

IMPACT EVALUATION FRAMEWORK FOR NITRIFICATION INHIBITOR APPLICATIONS

Technical Report from DCE – Danish Centre for Environment and Energy No. 406 2026

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Abstract:	Recommendations from the amoA project to the risk evaluation that any synthetic nitrifications inhibitor should undergo to be assessed as posing minimal environmental risk to groundwater and soil microbiome when used as a greenhouse gas mitigation compound.

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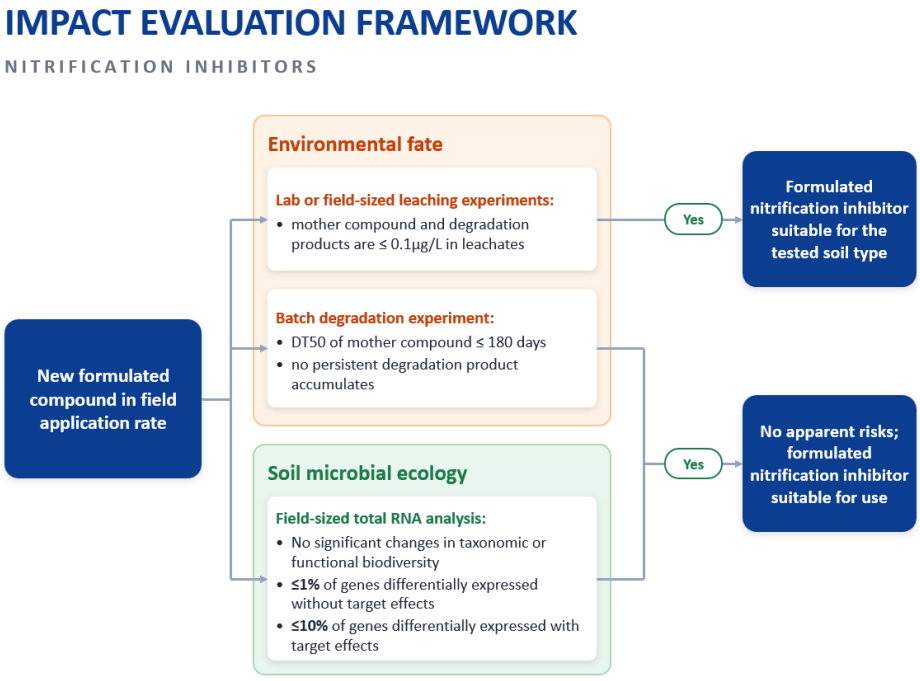
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Background

Nitrification inhibitors have been tested in Denmark in many studies to find out whether they reduce N_2O emission from agricultural fields. All studies agree that the inhibition effect comes with a high variation. This is likely because the compounds inhibit a biological system whose function is highly dependent on soil water content and soil temperature. Nevertheless, an unpublished meta-study by SEGES found N_2O emissions are reduced by 40 % on average when the nitrification inhibitors are applied in relevant Danish soil/crop systems.

Chemical nitrification inhibitors are used precisely because they inhibit a biological process in soil, i.e. the nitrification. In this sense, they act as biocides to control unwanted organisms and thereby their function. Yet, nitrification inhibitors are not regulated under the EU biocide regulation (Regulation (EU) No 528/2012) or the plant protection products regulation (Regulation (EC) No 1107/2009), but under the fertilizer legislation (Regulation (EU) 2019/1009) and are evaluated under REACH (Regulation (EC) No 1907/2006) (Horstmann et al., 2025). This means that the regulation is superficial compared to the biocide- and/or the pesticide-regulation.

The group behind this report recommends that chemical nitrification inhibitors are evaluated with the simple assessment proposed below – to evaluate the likelihood that chemical compounds can be used as nitrification inhibitors to avoid: 1) groundwater contamination and 2) harmful effects on soil biodiversity and ecosystem function. The evaluation of biocides and pesticides addresses a multitude of other risks that will not be covered by this impact evaluation framework.



The two principal areas to evaluate a nitrification inhibitor

The project partners behind this "Impact Evaluation Framework for Nitrification Inhibitor Applications" have made the current suggestion for nitrification inhibitor (NI) regulation that is much cheaper to perform compared to the biocide/pesticide regulation.

The rationale for our suggestions is that the three tests we propose provide the minimum knowledge base for assessing whether a compound is 1) persistent in soil, 2) might contaminate groundwater, or 3) harm other microbial biodiversity or microbially driven processes in soil other than the nitrification.

