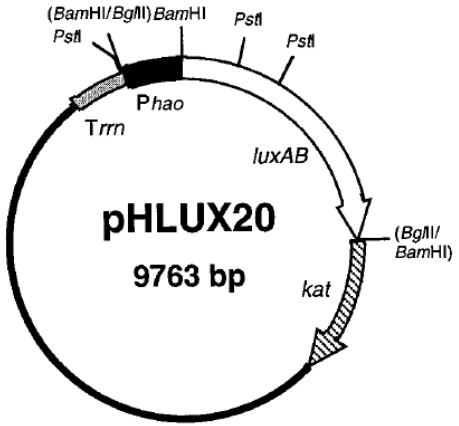
Bachtsevani, Kolovou et al. Environmental Science & Technology (Under review)

- ✓ Established protocols => comparative results
- ✓ Applicable to a broad range of soil representative nitrifiers
- ✓ Suitable for screening both SNIs & BNIs
- ✓ Large culture volumes allow monitoring across multiple growth phases
- ✓ Can detect solubility issues with test NIs

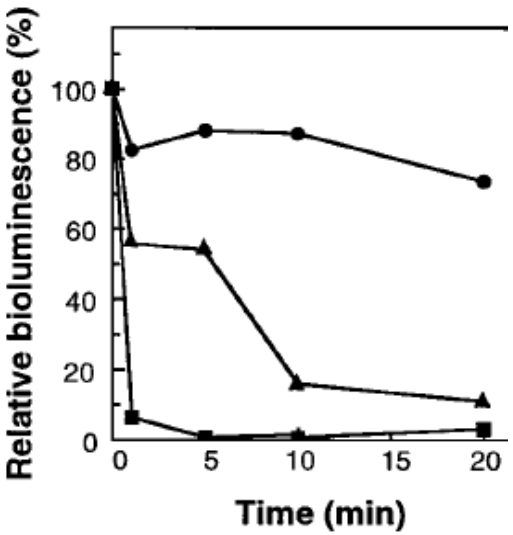
- ○ **Low throughput**
- Time consuming
- Resources and space demanding
- Labor intensive

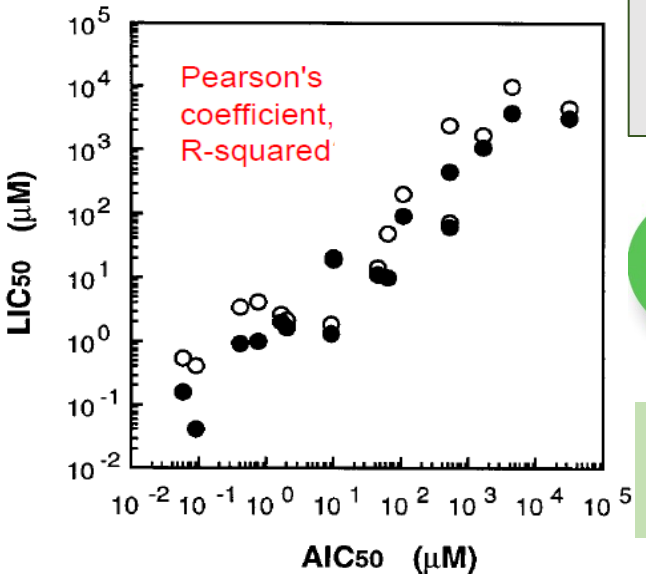
Current methodologies in BNIs screening

2 Bioluminescent *Nitrosomonas europaea* bioassay



A fluorescent AOB reporter strain constructed by inserting the LUX gene from *Vibrio harveyi.*





Linear correlation between bioluminescence & ammonia-oxidation inhibition

High throughput

Discovery of many known BNIs

✓Parallel functional differentiation of inhibitory modes (AMO vs. HAO)

- ✓ **Developed for monitoring the nitrification process in wastewater treatment plants ->** *Iizumi et al. 1998 Appl Environ Microbiol 64:3656–3662*
- ✓ **Adopted to detect and quantify natural nitrification inhibitors in plant–soil systems ->** *Subbarao et al. 2006 Plant Soil 288:101–112*

Current methodologies in BNIs screening

2 Bioluminescent *Nitrosomonas europaea* bioassay

Use of a genetically modified reporter bacterium

- ✓ Not available in microbial culture collections
- ✓ Requires regulated (PC2) lab conditions for GMO cultivation

Nitrosomonas europaea is not a good indicator of BNI inhibition

- ✓ Common in sediment and wastewater plants; less common in soil
- ✓ Lower representativeness of natural soil AOM communities

