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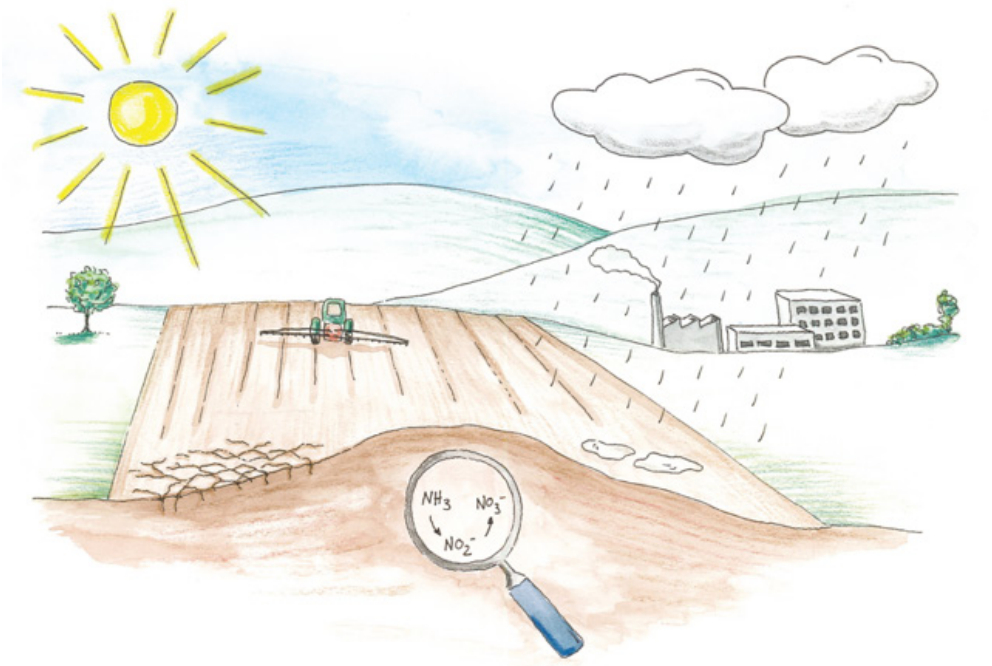
Ecotoxicity of pollutants on soil nitrifiers under a climate change scenario

Laura Johanna Müller



Ecotoxicity of pollutants on soil nitrifiers under a climate change scenario

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Abstract

Soil microorganisms are essential for soil functioning but face increasing stress due to e.g. pollution and climate change. Yet, a scientific basis for how to assess impacts of environmental stressors on soil microorganisms is lacking. Nitrification, the oxidation of ammonia via nitrite to nitrate, is a process commonly shared between ammonia oxidising archaea (AOA) and bacteria (AOB), and nitrite oxidising bacteria, primarily *Nitrobacter* (NIB) and *Nitrospira* (NIS). Although these guilds are suggested as indicators of soil functioning, little is known about their combined responses to stressors, especially to multiple stressors, and effects on associations between ammonia and nitrite oxidisers. This thesis aimed to determine how soil microorganisms, particularly nitrifiers, respond to single and multiple stressors, using a resistance and resilience framework. Soils were exposed to contamination of herbicides, polycyclic aromatic hydrocarbons (PAH), or copper (Cu), and to drying-rewetting cycles in meso- and microcosm experiments. The results show minimal herbicide effects, but PAH altered total prokaryotic, AOA and AOB community composition at high contamination levels, while Cu caused major decreases in ammonia oxidation and community shifts even at low contamination levels. Drying temporarily decelerated ammonia oxidation and altered AOA community composition, indicating low resistance. However, rewetting restored ammonia oxidation, indicating resilience, but caused persistent shifts in NIS community composition, suggesting low resistance and resilience. Network analysis revealed drought effects on co-associations between ammonia and nitrite oxidisers, which could suggest a destabilised interaction. Drought effects were influenced by soil properties and contamination legacy. While herbicides affected the subsequent responses of the nitrifier guilds to drought only marginally, PAH and Cu displayed moderate to strong legacy effects. Overall, the findings emphasise the importance to consider stressor effects on soil microorganisms and subsequent consequences for soil functioning and N fluxes under both single and multiple stressor scenarios.

Keywords: soil microorganisms, nitrification, ammonia oxidising microorganisms, nitrite oxidising bacteria, drought, herbicides, PAH, copper, multiple stressors, environmental risk assessment

Ekotoxicitet av föroreningar på nitrifierare i mark i ett scenario med klimatförändringar

Sammanfattning

Markmikroorganismer är viktiga för markens funktion men utsätts för ökande stress på grund av bl a föroreningar och klimatförändringar. Trots detta saknas vetenskaplig grund för hur effekter av miljöstressorer på markmikroorganismer bör bedömas. Nitrifikation, oxidationen av ammoniak via nitrit till nitrat, är en process som oftast delas mellan ammoniakoxiderande arkéer (AOA) och bakterier (AOB), och nitritoxiderande bakterier, främst *Nitrobacter* (NIB) och *Nitrospira* (NIS). Trots att dessa grupper har föreslagits som indikatorer för markfunktion, är kunskapen om deras kombinerade respons på stressorer, särskilt om de utsätts för flera, och effekter på interaktioner mellan ammoniak- och nitritoxiderare låg. Denna avhandling syftade till att undersöka hur markmikroorganismer, särskilt nitrifierare, svarar på enskilda och multipla stressfaktorer i kontexten av resistens och resiliens. Jord kontaminerades med herbicider, polycykliska aromatiska kolväten (PAH) eller koppar (Cu), samt utsattes för torka och återvätning i meso- och mikrokosmosexperiment. Effekter av herbicider var minimala, men PAH förändrade sammansättningen av de totala prokaryota, AOA- och AOB-samhällena vid höga kontamineringsnivåer, medan Cu minskade ammoniakoxidationen och orsakade omfattande samhällsförändringar även vid låga nivåer. Torka minskade ammoniakoxidationen och förändrade AOA-samhällenas sammansättning, vilket indikerar låg resistens. Återvätning återställde dock aktiviteten, vilket indikerar resiliens, men orsakade beständiga förändringar i NIS-samhällenas sammansättning, vilket tyder på låg resistens och resiliens. Nätverksanalys visade effekter av torka på associationer mellan ammoniak- och nitritoxiderare, vilket kan tyda på en destabiliserad interaktion. Effekter av torka påverkades av markegenskaper och förereningshistorik. Medan herbicider endast marginellt påverkade nitrifierarnas respons på torka, uppvisades måttliga till starka effekter i jordar med PAH och Cu. Sammantaget visar resultaten vikten av att beakta stressfaktorers effekter på markmikroorganismer och dess konsekvenser för markfunktioner och kväveflöden under scenarier med en och flera stressfaktorer.

Nyckelord: markmikroorganismer, nitrifikation, ammoniakoxiderande mikroorganismer, nitritoxiderande bakterier, torka, herbicider, PAH, koppar, multipla stressfaktorer, miljöriskbedömning

Dedication

Für Opa Hans.

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Müller L. J., Alicke M., Romdhane S., Pold G., Jones C. M., Saghaï A., Hallin S. (2025). Resistance and resilience of co-occurring nitrifying microbial guilds to drying-rewetting stress in soil. *Soil Biology and Biochemistry*, 208, 109846, https://doi.org/10.1016/j.soilbio.2025.109846

II. Müller L. J., Saghaï A., Jones C. M., Hallin S. (2025). Effects of pesticides on functional groups involved in nitrification and their subsequent resistance to drought exposure. (Manuscript)

III. Jones C. M., Müller L. J., Orozco-Hidalgo M. T., Enell A., Larsson M., Taylor A., Viketoft M., Weiss J., Dahlberg A. K., Wiberg W., Bergren-Kleja D., Hallin S. (2025). Resistance and resilience of microbial communities to drought in contaminated soils. (Manuscript)

The contribution of Laura Johanna Müller to the papers included in this thesis was as follows:

I. Designed the study with support from co-authors. Conducted the experiment and most of the lab work with support from co-authors. Analysed the data and interpreted results with support from co-authors. Wrote the first manuscript draft and made revisions with support from co-authors.

II. Designed the study with support from co-authors. Conducted the experiment and lab work with support from co-authors. Analysed the data. Interpreted results with support from co-authors. Wrote the first manuscript draft and made revisions with support from co-authors.

III. Contributed to lab work, data analyses, interpretation of results, and manuscript writing.

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Abbreviations

AOM	Ammonia oxidising microorganisms
NOB	Nitrite oxidising bacteria
AOA	Ammonia oxidising archaea
AOB	Ammonia oxidising bacteria
NIB	*Nitrobacter*-type nitrite oxidising bacteria
NIS	*Nitrospira*-type nitrite oxidising bacteria
Comammox	Complete ammonia oxidising bacteria
AMO	Ammonia monooxygenase
amoA	Gene coding for the A subunit of the ammonia monooxygenase
nxrB	Gene coding for the B subunit of the nitrite oxidoreductase
ERA	Environmental risk assessment
NH_4^+	Ammonium
NH_3	Ammonia
NO_2^-	Nitrite
NO_3^-	Nitrate
ASV	Amplicon sequence variant
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction

1. Introduction

1.1 Soil in the anthropocene

With the onset of the industrial revolution in the late 18th century, human activity and its effect on the Earth's biodiversity and biogeochemical cycles has become clearly noticeable. Since then, in the ongoing time period often referred to as the Anthropocene[1] (Crutzen 2006), human activity has resulted in the transgression of several planetary boundaries – thresholds that define a "safe operating space" for humanity within which the Earth system can remain stable and resilient. Boundaries of processes such as those regulating biogeochemical flows (e.g., nitrogen cycle) and climate have been crossed, partly through perturbations caused by novel entities, that is, entities like synthetic chemicals and pollutants that would not be present in the Earth system without human activity. This significantly increases the risk of triggering large-scale environmental change (Richardson et al. 2023).

The biological and physical integrity of soils is directly threatened due to urbanisation, soil sealing, deforestation, mining activities, or intense agricultural practices. Additionally, consequences of climate change, including more frequent extreme weather events such as droughts, are increasingly impacting soil systems. This thesis focuses on the impacts of contamination, drought, and the combination of these stressors on soil microorganisms.

[1] Throughout this thesis, the term "Anthropocene" is used to refer to the current time with profound impacts of human activity causing climate change, biodiversity loss, or the manipulation of biogeochemical cycles. However, it needs to be mentioned that the term is debated for failing to acknowledge historically and globally unequal contributions, i.e. the role of industrial, capitalist, and colonial systems in driving planetary changes. As an alternative, terms like "Capitalocene" have been proposed to better highlight inequalities (Moore 2016). Nonetheless, I am using the term "Anthropocene" here due to its wider use in the field of ecology and therefore its relevance to the environmental impacts addressed in this thesis.