The suggested requirements for approving the use of a nitrification inhibitor that the project group has defined as a minimum are the following tests in all cases with the formulated compound (the sales product):

- The compound does not have a DT50 value above 180 days, and the compound or any degradation products must not leach to groundwater in concentrations above 0.1 µg per liter, in line with the thresholds applied to pesticides.
- The compound must not affect the soil biodiversity (Shannon and Inverse Simpson indeces) of the active soil microbiome.
- The compound must not affect the active soil microbiome outside the target organisms.

Ad 1) Degradation of the compound should be followed in a batch soil degradation experiment according to OECD TG 307 (OECD, 2002) or using a similar setup as described by Albers et al. (2026), and DT50 values of the parent compound should not exceed 180 days. If the compound is microencapsulated or otherwise formulated for controlled release, the degradation experiment should be performed with the formulated product. Further there should be no accumulation of a persistent degradation product. The leaching experiment should in all cases be done with the sales ready compound, i.e. the formulated compound. For approval on sandy soils without macropores, the leaching can alternatively be done in the laboratory under controlled conditions, as described by Albers et al. (2026), if field or lysimeter studies are not feasible.

In case that the nitrification inhibitor is to be used on all soil types in Denmark, a test should be done on two clay fields in a PLAP-type field system (Badawi et al., 2023, 2025) https://www.vap.dk/language/en/). The monitoring should run for two years, and leaching must not exceed 0.1 µg per liter in the water.

Ad 2) The evaluation of effects of the nitrification inhibitors on the soil microbial community should be carried out under field conditions using the formulated compound mixed with manure in the manufacturer recommended dose. The experiment should compare manure with manure + nitrification inhibitor in a field experiment using a composite sampling technique including at least three biological replicates (European Commission JRC, n.d.). In brief: min. 10 topsoil samples are pooled and homogenized into one composite sample and sieved (4 mm). The sampling depth should be adjusted to the application

depth of the manure, and the number of cores pooled into each composite sample may need to be increased depending on plot size. Sampling should be carried out at a minimum of six time points during the growing season, covering initial effects (day 3–7 and day 14–21), intermediate effects (day 28–35 and day 50–60), and long-term or rebound effects (day 90–120 and at the end of the season or after harvest).

Soils are frozen in liquid nitrogen in the field and freeze dried prior to co-extraction of RNA and DNA using a commercial kit (Bang-Andreasen et al., 2020). RNA is prepared as cDNA for short read Illumina sequencing aiming for at least 10 gigabase sequencing depth per sample. After sequencing the total RNA dataset is processed using a bioinformatic pipeline (Jiménez, 2023) separating into ribosomal RNA (rRNA) and messenger RNA (mRNA) to allow for assessment of taxonomic and functional changes. rRNA sequences are assigned taxonomy across bacteria, archaea, fungi, protists and metazoa, and mRNA reads annotated against appropriate functional databases (e.g. eggNOG). Both rRNA and mRNA counts are normalized for differences in sequencing depth.

For the rRNA data, alpha diversity (Shannon and Inverse Simpson indices) and overall community composition, assessed using a PERMANOVA based on an ordination plot (e.g. NMDS), should not differ significantly between manure and manure + NI treatments at any time point. If differences occur, the community in the manure + NI treatment should return to that of the manure treatment by the end of the growing season.

Functional (mRNA) alpha diversity is calculated on annotated and unannotated mRNA contigs following previous approaches (Finn, 2024) and should also not show significant difference between manure and manure + NI treatments.

Differential expressions of mRNA genes are tested using the DEseq2 (Love et al., 2014) package in R. In general, differential expression analysis should reveal significant up- or downregulation ($|log2FC| > 1$; $padj < 0.05$) in no more than 1 % of all tested genes (annotated and unannotated) when comparing manure + NI treatments with manure alone at each time point. If the number of downregulated genes exceed 1 %, a significant effect on the nitrifying organisms needs to be proven on either taxonomic or functional level. A significant up or downregulation of more than 10 % of all tested genes is considered unacceptable, regardless of any demonstrated target-specific effects.



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Recommendations from the amoA project to the risk evaluation that any synthetic nitrification inhibitor should undergo to be assessed as posing minimal environmental risk to groundwater and soil microbiome when used as a greenhouse gas mitigation compound

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