Limited ecological relevance

Current methodologies in BNIs screening

2 Bioluminescent *Nitrosomonas europaea* bioassay

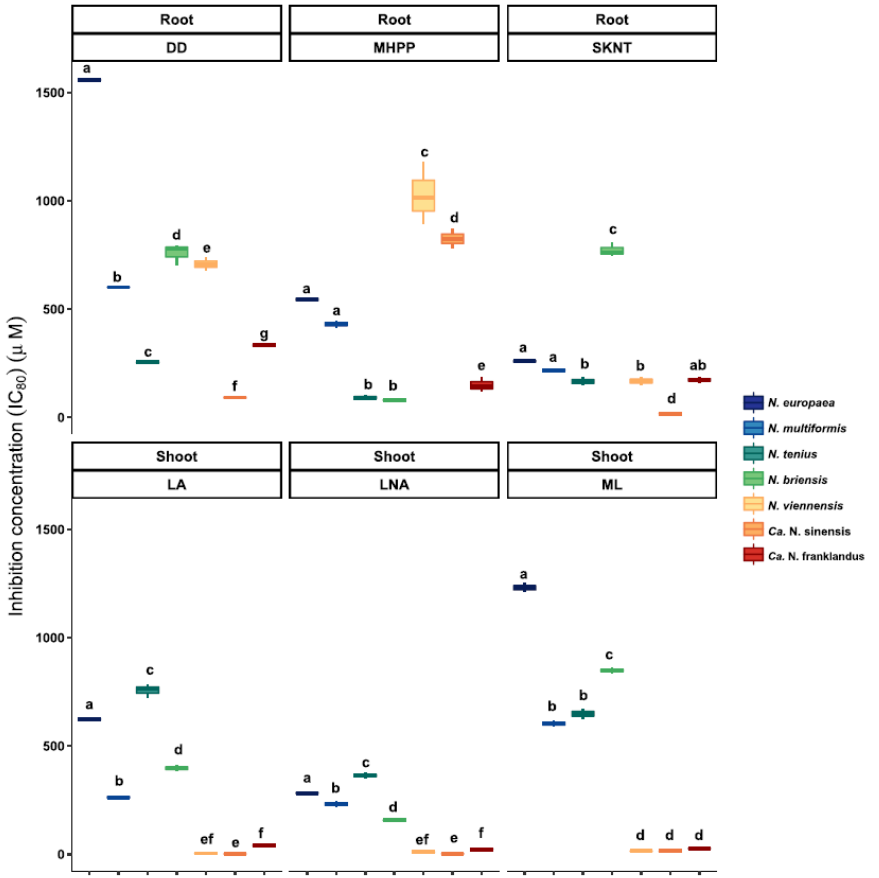
N. europaea is not a good indicator of BNI inhibition

✓Variable *in vitro* response compared to strains that are more representative of natural soil AOM communities

	N. europaea	*Nitrobacter* sp.	*N. multiformis*	*Ca.* N. sinensis	*Ca.* N. franklandianus
Quinic acid	1596.6 (±123.1,aA)	> 3000 (**)	< 3 (*)	84.3 (±14,bB)	>300 (**)
MHPP	339.3 (±32.4,cB)	7.4 (±1.3,fC)	104 (±12.8,bcdC)	268.2 (±3.8,aB)	877.7 (±165.3,bA)
Shikimic acid	> 5000 (**)	238.99 (±25.9,bcB)	191.2 (±42,bcB)	334.9 (±21,aB)	1420.4 (±167.8,aA)
Chlorogenic acid	777.5 (±49.9,bA)	71.9 (±8.9,efB)	81.2 (±9.4,bcdB)	65.5 (±6.9,bB)	148.9 (±80.3,dB)
1-Butyl-4-propyl-triazole	>500 (**)	377.7 (±19.8,aA)	407.3 (±63,aA)	237.4 (±44.8,aA)	460 (±86.1,cA)
1.4-Dibutyltriazole	> 500 (**)	290.6 (±35,abA)	> 500 (**)	283.6 (±71.7,aA)	284.8 (±46,cdA)
Zeanone	> 46 (**)	24.0 (±4.7,fA)	< 0.2 (*)	4.5 (±0.6,bB)	0.4 (±0.1,eB)
Caffeic acid	203.3 (±15.8,cA)	< 3 (*)	140.2 (±12.8,bcdB)	1.8 (±0.2,bC)	19.5 (±6,eC)
2-Methoxy-1.4-naphthoquinone	> 218 (**)	27.8 (±2.2,fA)	> 218 (**)	5.2 (±0.1,bB)	2.9 (±0.3,eB)
Sakuranetin	> 250	155.7 (±20.6,cdeA)	144.3 (±19,bcdA)	< 10 (*)	< 10 (*)
Simvastatin	> 100 (**)	>100 (**)	> 100 (**)	< 5 (*)	25.7 (±5.8,e)

1e+05 1e+04 1e+03 1e+02 1e+01 1e+00

Kolovou et al. 2023 Appl Environ Microbiol 89:e01380-23



Kaur-Bhambra et al. 2021, Biol. Fertil. Soils

Current methodologies in BNIs screening

2 Bioluminescent *Nitrosomonas europaea* bioassay

Use of a single AOB reporter strain

✓Significant differences in the inhibitory concentrations of BNIs across different microbial strains

✓ Bioassays should include a **wider range of representative organisms**, rather than relying on a single strain