1.1.1 Climate change – The increasing threat of drought

Climate change is already affecting large parts of the world. Changes in global precipitation regimes cause heavy rain and floodings on the one hand and the absence of rain and dry spells on the other hand (Coumou & Rahmstorf 2012). Extreme droughts are predicted to increase in severity and frequency even in areas previously not affected by severe dry spells (IPCC 2021). In Europe, especially the South is predicted to face increasing drought periods (Fig. 1). Drought is affecting agriculture and is considered one of the major threats to global food production (Lesk et al. 2016).

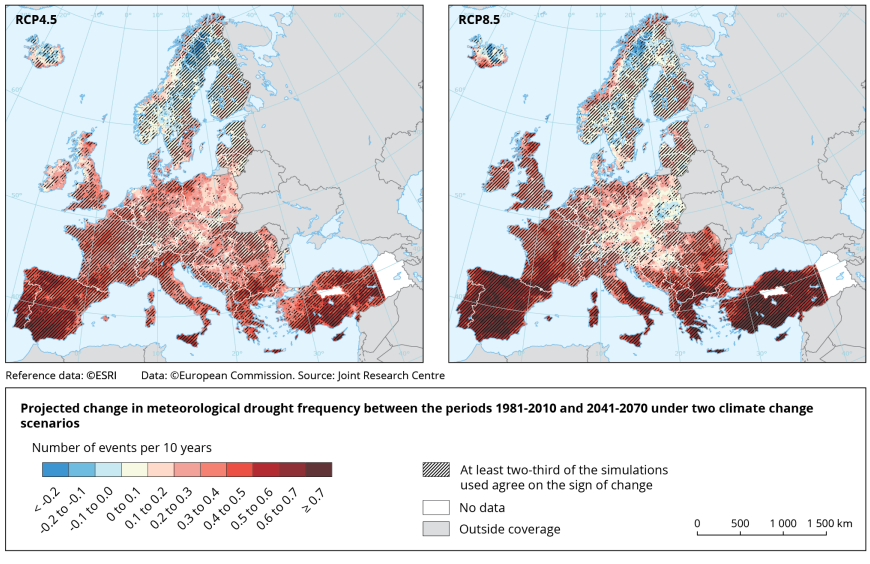


Figure 1. Change in meteorological drought frequency projected for 2041–2070 compared to the reference time period 1981–2010 according to two climate change scenarios RCP 4.5 and RCP 8.5[2]. The figure is reproduced from the European Environment Agency (2023). The material is licensed under CC BY 4.0 License (https://creativecommons.org/licenses/by/4.0/).

[2] RCP 4.5 is a moderate scenario, while RCP 8.5 is a high-emission scenario with continuously rising greenhouse gas emissions in the 21st century.

1.1.2 Soil contamination

Anthropogenic activity includes the intentional and unintentional deposition of a variety of substances into the environment. Current estimates indicate that there are over 2.5 million potentially contaminated sites across Europe (Ballabio et al. 2018). Until now, 342,000 contaminated sites have been identified, with heavy metals and mineral oil being the most common pollutants found in 59 % of the sites (Ballabio et al. 2018). Potential consequences of contamination include risks for human health and adverse effects on above- and belowground biodiversity. Some compounds unfold their toxicity after years of accumulation or after partial degradation, whereas others become toxic in concert with other compounds that can have been independently applied both temporarily and geographically (Fenner et al. 2013). In addition to chemical and photochemical processes, microbial degradation is of profound importance to remove pollutants from the environment (Fenner et al. 2013). However, some transformation products can be more toxic or more persistent than the parent compound (Fenner et al. 2013; Vasileiadis et al. 2018), and not all contaminants are degradable. Heavy metals, for instance, are non-degradable and thus very persistent in the soil. Environmental pollutants are posing a threat to agricultural soils and causing concerns about harmful effects on soil biota (Vieira et al. 2024), with consequences for associated ecosystem services. Soil contamination can lead to altered microbial communities; however, the effect strongly depends on the contaminant and – for some contaminants – on the levels of exposure (Tobor-Kapłon et al. 2005; Mertens et al. 2010; Tomco et al. 2016; Sim et al. 2022). Nevertheless, knowledge about effects and consequences of pollutants on soil microorganisms is limited, and the assessment of the toxicity on soil microorganisms is lagging behind that of soil fauna, despite their recognised role in soil ecosystem functioning (Bardgett & Van Der Putten 2014; Bünemann et al. 2018). This thesis specifically focuses on soil contamination with three different types of pollutants: herbicides, polycyclic aromatic hydrocarbons (PAH), and metals (copper (Cu)).

Contamination with herbicides

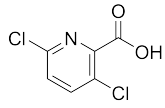
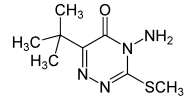
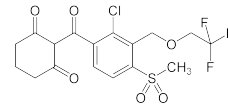
The use of agrochemicals contributes to soil contamination. In the last century, agricultural practices have been intensified worldwide with an overall increasing use of pesticides to combat weeds and other crop pests as well as pathogens (Heinrich-Böll-Stiftung et al. 2022). In a recent EU-wide

soil survey, 75 % of the 3,473 agricultural sites assessed contained residues of at least one pesticide, while 11 % contained residues from more than ten pesticides (Vieira et al. 2023)[3]. These findings highlight both the broad exposure of soils with pesticides and the importance of assessing toxicity effects on soil biota. However, a comprehensive framework for the assessment of pesticide toxicity on soil organisms, especially soil microorganisms, is lacking (see 1.1.4).

Paper II includes the investigation of effects of three herbicides on soil microorganisms (Table 1). Clopyralid is a selective post-emergence herbicide used in a range of crops, for example soy, wheat, and potato, to control broadleaved weeds (Lewis et al. 2016). By mimicking auxin, clopyralid can lead to an overdose in the plant, which results in deregulated and disorganised plant growth and finally death (Grossmann 2010). Clopyralid has no known relevant degradation products/metabolites, i.e. metabolites with potential or known toxicity (Lewis et al. 2016). Metribuzin was used to control weeds, for example in potato, but the approval in the EU was recently not renewed due to concerns for human health and a risk for bees (European Commission 2024). It kills plants by inhibiting photosystem II. Relevant metabolites according to Lewis et al. (2016) are diketo-metribuzin, desaminodiketo-metribuzin and desamino-metribuzin. Tembotrione is a post-emergence herbicide used for example in maize to control broadleaf weeds and grasses (Lewis et al. 2016). It acts as an inhibitor of the 4-Hydroxyphenylpyruvate dioxygenase (HPPD), causing the inhibition of carotenoid synthesis in the plant and therefore photosynthesis itself. HPPD is not only found in plants, but in nearly all aerobic organisms as part of the tyrosine catabolism (Moran 2005). Relevant metabolites are 2-chloro-4-(methylsulfonyl)-3-[(2,2,2-trifluoroethoxy)methyl]-benzoic acid and 2-chloro-4(methylsulfonyl)-3-((2,2,2-trifluoroethoxy)methyl)phenol (Lewis et al. 2016).

[3] Since 2021, there was a slight decrease in pesticide sales in the EU, reaching a relative low of 292 000 tonnes in 2023 (Eurostat 2025). However, due to accumulation and in some cases high half-life times of compounds or their transformation products, the contamination level of soils will only decrease very slowly.

Table 1. Information on the herbicides clopyralid, metribuzin, and tembotrione. Table modified from **paper II**, Table 1.

	Clopyralid	**Metribuzin**	**Tembotrione**
Molecular structure			
Molecular formula	$C_6H_3Cl_2NO_2$	$C_8H_{14}N_4OS$	$C_{17}H_{16}C_lF_3O_6S$
IUPAC name	3,6-dichloropyridine-2-carboxylic acid	4-amino-6-tert-butyl-4,5-dihydro-3-methylthio-1,2,4-triazin-5-one	2-{2-chloro-4-mesyl-3-[(2,2,2-trifluoroethoxy)methyl] benzoyl}cyclohexane-1,3-dione
Substance class	pyridine carboxylic acid	triazinone	triketone
Mode of action	synthetic auxin	photosystem II inhibitor	4-Hydroxy-phenylpyruvate dioxygenase/HPPD inhibitor

Contamination with polycyclic aromatic hydrocarbons

In **paper III**, polycyclic aromatic hydrocarbons (PAH) were selected as an example of comparatively stable contaminants that accumulate in soil over time. They are relevant soil contaminants in Europe, where PAH were reported in 10.9 % of contaminated soils (Panagos et al. 2013). PAH occur naturally, but due to anthropogenic activity, a substantial part originates by now from incomplete fossil- and biofuel combustion or from creosote used to preserve wood. Contamination levels in soils are especially high in industrial and urban areas, thereby posing a threat to human health (Davie-Martin et al. 2017). PAH consist of two or more phenol rings, have low solubility in water, and are very stable. However, microorganisms can degrade PAH and have therefore been used for soil remediation purposes, including bioaugmentation (Hu et al. 2025).

Contamination with copper

Another stable and accumulating soil contaminant is copper (Cu), and its effects on soil microorganisms were also assessed in **paper III**. In Europe, Cu is found in 34.9 % of contaminated soils (Panagos et al. 2013).

Cu contamination can stem from both unintentional and intentional release, for instance due to mining activity or the widespread application of Cu-containing fungicides such as Bordeaux mixture, used predominantly in vineyards and orchards. This explains the high copper contamination of topsoil in Europe, especially in large parts of Italy and the South of France (Figure 2) (Ballabio et al. 2018). While Cu-based fungicides are very effective, affordable, and exhibit low mammalian toxicity, they accumulate in soil and have known negative effects on soil biota (Lamichhane et al. 2018) (see 1.3.2).

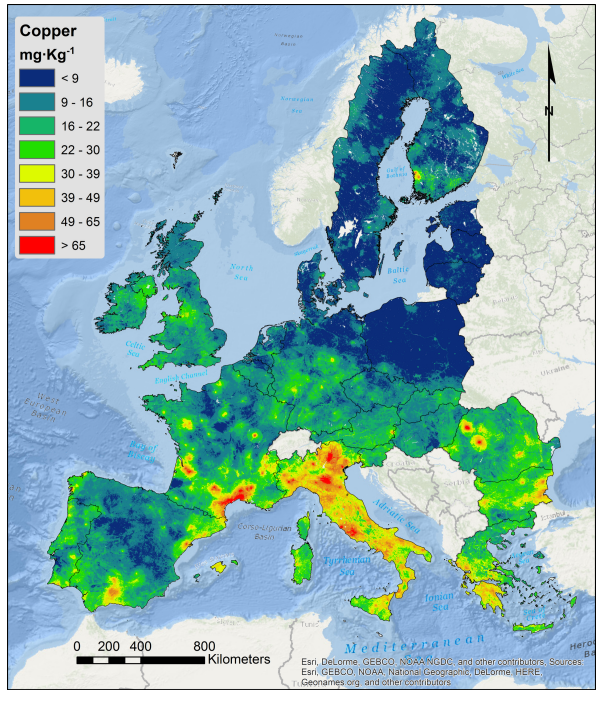


Figure 2. Copper distribution in European Union topsoil based on LUCAS points. Reproduced from C. Ballabio et al. (2018), *Science of the Total Environment*, 636: 282–298. © 2018 The Authors. Published by Elsevier B.V. This is an open access article under CC BY 4.0 License (https://creativecommons.org/licenses/by/4.0/).