N. europaea	*Nitrobacter* sp.	*N. multiformis*	*Ca.* N. sinensis	*Ca.* N. franklandianus	
1596.6 (±123.1,aA)	> 3000 (**)	< 3 (*)	84.3 (±14,bB)	>300 (**)	Quinic acid
339.3 (±32.4,cB)	7.4 (±1.3,fC)	104 (±12.8,bcdC)	268.2 (±3.8,aB)	877.7 (±165.3,bA)	MHPP
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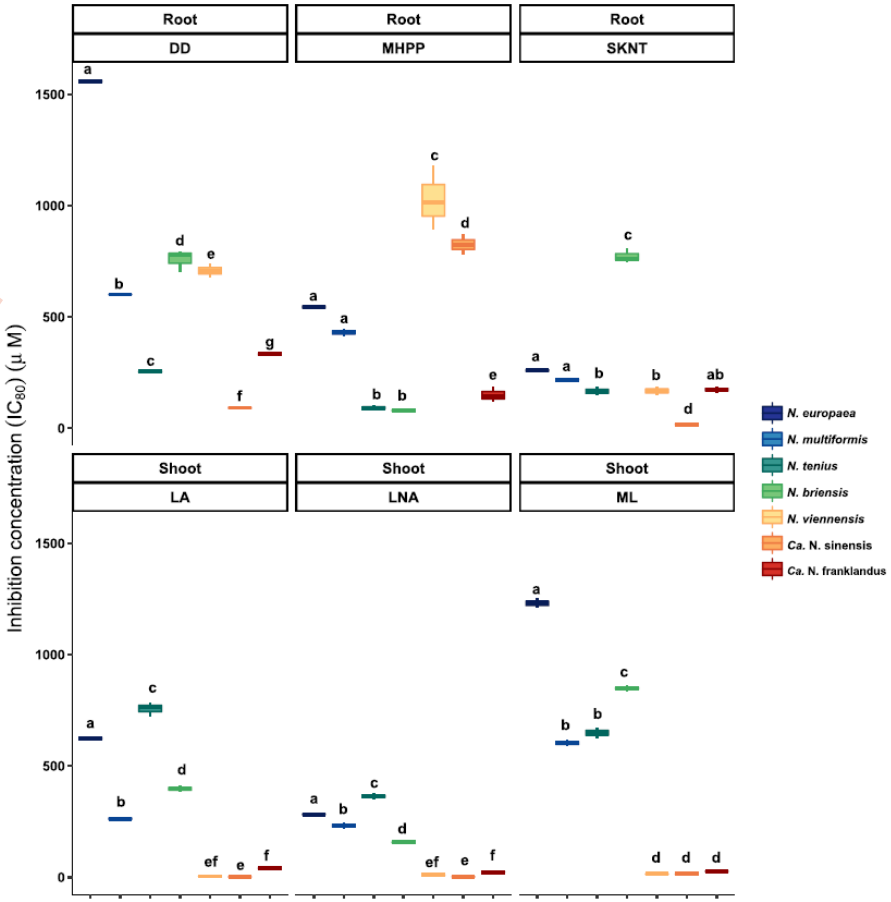
203.3 (±15.8,cA)	< 3 (*)	140.2 (±12.8,bcdB)	1.8 (±0.2,bC)	19.5 (±6,eC)	Caffeic acid
> 218 (**)	27.8 (±2.2,fA)	> 218 (**)	5.2 (±0.1,bB)	2.9 (±0.3,eB)	2-Methoxy-1.4-naphthoquinone
> 250	155.7 (±20.6,cdeA)	144.3 (±19,bcdA)	< 10 (*)	< 10 (*)	Sakuranetin
> 100 (**)	>100 (**)	> 100 (**)	< 5 (*)	25.7 (±5.8,e)	Simvastatin

1e+05 1e+04 1e+03 1e+02 1e+01 1e+00

Kolovou et al. 2023 Appl Environ Microbiol 89:e01380-23



Kaur-Bhambra et al. 2021, Biol. Fertil. Soils

Current methodologies in BNIs screening

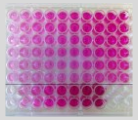
3 Other recent high-throughput bioassays based on concentrated AOB cultures

Plant Soil (2017) 413:275–287
DOI 10.1007/s11104-016-3100-1

REGULAR ARTICLE

A colourimetric microplate assay for simple, high throughput assessment of synthetic and biological nitrification inhibitors

- **AOM used:** *Nitrosomonas europaea* and *Nitrosospira multiformis* (AOB) in pure culture
- **Assay setup**:
  - ✓ **50 mL assay** vials with NH_4^+-based media| washed, overconcentrated AOB cells (~10^8 cells mL^{-1})
  - ✓ Sampling hourly over **9 hours**, nitrification stopped with DCD



Detection: Colorimetric NO_2^- quantification via Griess reaction (490 nm)

- **Inhibition measured as**:
  - % reduction in nitrification rate relative to control
  - **IC_{50} values** calculated from dose–response curves

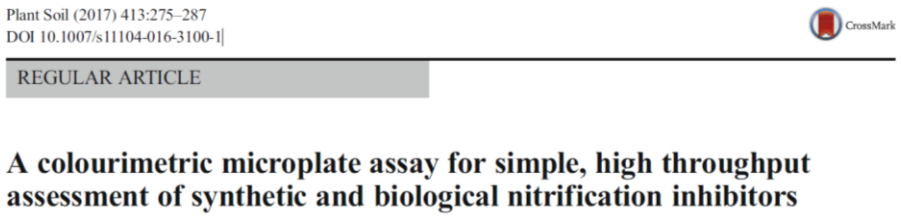
O'Sullivan et al. 2017, Plant Soil 413:275–287, doi:10.1007/s11104-016-3100-1

Proof of concept

- Used to assess **BNI** activity of root exudates from *Brachiaria humidicola* compared to low-BNI species (*Triticum aestivum* cv. Janz)

Current methodologies in BNIs screening

3 Other high-throughput bioassays based on concentrated AOB


Plant Soil (2017) 413:275–287
DOI 10.1007/s11104-016-3100-1

REGULAR ARTICLE

A colourimetric microplate assay for simple, high throughput assessment of synthetic and biological nitrification inhibitors

O'Sullivan et al. 2017, Plant Soil 413:275–287, doi:10.1007/s11104-016-3100-1

High-throughput potential: ≥ 50 samples/day in triplicate; **9 h turnaround**

- ✓ **Validated** using established SNIs and *B. humidicola* root exudates
- ✓ **Useful for phenotyping**: Useful for screening plant genotypes for BNI traits

Only AOB tested → *N. multiformis, N. europaea* -> **Low ecological relevance**

- **Relatively large culture volumes (20 mL/vial) → reduced throughput** compared to miniaturized formats
- Requirement for **manual sampling (hourly for 9 h)** -> **labor-intensive**, limits scalability

Current methodologies in BNIs screening

3 Other high-troughput bioassays based on concentrated AOB cultures

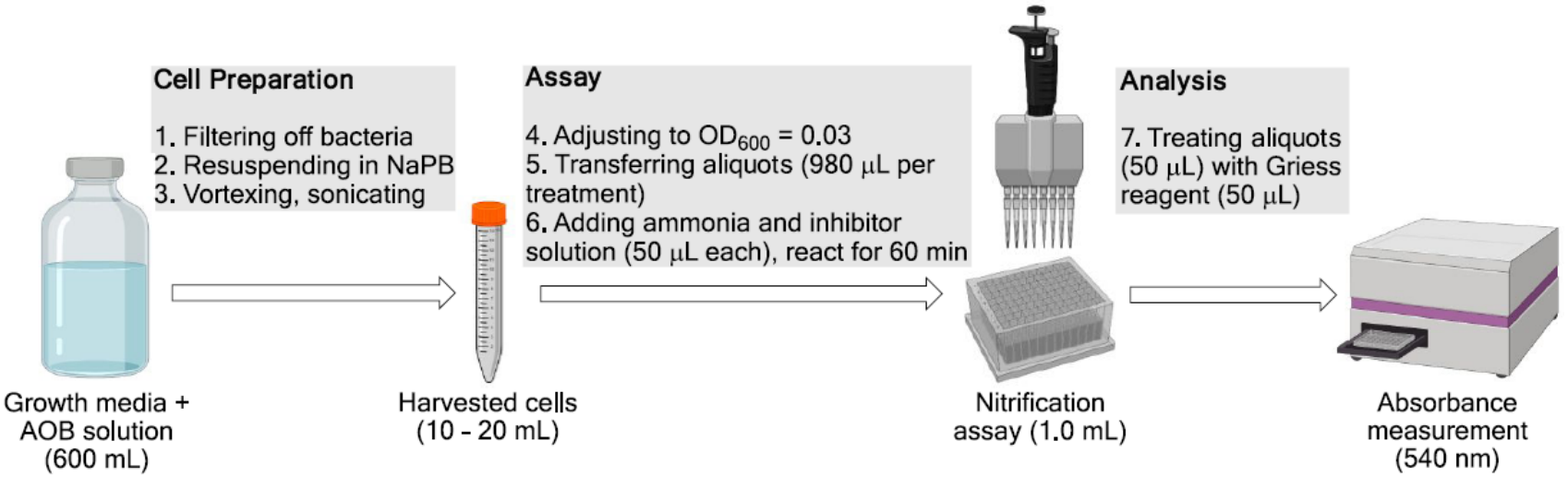
- **AOM used**: *N. europaea* and *N. multiformis* (AOB) grown in 1 L DURAN bottles (600 mL MSM)
- **Assay setup**: Conducted in **deep 96-well plates (2 mL capacity)**

ACS AGRICULTURAL SCIENCE & TECHNOLOGY

pubs.acs.org/acsagscitech Article

Rapid and Inexpensive Assay for Testing the Efficiency of Potential New Synthetic Nitrification Inhibitors

Sibel C. Yildirim, Robert M. Walker, Ute Roessner, and Uta Wille*



Yildirim et al., 2023, ACS Agric. Sci. Technol. 2023, 3, 260–269, https://doi.org/10.1021/acsagscitech.2c00229

Current methodologies in BNIs screening

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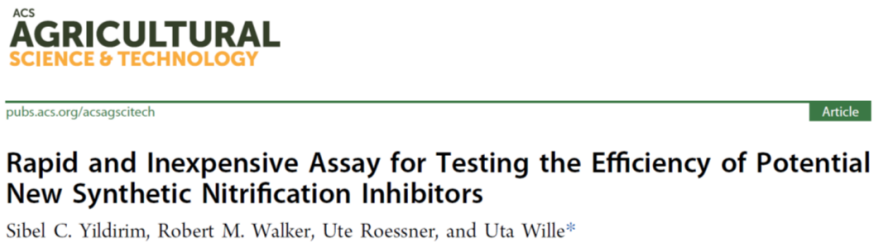
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Fast-track: **60-minute** assay post-inoculation

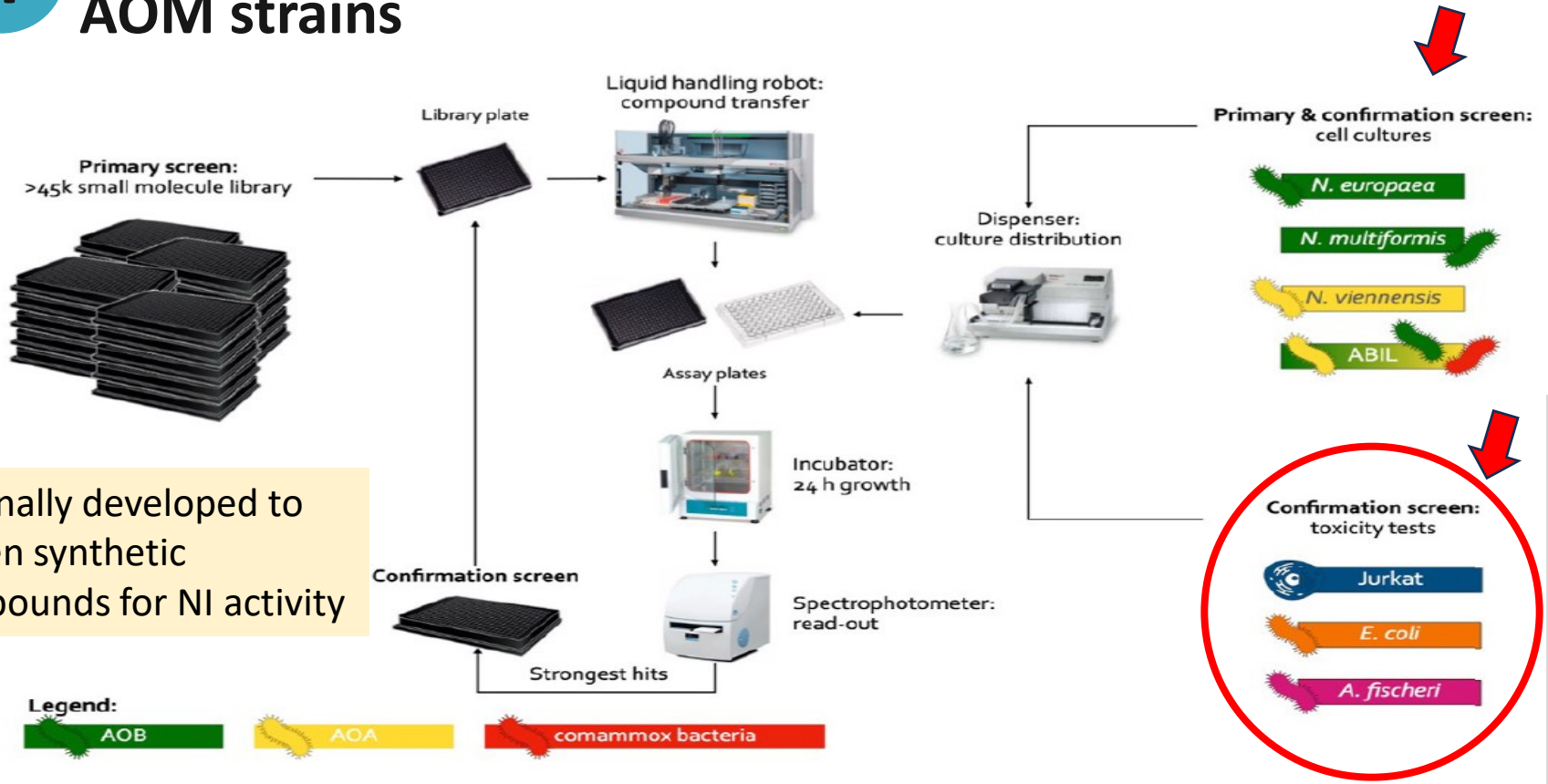
✓ **Miniaturized format (96-well deep plates)** allows **medium-throughput screening**