1.1.3 Multiple stressor effects on microbial communities

Ecosystems are increasingly affected by anthropogenic activities like the destruction of habitats, introduction of invasive species, and – as mentioned in the sections above – pollution and intensifying effects of climate change. Overlaps in time and space of two or more stressors are becoming more frequent and these multiple stressor scenarios affect terrestrial ecosystems including the microorganisms they harbour (Holmstrup et al. 2010; Rillig et al. 2019; Philippot et al. 2021).

What is a stressor?

Following Piggott et al. (2015), a stressor is a variable that varies stronger than within its normal range due to human activity and by that poses an effect – negative or positive – on parts of or whole ecosystems and/or associated functions. Depending on the research field, terms like "factor" or "driver" are used instead (Orr et al. 2020). Generally, stressors are disturbances and depending on their duration, they can be classified as "pulse" or "press" (Bender et al. 1984). A pulse disturbance is an abrupt and short-term change in a condition, such as a heatwave, drought, or fluctuation in oxygen availability, that often affects resource availability (Jentsch & White 2019). Drought, for instance, has been shown to cause profound shifts in bacterial communities and their function (Fierer & Schimel 2002; de Vries et al. 2018; Séneca et al. 2020; Cordero et al. 2023). When the amplitude of a disturbance decreases and the duration increases, a pulse disturbance becomes a press disturbance, as those are characterised by causing continuous and long-term exposure. Examples of press disturbances impacting terrestrial ecosystems are the climate change induced increase in global temperature and contamination.

Resistance and resilience

The interpretation of stressor effects using ecological concepts, such as resistance and resilience, can facilitate the understanding of the severity of effects. In this thesis and throughout **paper I–III**, resistance is defined as the extent to which a variable remains stable after a disturbance, meaning the stronger the immediate change upon stressor exposure, the lower the resistance. Resilience is defined as the capacity of a variable to return to the original state after a disturbance effect, i.e. the capacity to recover. Ecological systems often respond in a nonlinear and abrupt way to perturbations (Clements & Rohr 2009). The estimation of the threshold at

which a change is finally triggered, e.g. the level of contamination that triggers a shift in microbial community composition, is therefore highly valuable in ecotoxicological research as it improves the predictability of stressor effects. The situation becomes more complex when an additional stressor occurs before a system has returned to its initial state.

Concepts in the assessment of multiple stressors

The estimation of realistic effects of stressors can be erroneous when based on experimentally assessed individual stressors, as a stressor seldom acts in isolation. Multiple stressors can interact in complex ways, resulting in effects that differ from the sum of the individual effects (Galic et al. 2018). If an effect exceeds the sum of the individual effects, it is described as synergistic, while an effect smaller than the sum is described as antagonistic (Folt et al. 1999; Piggott et al. 2015). There is increasing consideration of potential interactions between multiple types of stressors, either concurrent or temporally separated, when assessing their effects on soil functioning (Schaeffer et al. 2016; Rillig et al. 2019).

Higher biodiversity is associated with more variability, hence more options to cope with a given stressor, in particular under unstable conditions. As mentioned in the previous section, the resistance and resilience towards a stressor can be modified by a preceding stressor. This emphasises that multiple stressor dynamics depend on the constitution of the studied system or the state of its recovery before every additional stressor (Tobor-Kapłon et al. 2005). Consequently, the number of stressors and their order matters (Calderón et al. 2018; Rillig et al. 2023).

In addition to the experimentally applied stressors, the choice of complexity of the system under study is crucial as well. Conclusions of a stress impact on a population or community drawn from assessments based on few individuals or species can be incorrect due to, for instance, competitive or mutual interactions between organisms (Holmstrup et al. 2010). However, toxicity studies are often conducted both under “optimal conditions” and on specific populations or model organisms rather than natural communities (EFSA PPR Panel 2013). While this approach is highly valuable for the understanding of specific effects like the dose-response relationship of an individual to one compound or the underlying mechanisms, it rarely resembles a realistic scenario. To bridge this gap between standardised tests and realistic scenarios, risk assessments include “safety” or “uncertainty” factors (Chapman et al. 1998). As both the over-

and underestimation of a risk can have negative effects, it is necessary to improve the scientific basis for the estimation of these factors by increasing the realism of ecotoxicological studies, including soil microorganisms, and – as mentioned above – multiple stressor scenarios (Holmstrup et al. 2010).

1.1.4 Environmental risk assessment in the EU

The European Commission (EC) has implemented an environmental risk assessment (ERA) scheme for compounds intentionally added to the environment. This is following an *a priori* approach, meaning that compounds must be assessed and approved before their release into the market and with that into the environment. The assessment and approval of pesticides – in EC terminology plant protection products (PPPs) – is directed by Regulation (EC) No 1107/2009 (European Commission 2009) and was last updated in November 2022. To be approved, an active ingredient contained in a PPP shall not have "unacceptable effects on the environment", specified as "contamination of surface waters, […] groundwater, air and soil", "impact on non-target species", and "impact on biodiversity and the ecosystem", while exhibiting sufficient effectiveness (ibid., *Article 4*).

For aquatic environments, a comprehensive tiered system, based on a range of OECD tests, guides the ERA (EFSA PPR Panel 2013). The assessment considers both acute and chronic effects, essentially resembling pulse and press disturbance scenarios (see 1.1.3). From Tier 1 to Tier 4, conservatism decreases while ecological realism, and the amount and complexity of data accumulated per tier increases. Tier 1 is based on single-species tests of three fish species, a daphnia, and a green alga, considering, for instance, their reproduction and growth. Conservative assumptions and worst-case scenarios are applied to minimise risks. If toxic effects exceed the acceptable range[4], it is necessary to proceed with higher-tier tests. Tier 2 increases complexity by assessing more species, refining PPP exposure, and considering PPP bioaccumulation. Furthermore, tier 2 includes modelling tools to simulate PPPs' fate and behaviour on and in the organism (so-called TK/DK models[5]). Tier 3 proceeds to experiments at the population and

[4] The definition of an "acceptable range" or a threshold for toxicity is a complex problem and highly critical for the assessment process and exceeds the scope of this thesis.

[5] Toxicokinetic/toxicodynamic models

community level, including models, while Tier 4 is based on field studies and landscape-level models, offering information on the most realistic scenario. However, there is no adequate counterpart of this approach for terrestrial environments. [6]

Call for improvements

While the European Commission provides a framework for ERA of aquatic organisms, requirements for the assessment of terrestrial microbial parameters are scarce, relying on the OECD 216 Nitrogen Transformation Test (OECD 2000). This test has been criticised for being outdated and only suitable to gain rough estimates of possible ecotoxicological effects (Martin-Laurent et al. 2013; Karpouzas et al. 2016), as well as more recently for being inconsistent and soil-dependent in its outcome (Sweeney et al. 2024).

In contrast to indicator organisms of higher order, such as fish or daphnia, it is not possible to pick one microbial species as a model organism representing all microorganisms and apply a similar type of test as those used for animals. This is because effects are highly context-dependent, and the taxonomic composition of microbial communities can vary substantially between soils. For that reason, it has been proposed to focus on specific functional guilds, and ammonia oxidising microorganisms (AOM), performing the first step in the nitrification process in which ammonia is oxidized via nitrite to nitrate, have been suggested as relevant microbial indicators of soil functioning and toxicity of pesticides and pollutants (Wessén & Hallin 2011; EFSA PPR Panel 2017). Beyond their key role in soil nitrification (see 1.2.1) and their sensitivity to external perturbations (Pereira e Silva et al. 2013), the availability of tools to measure their activity, abundance, and diversity has made AOM promising indicators (Pell et al. 1998; Nicol & Prosser 2011; Vasileiadis et al. 2018). Using AOM allows to unravel toxic effects on the soil microbial community with much higher sensitivity compared to the OECD 216 Nitrogen Transformation Test (Pedrinho et al. 2024). There is a call for standardisation of recently developed methods, especially including molecular tools (Thiele-Bruhn et al. 2020), and EFSA has recently published a technical report presenting an

[6] The content of his paragraph is partly based on a lecture by Theo Brock (Wageningen Environmental Research, The Netherlands) – "The tiered approach in aquatic ERA for pesticides", 7th December 2021

"Outline for the revision of the terrestrial ecotoxicology guidance document and for the development of an approach on indirect effects" (EFSA 2025).

The second step of nitrification involves nitrite oxidising bacteria (NOB), and while there is a large body of work on stress responses of AOM communities in soils, fewer studies have examined NOB communities. Their relevance as indicators in ERA is therefore still unclear, but many of the advantages of AOM mentioned above are true for NOB as well. Moreover, dynamics of co-associations between AOM and NOB could give important insights into stress effects on the assembly of cooperating communities involved in a key soil function, further elucidating potential disturbances in N-cycling. However, studies assessing these community-level effects including both AOM and NOB are rare (Fang et al. 2018; Lu et al. 2022; Rijk et al. 2023). The following Chapter introduces the process of nitrification and its organisms in more detail.

1.2 Nitrification in soil

Soil is inhabited by myriads of organisms, ranging from microscopic ones like bacteria and archaea, via fungi (greatly varying in size), protists, springtails and nematodes, up to earthworms. While some groups include pathogens or organisms contributing to unwanted processes like greenhouse gas emissions, a substantial part of soil organisms carry out processes supporting ecosystem function and services (Bardgett & Van Der Putten 2014; Bünemann et al. 2018).

Nitrification is an aerobic microbial process in the global nitrogen (N) cycle involving the oxidation of ammonia (NH_3) to nitrite (NO_2^-) and then to nitrate (NO_3^-; Figure 3). It plays an important role in determining the fate of N, especially in agricultural soils where high amounts of N are added through fertiliser application. While fertilisation is essential to ensure high crop yields in intensely managed agricultural systems, only less than half of the N inputs are used by plants (Lassaletta et al. 2014). As a result, the amounts of fixed N released to the environment are by far exceeding planetary boundaries (Richardson et al. 2023). Nitrate, being the end-product of nitrification, is mobile in soil due to its negative charge and similarly that of the soil particles. This leads to NO_3^- leaching, causing eutrophication and groundwater pollution (Kanter et al. 2020). If NO_3^- is further reduced via

denitrification, N is lost from the ecosystem as gaseous N, of which some can be in the form of the potent greenhouse gas nitrous oxide (N_2O).