Only AOB tested → *N. multiformis, N. europaea* -> **Low ecological relevance**

- Requires precise OD calibration and manual handling → **moderate labour demand**

Current methodologies in BNIs screening

4 Recent fast-track, miniaturized, and high-throughput bioassays using diverse soil AOM strains



Originally developed to screen synthetic compounds for NI activity

First high-throughput platform to include a soil-representative **AOA (*N. viennensis*),** a comammox enrichment (*Ca.* Nitrospira kreftii), and a complex nitrifying enrichment culture (**ABIL**)

- **24-hour turnaround**
- Normalized, **plate-wise data processing**
- Combines **relative nitrification**, **fold change**, and **z-score normalization** for compound selection

Beeckman et al. 2023 J Environm Manag 346:118996

Current methodologies in BNIs screening

Scalable high-throughput via robotic automation

Successfully screened **45,400 compounds** (selected from commercial compound libraries) using four parallel miniaturized assays

Beeckman et al. 2023 J Environm Manag 346:118996

✓ **Broader applicability across AOM diversity -> Covers key nitrifying guilds**

Includes *Nitrosomonas europaea* (AOB), *Nitrosospira multiformis* (AOB), ***Nitrososphaera viennensis*** **(AOA)**, comammox (*Ca. Nitrospira kreftii*, from bioreactor enrichment), and ABIL (a complex nitrifying enrichment culture: AOA > Comammox clade B > Comammox clade A > AOB)

✓ **Miniaturized culture volumes**

- **50 µL for AOB (384-well format)**
- **200 µL** for AOA and Comammox (96-well format)
- **135–150 µL** for ABIL (96-well format)

=> Enables low reagent use and large-scale screening

✓ **Rapid readout & short turnaround time (24 h)**

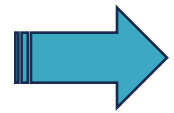
✓ **Inclusion of** DMP & PTIO **positive controls** for AOB and AOA, respectively, at one inhibiting concentration

✓ Compatible with parallel **functional differentiation** of inhibitory modes (AMO vs. HAO; Cu-binding)

Current methodologies in BNIs screening

Beeckman et al. 2023 J Environm Manag 346:118996

- **Single concentration + 1 replicate** per compound in the primary screen → limits dose-response insight and increases variability
- **Small well volumes (< 200 µL)** can lead to:
  - Evaporation and edge effects
  - Oxygen limitation
  - Compound solubility or dosing inaccuracies
- ABIL and *Ca.* N. kreftii enrichments are **not soil isolates**, which may **reduce ecological specificity**



Current methodologies in BNIs screening

Beeckman et al. 2023 J Environm Manag 346:118996

Off-Target Screening (Jurkat, *E. coli*, *A. fischeri*)

Advantages

- ✓ Screens for general cytotoxicity across human, bacterial, and marine models
- ✓ Helps triage toxic compounds early in the screening pipeline
- ✓ Uses established, scalable assays (e.g., CellTiter-Glo, OD600, luminescence)
- ✓ Compatible with high-throughput formats (96-/384-well plates)

Limitations

- Not soil- or plant-representative → **Limited ecological relevance**
- General toxicity ≠ environmental safety in soil/rhizosphere systems
- Jurkat cells (human immune cells) not metabolically comparable to environmental microbes

Current methodologies in BNIs screening

Bioassays based on high cell density AOM cultures

Very low turnaround time
30 min – 2h !

- ✓ High throughput
- ✓ Enabled the discovery of many known BNIs

Rely on very dense AOM cultures → May underestimate true NI potential

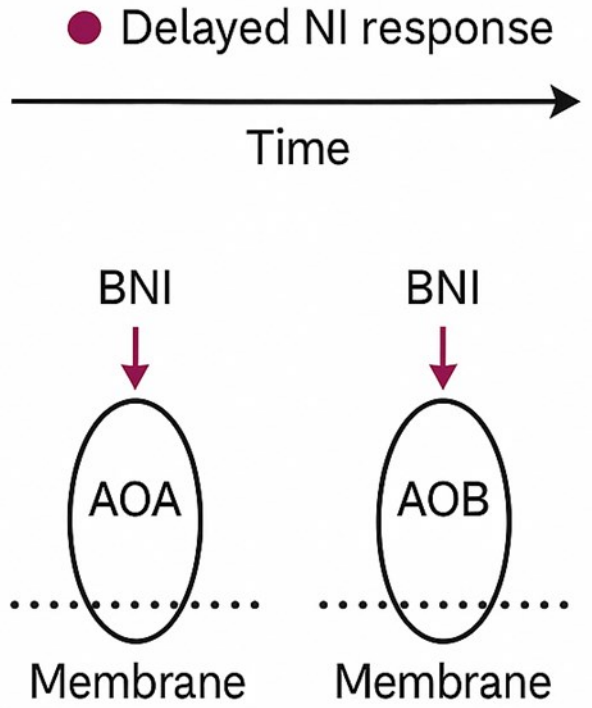
- High metabolic activity can mask inhibitory effects
- Multiple molecular targets complicate interpretation

…Use of indirect endpoints (i.e., bioluminescence) instead of direct nitrification measures

Why exposure time & culture density matters

- **" Inoculum effect":** Dense AOM cultures can reduce perceived NI efficacy (like with antibiotics)

  - High AMO levels may metabolize NIs faster lowering impact e.g., nitrapyrin → 6-chloropicolinic acid