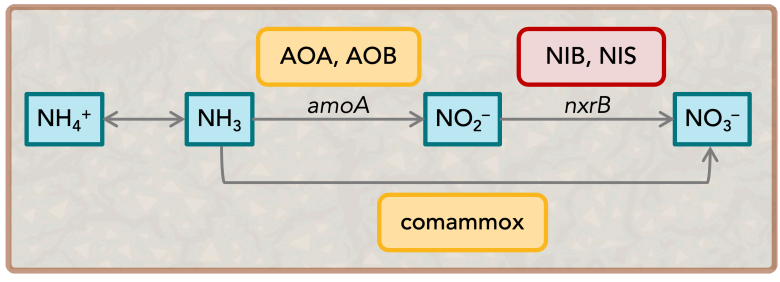


Figure 3. Nitrification in soil, simplified scheme. Ammonia (NH_3, in a pH-dependent equilibrium with ammonium, NH_4^+) is oxidised to nitrite (NO_2^-) by ammonia oxidising archaea (AOA) and ammonia oxidising bacteria (AOB). Homologs of the involved functional gene *amoA* are used as marker genes. Nitrite is further oxidised to nitrate (NO_3^-) by nitrite oxidising bacteria, in soil mainly belonging to the phyla *Nitrobacter* (NIB) and *Nitrospira* (NIS). Here, the involved functional gene *nxrB* is used as a marker gene in two variants. Complete ammonia oxidising bacteria (comammox) can perform the whole process.

In agricultural soils, nitrification is an unwanted process, and in fact, a huge effort is spent on the development of nitrification inhibitors (Lakshmanan et al. 2025) and agricultural practices aiming to improve N use efficiency. Furthermore, nitrification is extensively studied because disturbances of this process in soil can impact global N cycling, with potential environmental and climate implications.

1.2.1 Ammonia oxidising microorganisms

AOM carry out the first and rate-limiting step in nitrification and can be divided into three groups of chemolithoautotrophs, namely ammonia oxidising bacteria (AOB), ammonia oxidising archaea (AOA) and comammox bacteria (Kowalchuk & Stephen 2001; Prosser & Nicol 2008; Daims et al. 2015). The key enzyme in this process is the membrane-bound ammonia monooxygenase (AMO) found in all AOM. This enzyme enables the first step of ammonia oxidation, where ammonia is transformed to hydroxylamine (NH_2OH). The *amoA* gene, encoding subunit A of the AMO,

is used as a marker for AOM, with distinct homologs found in bacteria and archaea.

Ammonia oxidising bacteria (AOB)

AOB were the first organisms recognised for their importance in ammonia oxidation by Frankland and Frankland (1890) and Winogradsky in 1890. Terrestrial AOB are mainly found within Betaproteobacteria, a class within the phylum Pseudomonadota (former Proteobacteria). The bacterial AMO consists of three subunits, which are encoded by multiple gene clusters containing *amoA*, *amoB*, and *amoC* genes (Norton et al. 2008). While AmoA and AmoC are likely to be integral membrane proteins, AmoB is a periplasmic and copper-dependent protein (Balasubramanian et al. 2010), making copper crucial for bacterial ammonia oxidation (Ensign et al. 1993). The next step in this oxidation process is the conversion of hydroxylamine to nitric oxide (NO), catalysed by the enzyme hydroxylamine dehydrogenase (HAO). Within the "NH_2OH obligate intermediate model", N_2O was previously assumed to be the end product of HAO, making AMO and HAO the complete set of required enzymes. However, Caranto and Lancaster (2017) could show that NO rather than N_2O is the enzymatic product of HAO and that NO is even required as an intermediate in ammonia oxidation. For this reason, they suggest the necessity of a third enzyme, catalysing the conversion of NO to NO_2, and an updated model, the "NH_2OH/NO obligate intermediate model". In addition to ammonia oxidation, AOB also perform processes described as nitrifier denitrification, where they convert nitrite under aerobic conditions further to nitric oxide (NO), N_2O, or nitrogen gas (N2).

Ammonia oxidising archaea (AOA)

The role archaea play in ammonia oxidation was discovered much later than for bacteria. Treusch et al. (2005) first reported the existence of an archaeal version of the *amoA* gene, leading to the assumption that some archaea can perform the first step in nitrification. The same year, the first AOA isolate was obtained (Könneke et al. 2005), showing aerobic ammonia oxidation and thereby proving the earlier assumption. All known AOA belong to the class Nitrososphaeria within the phylum Thermoproteota (originally Crenarchaeota and later Thaumarchaeota as proposed by Brochier-Armanet et al. (2008)). Despite their ubiquitous appearance and extensive

phylogenetic diversity, Alves et al. (2018) showed that a few highly abundant taxa dominate AOA diversity on a global scale.

The mechanism for ammonia oxidation in AOA differs from that of AOB, as one of the AMO subunits (AmoB) is lacking a domain stabilising the copper in the active site, as it is the case for the bacterial AMO (Tolar et al. 2017). This leads to an inactive enzyme, at least when isolated (Lawton et al. 2014). However, the AOA genome encodes a fourth kind of AMO subunit, AmoX (Bartossek et al. 2012), where structural similarities to the bacterial amoB could indicate a contribution to copper stabilisation. In addition, homologs of the bacterial HAO are not present in archaea (Schleper & Nicol 2010) and it is not clear yet how hydroxylamine is converted to nitrite (Wright & Lehtovirta-Morley 2023). Copper-containing proteins, such as multicopper oxidases (MCOs) and/or proteins connected to a cofactor F420, which is present in all known AOA genomes (Kerou et al. 2016), are potential candidates.

Comammox

Comammox bacteria are a group of AOM capable of carrying out the complete nitrification process. They are a relatively recently discovered group of microorganisms, although their existence had already been presumed because of energetic advantages (Costa et al. 2006). Daims et al. (2015) discovered and cultivated the first comammox bacterium from the genus *Nitrospira*, the genus to which all currently known comammox species belong. Other *Nitrospira* species are nitrite oxidising bacteria (NOB) without having the capacity for ammonia oxidation, and some might have acquired the machinery for ammonia oxidation – i.e. *amo* and *hao* genes – via horizontal gene transfer from AOB (Daims et al. 2015; van Kessel et al. 2015). Due to their capability to perform all steps in the nitrification process, comammox *Nitrospira* are less dependent on the interaction with other involved microorganisms. Accordingly, genomic analysis revealed a higher diversity of urea transporters and the lack of genes for the ability to use external nitrite as a nitrogen source (Palomo et al. 2018). There are indications for the tolerance for low oxygen environments in comammox (Palomo et al. 2018).

1.2.2 Nitrite oxidising bacteria

The second step in nitrification, the oxidation of NO_2^- to NO_3^-, is carried out by NOB (Schleper & Nicol 2010), whose members are chemolitho-autotrophs found in the genera *Nitrobacter*, *Nitrospira*, *Nitrotoga*, *Nitrococcus*, *Nitrospina*, *Nitrolancea*, and '*Candidatus* Nitromaritima' (Daims et al. 2016). In terrestrial ecosystems, *Nitrobacter* and *Nitrospira* are the most prevalent (Daims et al. 2016; Li et al. 2018) and subsequently referred to as NIB and NIS, respectively. The key enzyme for NO_2^- oxidation is the nitrite oxidoreductase, probably consisting of the three subunits A, B, and C (Sundermeyer-Klinger et al. 1984; Lücker et al. 2010), of which the substrate-binding subunit A is located in the periplasm in NIS, while it is located in the cytoplasm in NIB. For both genera, a distinct version of the gene encoding subunit B, *nxrB*, is used as a marker. The high phylogenetic diversity of NOB is likely caused by lateral gene transfer of the gene coding for the nitrite oxidoreductase (Daims et al. 2016).

Nitrobacter-*type (NIB) and* Nitrospira-*type (NIS) nitrite oxidisers*

The genus *Nitrobacter* belongs to the phylum *Pseudomonadota* (former Proteobacteria), class Alphaproteobacteria. NIB occur in soil, freshwater, marine, and subsurface environments, as well as in engineered systems such as waste water treatment plants (Daims et al. 2016).

The genus *Nitrospira* – containing canonical nitrite oxidisers as well as comammox bacteria – belongs to the phylum Nitrospirota (former Nitrospirae). They consist of seven sub-lineages occurring in soil, freshwater, and marine environments, as well as geothermal springs, subsurface and in engineered systems (Daims et al. 2016). NIS do not only contribute to the N cycle by the oxidation of NO_2^-, as they can also be involved in a so-called reciprocal feeding interaction with AOM by converting urea to ammonia, thereby providing AOM with the substrate NH_3 and subsequently oxidising the product of ammonia oxidation (Koch et al. 2015).

1.2.3 Ecology of nitrifiers

When nitrification is a two-step process, NOB interact with AOM. These interaction patterns depend on multiple factors, including substrate and oxygen availability (Stempfhuber et al. 2016). While there is a large body of literature on AOM communities in soils, fewer studies have examined the

NOB communities and even less the interactions or associations among different groups of AOM and NOB. It is known that AOA are generally positively correlated to NIS, and AOB to NIB (Placella & Firestone 2013; Simonin et al. 2015; Stempfhuber et al. 2017) and that the spatial distribution of nitrifying communities in soil is shaped by associations between specific lineages (Jones & Hallin 2019). However, effects of individual or even multiple stressors on these co-associations are not well investigated.

Ecology of AOM in soil

AOA, AOB, and comammox coexist and exhibit niche differentiation in soils, driven by characteristics like ammonia affinity and mixotrophy (Verhamme et al. 2011; Kits et al. 2017; Palomo et al. 2018) as well as their responses to various edaphic factors (Wessén et al. 2011; Prosser & Nicol 2012; Banning et al. 2015). For some AOA, AOB, and comammox, urea and cyanate can be an alternative N source (Palatinszky et al. 2015; Lehtovirta-Morley et al. 2016). This can be of advantage in low ammonia and acidic environments, as the availability of these alternative substrates is – in contrast to ammonia – not pH dependent (Lehtovirta-Morley et al. 2016). Additionally, guanidine can be the sole source of energy, reductant and nitrogen for the comammox *N. inopinata*, which might be true for most other comammox as well (Palatinszky et al. 2024).