  - Some NIs show delayed AOM responses -> missed in short assays : e.g., 6-chloropicolinic acid: ~8 h
  - BNI uptake influenced by lipophilicity, polarity, and membrane differences (AOA vs. AOB)



✓ **Longer incubations better capture full cellular response and true BNI potential!**

Establishment of a refined fast – track BNI screening system

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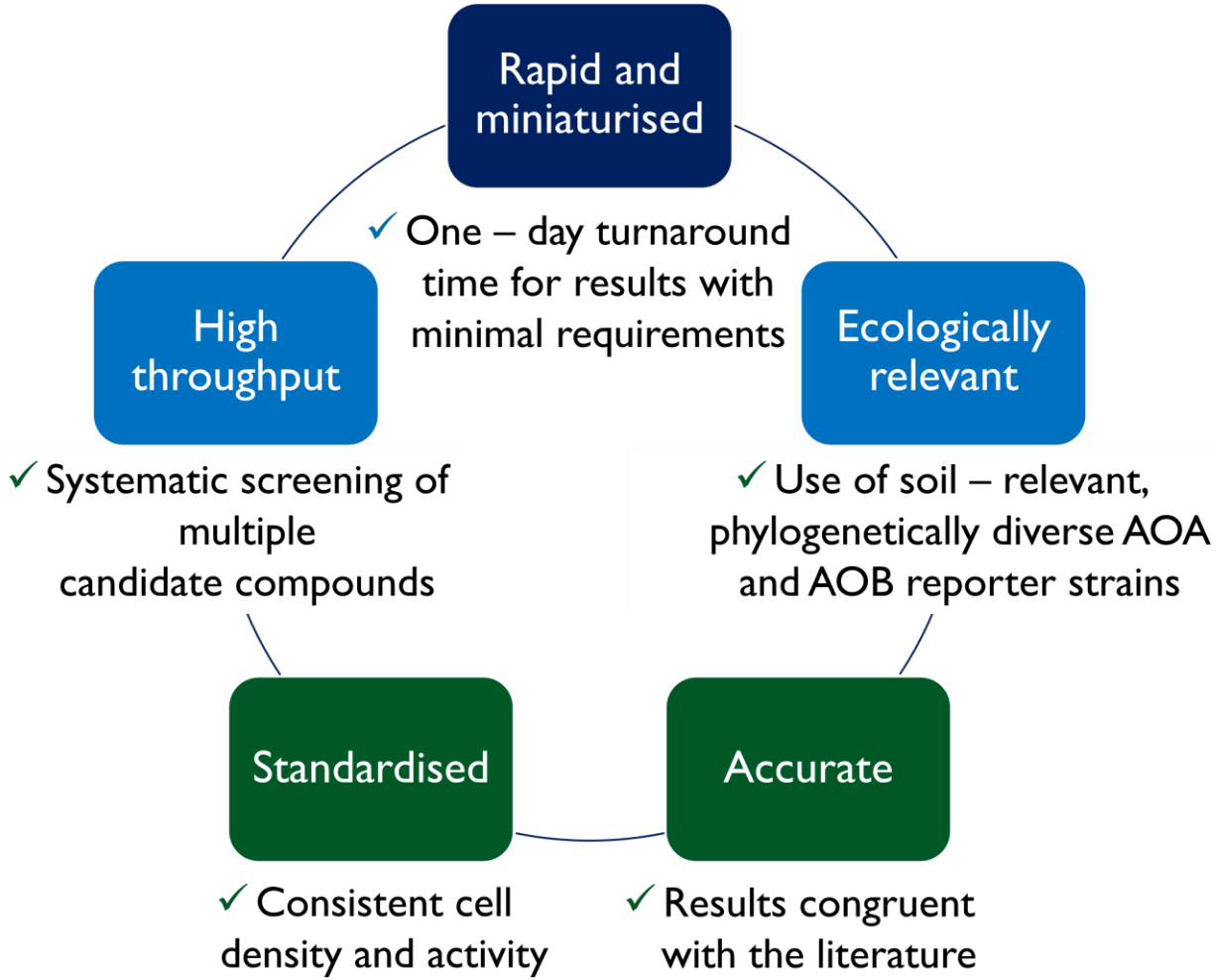
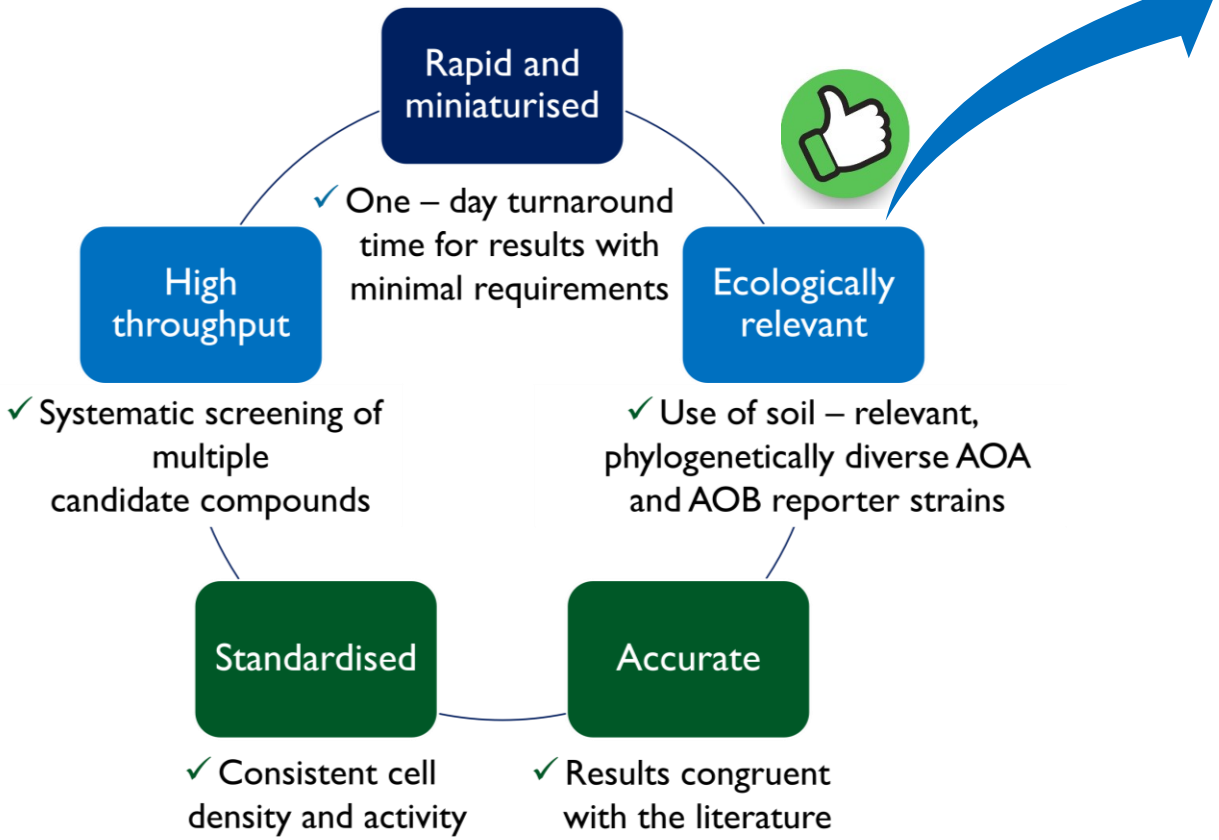
- **Our Aim**: Establish a refined fast – track high-throughput screening system, for the discovery of BNIs, **based on ecologically relevant ammonia oxidizing strains**