AOA and comammox generally have a higher substrate affinity and tolerance to different levels of substrate availability (Schleper & Nicol 2010), enabling them to grow at various N levels. There are, for instance, indications for an oligotrophic lifestyle of comammox *Nitrospira* (Palomo et al. 2018). Additionally, comammox seem to have a higher growth yield in comparison to canonical nitrifiers (Kits et al. 2017). AOB, in contrast to AOA and comammox, have a much lower substrate affinity. This was, for example, observed in a microcosm experiment, in which growth of AOB was only triggered at the highest level of N addition (Verhamme et al. 2011). Thus, AOB are particularly important in soils with high N content, typically agricultural soils with high inorganic N input. AOB appear to be sensitive to changes in soil pH, and AOA typically outcompete AOB at low pH (Zhang et al. 2012; Banning et al. 2015). The pH effect on ammonia oxidisers could also be linked to their different substrate preferences, as the equilibrium between ammonium and ammonia depends on the pH (pKa of 9.25 for ammonium). The availability of ammonia, the substrate that binds to the ammonia monooxygenase, therefore decreases with decreasing pH. Despite

general trends of niche differentiation between AOA and AOB, recent studies found a surprising variability within AOA, challenging the idea of a clear separation from AOB (Jung et al. 2021; Saghaï et al. 2022; Qin et al. 2024).

Ecology of NOB in soil

NOB are highly versatile and flexible in their metabolic capacity. They are autotrophs and can utilise – apart from nitrite – sulfide, formate, and hydrogen to acquire energy for growth (Daims et al. 2016). NIB and NIS coexist in soils, but they differ in their nitrite affinity and their energetic efficiency when oxidising nitrite. Due to a periplasmic nitrite oxidoreductase subunit A, there is an energetic advantage for NIS compared to NIB, which have a cytoplasmic subunit A (Lücker et al. 2010). This results in a better adaptation of NIS to low substrate conditions. NIS have been found in environments with both low and high substrate availabilities, but high nitrite concentrations select for NIB when conditions are more than temporary, as found in bioreactors (Nogueira & Melo 2006) or in soil after fertilisation (Wertz et al. 2012). In general, NIB show low substrate affinity and a rather high growth rate and maximum oxidation activity, while NIS have comparatively high substrate affinity and low growth rates (Blackburne et al. 2007; Nowka et al. 2015).

1.3 Nitrifiers under disturbance

1.3.1 Drought effects on soil microbes and N-cycling

Drought causes profound changes in the immediate surroundings of microorganisms by altering the physicochemical environment, with contrasting conditions during the drying and rewetting phases. When soil dries out, diffusion rates drastically decrease, restricting the availability of nutrients. This can be especially challenging for bacteria and archaea, as they are limited to their immediate environment and cannot reach nutrients from a distance, like fungi, and thus, largely depend on diffusion.

Besides resource shortage, high osmolality is an important source of stress for microorganisms during drought. Bacteria and archaea utilise a range of coping strategies, including the formation of cysts, dormancy, the build-up of biofilms, or the accumulation of salts (mainly restricted to

Halobacteriaceae) or so-called compatible solutes (Sleator & Hill 2002). Solutes – in contrast to salts – do not interfere with the cell's metabolism when highly concentrated in the cytoplasm and increase internal osmolality (Roeßler & Müller 2001). During rewetting, the external osmolality decreases rapidly, and microorganisms must dispose of the previously accumulated intracellular solutes to avoid cell lysis. The release of these solutes (Halverson et al. 2000) and the resuspension of material from dead cells lead to a nutrient flush upon rewetting, characterised by an increase of carbon compounds and reactive nitrogen (NH_4^+ and NO_3^-) in the soil (Birch 1964).

The recurrence of drying-rewetting cycles can affect the microorganisms' response. It has been shown that soil microbial communities regularly affected by drought are more resistant and/or resilient to recurrent drought (de Nijs et al. 2019; Pezzolla et al. 2019; Canarini et al. 2021; Leizeaga et al. 2022), meaning that microorganisms in soils with no prior exposure to drought can be more strongly affected by a drought event (Canarini et al. 2021).

In general, drying-rewetting cycles alter the composition and structure of microbial communities, affecting microbial respiration and growth (Cordero et al. 2023). Moreover, the contrasting conditions within a drought cycle can affect different microbial groups in various ways (de Vries et al. 2012, 2018; Barnard et al. 2013). The four guilds involved in shared nitrification (AOA, AOB, NIB, NIS) exhibit quite different responses to this stress. Among the strict ammonia oxidisers, AOA are generally more sensitive than AOB (e.g. Thion & Prosser 2014; Bello et al. 2019; Séneca et al. 2020). This can be explained by a generally higher substrate affinity in AOA compared to AOB (Schleper & Nicol 2010; Verhamme et al. 2011), leading to the fact that AOA can be outcompeted by AOB or inhibited upon rewetting. However, there are also studies reporting that AOM abundances are unaffected by drought, indicating high context-dependency, for instance, due to the soil used for the assessment and the duration and frequency of the drought events to which AOM are subjected (Hammerl et al. 2019).

Among the strict nitrite oxidisers, NIS exhibit higher substrate affinity and therefore dominate nitrite oxidation under low NH_4^+ or NO_2^- conditions (Blackburne et al. 2007). However, conditions become unfavourable when NO_2^- accumulates due to a drying-rewetting event (Gelfand & Yakir 2008). Few studies have, however, investigated effects of drought on NOB and are

mainly limited to gene abundances (Placella & Firestone 2013; Séneca et al. 2020; Hafeez et al. 2023). Especially, little is known about effects on the composition and abundance of the four guilds in the shared nitrification pathway and their associations. Likewise, the interplay between various factors, such as drought intensity and frequency, soil type and community composition, is unclear. Given the ongoing climate change and its effects on precipitation patterns, determining how drying-rewetting cycles affect nitrifiers is crucial for the ability to understand and predict global N fluxes (Schimel 2018; Williams & de Vries 2020).

1.3.2 Contamination effects

In comparison to drought, soil contamination is a much more diverse type of stressor, as effects vary substantially between contaminant types and depend on the degree of exposure, that is, the extent to which organisms come into contact with contaminants in the soil. Exposure can be influenced by the contamination level, but also depends on the contaminant's water solubility and adsorption to soil particles (Hu et al. 2025), the latter being influenced by soil pH (Fernández-Calviño & Bååth 2016). Potential toxic effects on soil microorganisms also depend on environmental conditions (Holmstrup et al. 2010) and on the composition of microbial communities (Hallin et al. 2012), guiding direct but also indirect effects (Meyer et al. 2024).

The reduction of contaminant load in soil is influenced by biotic and abiotic soil characteristics as they affect chemical, photochemical and microbial degradation (Fenner et al. 2013). For the latter, a contaminant or its degradation products may also serve as an energy source to the organism performing the degradation or to other community members. If a compound is not degradable, microorganisms can utilise several other strategies. In response to contamination with metals, for instance, they can decrease cell wall or membrane permeability, increase active removal by synthesising efflux systems, bind toxic compounds by intra- or extracellular sequestration or decrease their sensitivity using repair mechanisms (Bruins et al. 2000).

The toxic effect of pesticides depends on their mode of action (Karpouzas et al. 2022). The herbicides clopyralid, metribuzin, and tembotrione used in this thesis have been reported to have no effects on AOM or NOB in pure culture at comparable contamination levels (Bachtsevani 2024). Effects in soil might differ due to reasons described above. However, clopyralid has been reported to not affect AOM abundances in microcosm experiments

(Sim et al. 2022:20; Drocco et al. 2025). Meanwhile, studies assessing effects of metribuzin and tembotrione on natural AOM and NOB communities are rare and show high soil-dependency (Sim et al. 2022).

The toxicity of PAH to microorganisms is assumed to be rather low, as PAH molecules are characterised by low bioavailability in soil, due to their low solubility in water and high adsorption to soil particles (Heipieper & Martínez 2010). Low soil moisture, as caused by drought periods, can reduce the availability of PAH further (Johnsen et al. 2005). However, high PAH levels reportedly affect community composition and cause a decrease in microbial degradation rates (Sun et al. 2023). Furthermore, polycyclic aromatic compounds, including PAH, have been reported to negatively affect nitrification (Sverdrup et al. 2002). Community-level analyses, including AOM and NOB communities, are, however, limited.

Cu is a vital nutrient for AOM, due to its involvement in electron transport in AOA (Hosseinzadeh et al. 2016) and the Cu-dependency of the bacterial AMO (Balasubramanian et al. 2010). However, negative impacts on soil nitrifier function and abundance have been found in Cu-contaminated soil (e.g. Mertens et al. 2009; He et al. 2018; Lu et al. 2022; Rijk et al. 2023) – illustrating the fact that "The dose makes the poison". Despite a relatively large body of literature on Cu contamination effects on overall microbial communities, knowledge about Cu effects on the composition and abundance of nitrifying communities, especially NOB, is limited.

Overall, relatively few studies focus on nitrifiers' response to stress, and very little is known about possible legacy effects of contaminants on microbial communities exposed to an additional stressor like drying-rewetting.

2. Aim of this thesis

The overall aim of this thesis was to assess the effect of single and multiple stressors, applied sequentially, on soil microorganisms, with a special focus on AOM and NOB. Drying-rewetting is used as an example of a stress of increasing concern throughout all papers, where **paper I** focuses solely on this phenomenon, while **paper II** and **paper III** include it as an additional stressor on microorganisms in soil contaminated with herbicides, copper (Cu) and polycyclic aromatic hydrocarbons (PAH). The aim goes beyond examining direct effects of stressors on AOM and NOB, as both resistance and resilience to stress exposure are addressed as well as legacy effects of contaminants on additional stress.

In **paper I**, we focused on drying-rewetting dynamics in four distinct soils and assessed their effect on function, composition and co-associations of AOM and NOB communities. The hypotheses were that different nitrifying guilds would exhibit distinct responses to drying-rewetting stress, with higher substrate availability favouring AOB and NIB over AOA and NIS, respectively, due to their differing environmental preferences. We consequently expected the resulting shifts in community composition to modify co-association among lineages from different nitrifying guilds. Finally, we anticipated these community changes to affect overall nitrification activity. Specific objectives were to identify:

(i) effects of drying and rewetting on the structure and composition of AOM and NOB communities
(ii) effects of drying-rewetting cycles on co-associations between AOM and NOB
(iii) effects of drying and rewetting on ammonia oxidation rates
(iv) general trends across contrasting soil types

In **paper II**, we conducted a two-phase microcosm experiment to assess the effect of the herbicides clopyralid, metribuzin, and tembotrione on the abundance and activity of AOM and NOB (phase I) and then tested whether herbicide exposure affected the resistance and resilience of these functional groups when subjected to subsequent drying-rewetting cycles (phase II). For the latter, we hypothesised that subsequent drying and rewetting will differentially alter nitrifier abundances due to the variation in niche

preferences among nitrifiers, with AOA and NIS being more affected than AOB and NIB by the increase in nutrient content following rewetting (Verhamme et al. 2011; Nowka et al. 2015), and that nitrifying communities negatively affected by the herbicide treatments will be more sensitive to additional stress and thus respond stronger to drying-rewetting than communities not previously exposed to herbicides. Specific objectives were to identify:

(i) effect of the herbicide's mode of action and dose on abundances of AOM and NOB and on the soil ammonium and nitrate pools, which reflect their activity
(ii) legacy effects of herbicide application on drying-rewetting effects on abundances of AOM and NOB, and on the soil ammonium and nitrate pools

Paper III investigated the effect of soil pollution in the form of copper (Cu) and polycyclic aromatic hydrocarbons (PAH) on the total prokaryotic and ammonia oxidising community regarding composition and abundance as well as ammonia oxidation potential in a mesocosm experiment representing phase I. In phase II, contaminated soils were subjected to multiple drying-rewetting cycles. We hypothesise that the application of increasing PAH or Cu levels as press contamination stresses results in distinct compositional shifts in total and ammonia oxidising microbial communities for each contaminant, and that responses to pulse-type drying-rewetting stress are dependent on the nature of the initial contamination stress. Specific objectives were to identify:

(i) resistance and resilience of the total prokaryotic community, as well as ammonia oxidisers in soil to Cu and PAH pollution at different contamination levels
(ii) legacy effects of Cu and PAH pollution on drying-rewetting effects on the composition of total prokaryotic and AOM communities, as well as their relative abundances
(iii) drought effects after multiple cycles
(iv) general trends and differences in effects between contrasting soil types

3. Experimental approach

Soil is a complex matrix, with enormous variation at the global scale due to differences in for example climatic conditions, parent material, soil age, and micro- and macroorganisms (Fierer 2017). However, conditions can vary on a much smaller spatial scale. For instance, soil aggregates contain anaerobic microsites with oxygen concentrations < 1 % whereas the concentration a few millimetres away reaches 20 % (Sexstone et al. 1985). Conditions can also change over both shorter and longer time scales, for example, due to weather events, the activity of organisms, and land-use change. It is assumed that the most important factor that structures soil microbial communities is pH, followed by the quality and quantity of organic carbon, oxygen availability and redox status, soil moisture, the availability of N and P, texture and structure, as well as temperature (Fierer 2017). The plant community, predator-prey dynamics (including protists and nematodes) and effects of viruses are important biotic factors (ibid.).

When studying responses of soil microbial communities to perturbations, acknowledging the high natural variability but simultaneously disentangling it from the effects caused by stressors is a challenge (Caruso & Bardgett 2021). It becomes increasingly difficult to isolate the effect of a specific variable as the number of variables allowed to naturally vary increases. Thus, there is a trade-off between the opportunity to understand specific effects and mechanisms at a smaller scale on the one hand, and generalisability and realism on the other hand. In other words, focusing on specific variables can require artificial controls or conditions and stabilisation of other variables.

3.1 Experimental drought

In studies assessing the effect of drought on soil microorganisms, experiments span a wide range of approaches. Although the lack of standardisation of imposed drought treatments that can be observed across studies (Naylor & Coleman-Derr 2018) can hamper comparisons, drought length and severity vary substantially depending on climate or weather conditions or the soil itself under natural conditions as well. Among the most controlled drought-effect experiments are those exposing microorganisms in liquid cultures to increasing salinity, mimicking increasing osmotic pressure in drying soil (Ilgrande et al. 2018). This reductionistic approach allows to

assess ammonia and nitrite oxidation under different levels of osmotic pressure at the single-species level and in small synthetic communities. When experiments are conducted in closed environments (batch), microorganisms affect the environment by decreasing the concentration of the substrates and increasing the concentration of the products, thereby changing environmental conditions. However, experiments conducted in open systems (chemostats) can offer valuable insights into the physiology of organisms or the ecophysiology of communities. Nevertheless, effects observed in liquid cultures likely do not resemble those in natural settings. Laboratory microcosms under controlled conditions with bare soil (Cordero et al. 2023) or with plants (Munoz-Ucros et al. 2022) gain realism by using soil as a matrix while fully controlling external conditions, whereas rain shelter plots in the field (Beier et al. 2004; Tóth et al. 2017; Bintarti et al. 2025) reduce the control of conditions other than soil moisture. An alternative are outdoor mesocosms (de Vries et al. 2018) that try to bridge the gap between microcosms and the complexity of field experiments. Finally, sampling campaigns along precipitation gradients have also been used (Bachar et al. 2010), but are not actively manipulating or controlling any variables. While geographic, soil, temperature, or other conditions, as well as the soil communities, can vary substantially across natural gradients and need to be considered in the analysis, these investigations offer the highest level of realism, but also face the challenge of disentangling the stressor effects from other effects (Caruso & Bardgett 2021).

*Experimental drought in **paper I–III***

Drought experiments were performed using different types of agricultural soil. In **paper I**, four arable soils with contrasting properties were selected, of which one was also used in **paper II**. Here, the drought experiment was conducted using uncontaminated and herbicide-treated soils. Similarly, in **paper III**, uncontaminated and contaminated soils from two pastures were used. All drought experiments were performed in microcosms incubated under controlled conditions. This level of control was selected as it allowed for assessing a microbial community similar to that in the field while controlling other parameters, such as temperature, humidity, and light, in a climate chamber. Controlled conditions and frequent soil moisture monitoring minimised variation across drought cycles and improved reproducibility as well as comparability between soils. This approach is particularly valuable when working with soils from different geographic

locations, as it allows for the standardisation of external conditions that are otherwise challenging to achieve in field experiments conducted across multiple sites. To further reduce the complexity of the soil system, no plants were added to the microcosms in this thesis. This is a major difference to natural settings, as plants are known to shape microbial communities under drought (Williams & de Vries 2020) and, for example in pastures and grasslands, drought is also shaping the plant community (de Vries et al. 2018). However, by not including plants, interaction effects with drought-stressed plants on the microorganisms are prevented. This decision is further justified by the assumption that nitrifiers are not directly associated with the rhizosphere, because as autotrophs, they do not rely on exudates as a carbon source (Daims et al. 2016; Lehtovirta-Morley 2018).

To keep the drying process in the microcosms as realistic as possible, soils were allowed to dry out naturally during drought periods, without acceleration by elevated temperature or the use of fans, or deceleration by the addition of small amounts of water during drying. The duration of the drought periods was chosen to allow the soil to dry out to the possible minimum soil moisture (**paper I, II**) or to a certain low soil moisture (**paper III**), based on preliminary tests.

3.2 Experimental contamination/toxicity tests

Studies assessing the toxicity of various compounds follow similar approaches as those discussed previously for drought experiments. The most artificial but highly specific laboratory experiments are *in vitro* tests with single species (Bachtsevani 2024), allowing for the assessment of cell-specific effects and the generation of, for instance, dose-response curves and EC_{50} (effective concentration, concentration causing 50% inhibition of test organisms) values. In the context of ecotoxicity tests, this approach is valuable due to the possibility of standardisation and high reproducibility. However, the transferability of the outcome to in-soil effects is limited, as exposure can differ in soil compared to liquid medium, for instance, depending on a compound's adsorption to soil particles, degradation, or fluctuations in soil moisture. Additionally, only direct effects are considered in single-species tests. It was recently shown that toxic effects in communities are mainly indirect, mediated by other microorganisms (Meyer et al. 2024). In microcosms, this is accounted for by using soil as a matrix

(Sim et al. 2022; Meyer et al. 2024) while still controlling external conditions. Internal conditions that change especially in closed systems, for instance, due to the production of nitrate, can, however, not be controlled. Outdoor mesocosms (Rijk et al. 2023), and even more so field experiments provide the highest level of realism, but again, stressor effects can be difficult to interpret. For risk assessments, studies combining effects of specific compounds across levels of environmental complexity in a "lab-to-field approach" (Karas et al. 2018) could be the most relevant – reflecting the tiered risk assessment introduced in Chapter 1.1.4.

*Experimental contamination in **paper II–III***

Approaches for testing the effects of soil contamination on soil microbial communities and functions differed between studies in this thesis. In **paper II**, herbicide effects were assessed in microcosms under controlled conditions, in the same way as the drought microcosms were set up in **paper I** and **II**. This combines the realism of testing in a soil, with an accepted bias, for instance, due to sieving of the soil, with controlling the external conditions, e.g. soil moisture and the initial amount of herbicide per volume of soil. In contrast to more simplified experiments, microcosm experiments allowed for community analysis, which is considered a valuable "midpoint" regarding the level of ecological organisation, between individuals or populations and whole ecosystems (Clements & Rohr 2009). The recommended and 10 × of recommended dose were added following a standard approach to resemble the realistic dose plus a "worst case scenario". As herbicides were known to have relatively low half-life times in soil (Lewis et al. 2016), samples were taken destructively 30 and 70 days after herbicide application.

In **paper III**, another approach compared to the one in **paper II** was chosen due to the different properties of the contaminants. Both PAH and Cu accumulate in soil over long time spans, which affects adsorption and bioavailability of contaminants (Alexander 2000). Two pasture soils were spiked with soil containing high levels of PAH or Cu that had been aged previously to avoid acute toxic effects. To mimic rather long-term conditions, soils were incubated in outdoor mesocosms planted with a grass mixture. Samples were taken on three occasions during 16 months.

*Legacy effects of contamination – Multiple stressor scenarios in **paper II–III***

Experiments in **paper II** and **paper III** examined legacy effects of contamination when subject to a second stressor and included two phases, with the contamination phase I followed by a phase II where uncontaminated soil and soil contaminated with 10 × metribuzin and 10 × tembotrione was subjected to drying and rewetting (drought treatments are discussed in Chapter 3.1). Effects of sequential, multiple stressors are difficult to predict from the assessment of individual stressors, as discussed in Chapter 1.1.3, and contamination and drought stress can interact in various ways. For example, if drought selects for physiological adaptations such as adjustments of the cell membrane composition, previous or simultaneous exposure to contaminants interfering with a cell's ability to make these adjustments will strongly reduce its chances to adapt to and survive drought (Holmstrup et al., 2010). Additionally, microbial activity is strongly influenced by soil moisture, and drought can affect the degradation and fate of pollutants (Johnsen et al. 2005). As a consequence, the persistence of compounds might be prolonged in dry soil, which implies longer exposure times for soil organisms and a possible increase in toxic effects (Franco-Andreu et al. 2016).