ACTION Nr

Establishment of a refined fast – track BNI screening system



Ammonia-Oxidizing Bacteria

Nitrosospira multiformis

- ✓ *Nitrosospira* is the most abundant soil AOB genus
- ✓ Model AOB
- ✓ Employed before in bioassays

Nitrosomonas ureae* and *Nitrosomonas communis

- ✓ *Nitrosomonas is* the second most abundant soil AOB genus
- ✓ Soil abundant representatives compared to *N. europaea*

Ammonia-Oxidizing Archaea

Nitrososphaera viennensis

- ✓ Important contribution to N_2O emissions
- ✓ One of the best studied soil AOA
- ✓ Employed before in bioassays

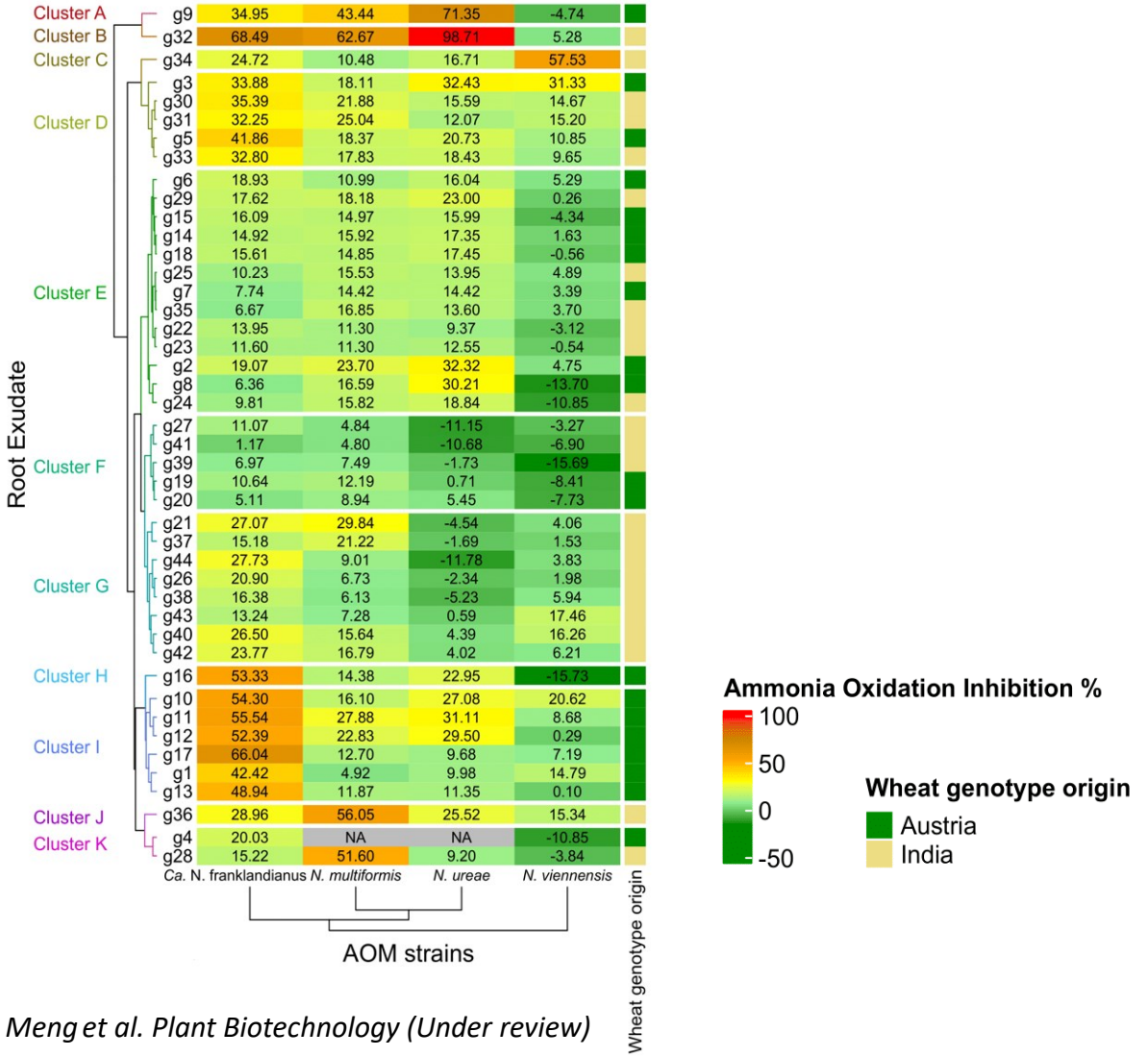
***"Candidatus* Nitrosocosmicus franklandianus"**

- ✓ AOA genus with high autotrophic growth in soil
- ✓ "*Ca.* N. franklandianus" employed before in bioassays
- ✓ *Nitrosocosmicus* are key nitrifiers in the rhizosphere of important crops and distinct to *Nitrososphaera*

Results: Screening of wheat root exudates for BNI activity

Root exudates from 44 diverse wheat genotypes from Austria & India were tested in the AOM fast – track bioassay

Distinct inhibitory patterns grouping root exudates into several clusters.



Ghatak, Kanellopoulos, López-Hidalgo, Malits, Meng et al. Plant Biotechnology (Under review)

Concluding remarks

This platform offers a **reliable and efficient** approach **to identify** plant genotypes and compounds with BNI potential in a more **ecologically relevant** context.

When used alongside more complex testing environments, it can significantly **speed up** the discovery of new BNI compounds and plants that help reduce nitrogen loss, pollution, and greenhouse gas emissions in agriculture.

Future Perspectives

- To enhance ecological relevance, **future versions** of the assay could **incorporate acidophilic AOA strains,** such as *Nitrosotalea sinensis* from the *Nitrosotaleales* lineage.

- Integration of **synthetic ammonia- and nitrite-oxidizing communities** would further mimic real-world microbial interactions, improving the predictive power of the platform.

- Exploring the effects of BNI compounds on **off-target BUT environmentally relevant microbes,** like nitrite oxidizers and methanotrophs, could help identify any unintended ecological consequences.

Key Takeaways

BNI bioassays should incorporate **a broader range of representative microbial strains** to better reflect natural soil diversity and improve ecological relevance.

Longer incubation times are crucial **to capture full cellular responses** and avoid underestimating the true BNI potential of candidate compounds.

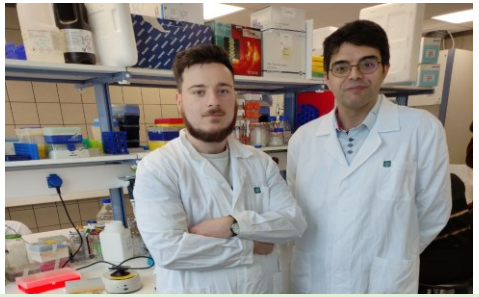
Validation of high-throughput BNI assays is essential and **should include benchmarking estimated inhibition thresholds against known nitrification inhibitors** to ensure accuracy and reliability.

Robust BNI bioassays require **well-established positive and negative controls**, including characterized plant genotypes and known BNI compounds, to ensure assay reliability and comparability.

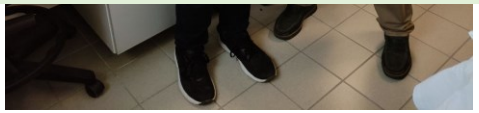
Effective high-throughput BNI bioassays must **balance assay speed with biological depth** to avoid sacrificing accuracy for throughput.

Acknowledgments

Dr. Andrea Malits



Dr. Hugo Ribeiro
MSc Alexandros Kanellopoulos



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Plant & Environmental Biotecnology Laboratory

Dr. Arindam Ghatak

Dr. Palak Chaturvedi

Grantham Foundation for the Protection of the Environment

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Bonus Slide: Critical Thinking Challenge

Can you identify potential advantages and limitations of our in-house BNI screening assay?

???

???

THANK YOU