4. Nitrifying communities under pressure – New insights

Studies conducted in **paper I–III** showed rather low toxicity of the tested organic contaminants, i.e., the herbicides and PAH, while Cu exhibited stronger effects. Drying and rewetting modified nitrification and microbial communities in a distinct way, and effects changed especially in soils contaminated with PAH and Cu. Overall, results indicate a strong context-dependency of disturbance effects.

4.1 Single stressor effects

4.1.1 The context-dependency of drought effects

Drought is often referred to as a single stressor, although conditions in soil change drastically between the stages of drying and rewetting. During drying, osmotic pressure is high while diffusion rates are low, causing nutrients to be potentially out of reach. When soil is rewetted, diffusion is rapidly re-established and nutrient availability is high. Moreover, oxygen levels and redox conditions fluctuate during drying and rewetting. The frequency of drought also matters, as microbial communities in soils exposed to regular drought have been observed to cope better with additional drought (de Nijs et al. 2019; Canarini et al. 2021)

To evaluate the effect of drying and rewetting across soils on the resistance and resilience of the functional potential for ammonia oxidation (PAO) as well as the abundance and community composition of the functional guilds involved in nitrification and their potential interactions, four contrasting agricultural soils were subjected to different drought treatments in **paper I**, representing the most detailed investigation of drying and rewetting in this thesis. Soils were either kept at constant soil moisture, subjected to one long drought, followed by rewetting, or two shorter droughts (**paper I**, Fig. 1). Insights from **paper I** can be complemented with results from experiments conducted in uncontaminated soils of **paper II** (**paper II**, Fig. 1) and **paper III**. However, in **paper III**, samples were taken only after rewetting in each drought cycle (**paper III**, Fig. S1), which reflects the combined effects of drying-rewetting, and the number of drought cycles was

increased to three to assess effects of recurrent drought. Pronounced differences between drought stages, but also between specific nitrifying guilds, were found across **papers I-III**.

Drought can decelerate nitrification

The nitrate levels measured after drought in **paper I** and **paper II** (phase II) indicated deceleration of nitrification rates. In **paper I**, nitrate levels were significantly lower during drying in the 1 × drought treatment (**paper I**, Fig. S1). However, one soil ('soil S') was unaffected. This was a sandy soil (**paper I**, Table 1), which potentially harboured communities better adapted to drought periods due to higher drying speed in sandy soils and consequently a history of more frequent moisture fluctuations (Peralta et al. 2013; Placella & Firestone 2013). Alternatively, the very low ammonia oxidation potential in this soil (**paper I**, table S3) hampered the detection of a potential community response. An adapted community seems unlikely given the results from **paper II**, where the same soil was indeed affected, showing less nitrate accumulation over time when subjected to drought compared to the moist control soil (**paper II**, Fig. 4, control soil). Similar trends of drought-induced decreases in nitrate levels have been observed in field experiments (Canarini et al. 2021). Bintarti et al. (2025) also reported decreased nitrate levels during drought in organically managed fields but a strong increase in conventionally managed fields with inorganic fertiliser input. Overall, this suggests that effects of drought on soil nitrate levels are context-dependent, potentially shaped by resource levels in the soil.

Even in the absence of plants, nitrate pools are influenced not only by nitrification, but also by processes such as denitrification, dissimilatory nitrate reduction to ammonium (DNRA), assimilation, leaching, or – indirectly – mineralisation. A more targeted approach is therefore the estimation of potential nitrification rates. For this, the so-called 'soil-slurry method' is a standardised approach (Belser & Mays 1980; ISO 2012). However, potential ammonia oxidation (PAO) rates must be interpreted with care. The addition of excessive substrate for nitrification – necessary to avoid substrate limitation during incubation and allow for maximum activity – can cause the inhibition of AOA that are typically active under low substrate levels due to high substrate affinity of the archaeal AMO. If substrate concentration is lower, AOB might not oxidise at their highest possible rates. The full ammonia oxidation potential in soil might therefore never be measured with this method (Hazard et al. 2021). In an attempt to circumvent

the issues with the PAO method, potential rates were estimated using three substrates with increasing complexity (i.e., decreasing levels of direct availability) in individual assays in **paper I**. Regardless of the substrate type, it could be shown that the decrease in nitrate agreed with lowered potential for ammonia oxidation in the 1 × drought treatment (**paper I**, Fig. 2). However, this decrease was not observed in the 2 × drought treatment, possibly indicating a shift in the microbial community after the first drought that increased their resistance to another drought, as observed previously (Canarini et al. 2021).

The functional potential for ammonia oxidation was supplemented with an assessment of the genetic potential. For this, amplicons of functional genes coding for proteins involved in ammonia oxidation (*amoA* gene) and nitrite oxidation (*nxrB* gene, see chapter 1.2) were used to determine the abundance and composition of the AOA, AOB, NIB and NIS communities. Even though DNA-based methods cannot discriminate whether a cell is alive or dead, active or inactive, the analysis of DNA-based amplicons provides a comprehensive overview of the state of a microbial community (Knight et al. 2018). AOA, but not AOB communities, shifted during drought (**paper I**, Fig. 3), and a relatively high percentage of the amplicon sequence variants (ASVs) changed in relative abundance (Figure 4). AOB seem to be more resistant to dry conditions, which is consistent with studies reporting higher sensitivity of AOA compared to AOB to osmotic pressure in pure culture (Bello et al. 2019) and to drought in soil in both microcosms and field experiments (Fuchslueger et al. 2014; Thion & Prosser 2014; Bello et al. 2019; Séneca et al. 2020; Bintarti et al. 2025). The concurrence of decelerated ammonia oxidation and impacted AOA communities suggests an important role of AOA in driving nitrification in the tested soils. As these soils had rather low ammonia content, the findings align with observations that AOA rather than AOB dominate ammonia oxidation in nitrogen-poor soils (Verhamme et al. 2011; Sterngren et al. 2015). Ammonia oxidation in pure cultures or co-cultures involving two strains was not influenced by salinity (Ilgrande et al. 2018). This further supports the hypothesis that nitrogen availability, rather than increased osmotic pressure, was responsible for the observed effects on AOA compared to AOB.

Continuing to the second step of nitrification, studies in **paper I** and **II** revealed different effects of drying on the abundance and composition of NIB and NIS. The composition of NIS and NIB communities showed mini-

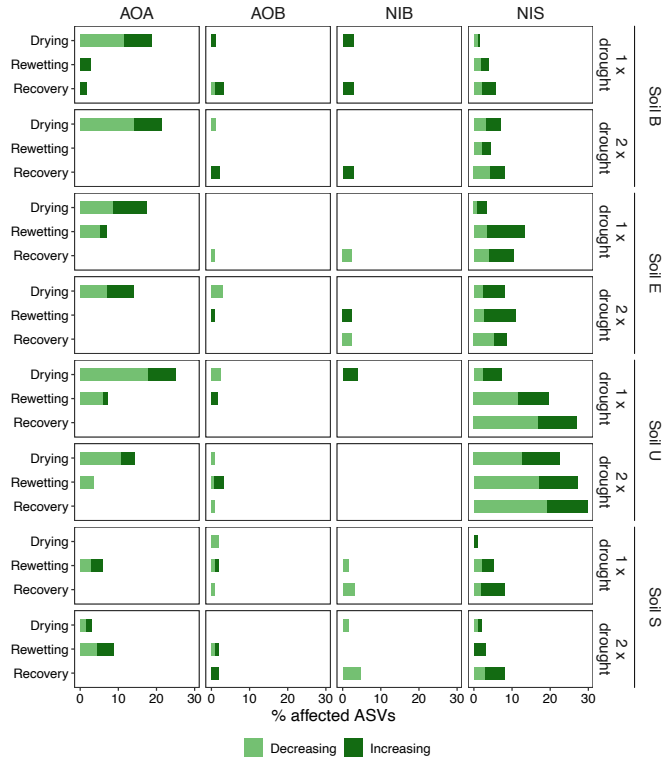


Figure 4. Percentage of amplicon sequence variants (ASVs) of the four functional guilds with significantly affected relative abundance after one long or two shorter drought periods in comparison to the moist control across four soils at the end of the drought treatments (day 42), after rewetting (day 49) and after the recovery phase (day 77). AOA: ammonia oxidising archaea; AOB: ammonia oxidising bacteria; NIB: *Nitrobacter* type nitrite oxidisers; NIS: *Nitrospira* type nitrite oxidisers. Figure reproduced from **paper I**.

minimal impact of drought (**paper I**). Possible strategies explaining a lack of effects might include dormancy (Roszak & Colwell 1987) or mixotrophic growth (Daims et al. 2001; Starkenburg et al. 2008). Nevertheless, the relative abundance of NIB within the overall prokaryotic community increased significantly during drying, whereas NIS was unaffected (**paper II**, Fig. 5). This could be a response to increased substrate concentrations assumed during drought, as NIB are supported by conditions with high nitrite availability (Wertz et al. 2012; Nowka et al. 2015; Simonin et al. 2015). There could also be differences between the two NOB guilds in terms of their capacity to cope with salt stress. However, there are no

indications of especially well-developed osmoadaptation in NOB (Wu et al. 2024).

Rewetting restored ammonia oxidation but negatively affected nitrite oxidisers

Potential ammonia oxidation rates that were reduced during drying recovered within seven days after rewetting in **paper I**. This aligns with reports on a fast metabolic switch of AOM in response to rewetting, indicated by increased expression of both bacterial and archaeal *amoA* genes within hours after drought was terminated (Placella & Firestone 2013). The PAO rates in samples collected two days after rewetting in uncontaminated soils in **paper III** were also not different from the control, but since they were not determined during the preceding drought, it is not known if this reflects recovery. In accordance with the unaffected PAO activity, abundances of AOA and AOB measured by qPCR in soils after rewetting did not differ in droughted soils compared to control soils (**paper III**, Fig. 3, uncontaminated soil). The percentage of individual AOM ASVs that changed in relative abundance was also rather low after rewetting (**paper III**, Fig. 6).

Similar to the ammonia oxidisers, the abundance of NIB showed quick recovery from drought when soils were rewetted, indicating high resilience (**paper II,** Fig. 5, uncontaminated soil). However, rewetting caused decreased abundance of NIS without any recovery (**paper II,** Fig. 5, uncontaminated soil). While some studies reported no or limited effect on NOB abundance (Placella & Firestone 2013; Séneca et al. 2020), Hafeez et al. (2023) observed a decrease and no recovery of NIS abundances while NIB was unaffected, in accordance with results in **paper II**. This difference between NIS and NIB could be due to the expected higher substrate levels, which can pose a disadvantage for NIS due to higher substrate affinity compared to NIB. Rewetting also resulted in strong shifts in community composition (**paper I**, Fig. 3) and relative abundances of NIS ASVs (Figure 4) that did not recover. The substantial share of increasing NIS ASVs challenges the commonly held view that NIS, compared to NIB, thrive in conditions with lower nitrite availability (Wertz et al. 2012; Nowka et al. 2015; Simonin et al. 2015). Rather, this supports the presence of ecological differentiation within NIS at a fine phylogenetic scale, of which not much is known yet (Maixner et al. 2006; Gruber-Dorninger et al. 2015; Jones & Hallin 2019).

The stability of nitrifier co-associations

As described above, drying and rewetting affected distinct nitrifier guilds. These patterns raise concerns about the stability of the shared nitrification pathway as it requires interaction via the provision and consumption of resources between AOM and NOB.

To complement the analysis of individual nitrifying guilds and to estimate the stability of co-occurrences, a network analysis of the most abundant AOM and NOB ASVs was conducted (Figure 5). Networks are increasingly used in soil microbial ecology to complement analysis of diversity and structure, and to improve the understanding of relationships between taxa (Röttjers & Faust 2018), considering, for instance, effects of ecosystem on

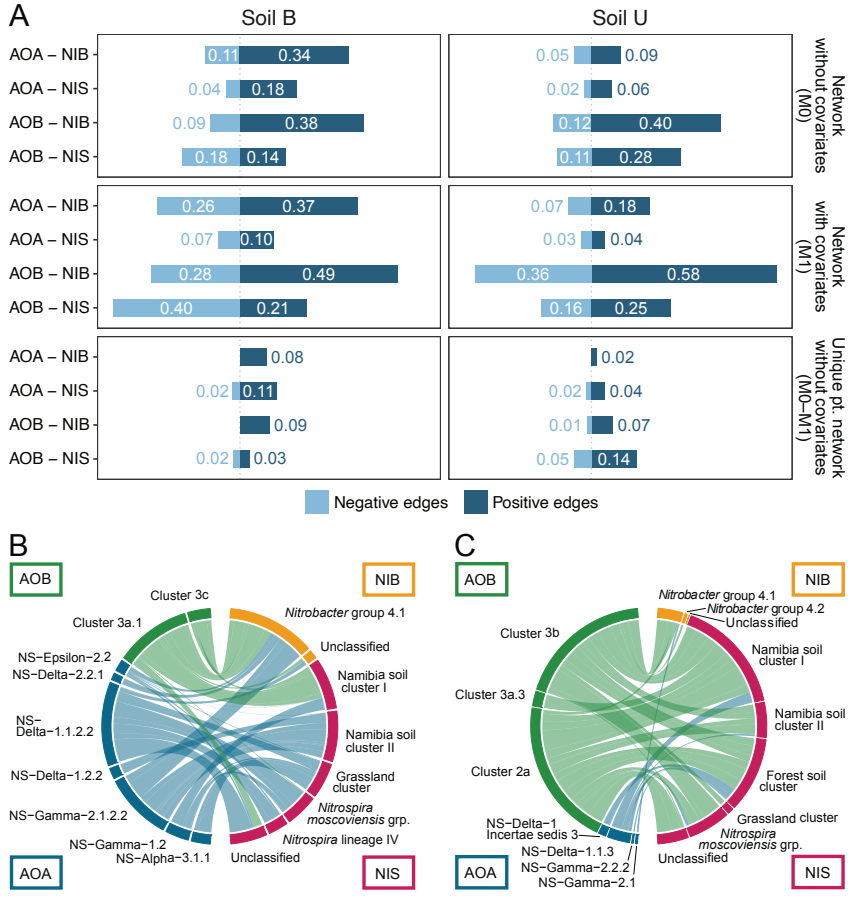


Figure 5. Network analysis. The figure caption can be found on the next page.

total prokaryotic (Barberán et al. 2012) or geographic location on nitrifying communities (Jones & Hallin 2019). In these cases, network 'nodes' represent microbial taxa, and 'edges' statistically significant associations between them. An 'edge degree' informs about the strength and the type of association, where a negative degree indicates inhibitory effects (two taxa fluctuate in opposite directions), and a positive degree indicates co-occurrence (two taxa fluctuate in parallel). In general, caution is advised when interpreting co-occurrences in inferred networks, as they cannot be directly interpreted as interactions (Blanchet et al. 2020). However, in **paper I**, microbial guilds that are known to interact were assessed and network analysis was rather used as a tool to estimate the stability of their interactions.

The network analysis in **paper I** is based on the most abundant AOM and NOB, due to methodological limitations (models cannot handle abundance data with high sparsity), and to indicate the major tendencies and shifts in the soil. Because of missing sequencing data for some samples in two soils, networks could only be built for the other two soils. Per soil, two networks M0 and M1 were built, where M0 was built without covariates, and M1 with the covariates treatment and timepoint, resulting in a model M1 where effects

Figure 5. Network analysis of dominant ammonia and nitrite oxidising microorganisms in two agricultural soils ('soil B' and 'soil U'). **A)** The proportion of edges per node (negative and positive) in the networks M0 (built without covariates) and M1 (effects of treatment and timepoint on nodes and edges were removed by including treatment and timepoint as covariates) and the non-overlapping part of the M0 network, M0-M1 (containing only the nodes and edges affected by treatment and timepoint, obtained by subtracting the nodes and edges not affected by the covariates from the network without covariates). Edges per node were obtained by dividing the edge count of a specific node by the average number of ASVs found in the respective functional group to which the two connected nodes belong (AOA: ammonia oxidising archaea; AOB: ammonia oxidising bacteria; NIB: *Nitrobacter* type nitrite oxidisers; NIS: *Nitrospira* type nitrite oxidisers). Colours denote positive and negative edges. **B, C)** Unique positive network edges (M0–M1), i.e. edges affected by timepoint and treatment, between ammonia oxidisers (AOA and AOB) and nitrite oxidisers (NIB and NIS), summarised per clade and visualised in chord diagrams. Soil B (B): 42 edges; soil U (C): 43 edges. Colours in the ring indicate the functional guilds and edge width indicates edge degree. Figure reproduced from **paper I**.

of the covariates are removed from the network (Chiquet et al. 2019, 2021). This dual-model approach allowed for the identification of edges affected by treatment and timepoint by subtracting those in M1 from the 'full' M0 model. The majority of associations between AOM and NOB were positive (**paper I**, Fig. S6), supporting the assumption that associations are mainly influenced by a mutualistic interaction. However, these co-occurrences were more strongly affected than negative associations by drying-rewetting, indicated by more positive edges per node in the M0-M1 model (Figure 5A). While this might indicate shared niche (Fuhrman & Steele 2008), the decrease in co-occurrences could also signal a destabilisation of associations and decoupling of AOM and NOB. This could, for example, lead to an accumulation of nitrite (Gelfand & Yakir 2008). Previous findings that AOA tends to be associated with NIS (Simonin et al. 2015; Stempfhuber et al. 2017; Jones & Hallin 2019) were not clearly supported here. All combinations were found, with co-associations between AOB and NIB (ibid.) being the most common regardless of the soil in both the 'full' M0 network and in the 'reduced' M1 (Figure 5A). In a soil-dependent manner, positive associations between NIS and both AOA and AOB were affected by drying-rewetting (Figure 5B and 5C), reflecting the high diversity of NIS and their ability to fill various niches (Daims et al. 2016).

4.1.2 Soil contamination

Soil contamination effects on soil microorganisms were assessed in **paper II** and **III**. To improve the understanding of effects depending on the mode of action and dose of herbicides on abundances of AOM and NOB, as well as their activity, a 70-day microcosm experiment was used in **paper II**. Three herbicides (Table 1) were applied to an agricultural soil at 1 × and 10 × of their recommended dose (**paper II**, Figure 1), according to EFSA guidelines. In **paper III,** the resistance and resilience of AOM communities and the total prokaryotic community to more persistent soil contaminants were assessed in outdoor mesocosms spiked with aged PAH- or Cu-contaminated soil studied over a 16-month period.

Due to the nature of the contaminants and resulting similarities in their effects, they are categorised below as organic, including herbicides and PAH, and inorganic, including Cu. Organic compounds are degradable by microorganisms and can potentially serve as an energy source, while Cu as an element is inert and a very stable contaminant in soil.

Organic pollutants – Herbicides and PAH

The organic pollutants decreased during the experiments. The three herbicides had almost completely dissipated ten weeks after application, except for the 10 × dose of clopyralid (**paper II**, Fig. 1). Similarly, PAH compounds had decreased almost entirely at the end of the mesocosm experiment and were likely degraded (**paper III**, Table S3). However, remaining amounts of PAH differed between soils, where especially in the highest contamination level, PAH decreased less in the sandy soil compared to the loamy soil, resulting in 2.5 times difference in content between the soils after 16 months.

After herbicide application, an overall increase in soil nitrate content during the experiment indicated nitrification activity in the soil (**paper II**, Fig. 2). Nitrate content was slightly elevated in soil treated with 10 × clopyralid compared to uncontaminated soil, but apart from that, there was no effect of herbicide addition on the activity. This is consistent with results from single-species assays demonstrating no effect of these herbicides at 1 × and 10 × of the recommended dose on ammonia or nitrite oxidation activity of selected sensitive AOA, AOB, and NIB strains (Bachtsevani 2024). Only the exposure to 10 × metribuzin caused partial inhibition of the AOA strain. Results also align with microcosm studies where AOA and AOB abundances were unaffected whether clopyralid was applied as a pure compound (Drocco et al. 2025), as in **paper II**, or in formulation (Sim et al. 2022). Applied in formulation in a field experiment, clopyralid did not show effects on the total bacterial community either (Tomco et al. 2016). Clopyralid is considered a xenobiotic, as it mimics the plant hormone auxin. Auxin is also involved in plant-microbe interactions – plant growth promoting microorganisms, for example, produce auxin to influence root growth and exudate release (Glick et al. 1998; Spaepen & Vanderleyden 2011). Even though this does not rule out possible adverse effects in different contexts, it suggests, in combination with our results, no direct effects even of high-dose synthetic auxin on nitrifier abundance or activity. Aligning with the results of metribuzin in **paper II**, Lewis et al. (1978) reported no changes in microbial respiration and decomposition rates in soil after application of the pure compound, and Junnila et al. (1993) observed no effects on nitrification activity in a field study spanning various soils. However, the majority of studies on metribuzin in soil focus on the impact of environmental or microbial factors on the pesticide activity and its degradation rather than its effects on soil

microorganisms and their activity (Allen & Walker 1987; Mutua et al. 2016). Despite the wide occurrence of the *hppd* gene also in microorganisms, the HPPD inhibitor tembotrione has shown no effect on the abundance, composition and diversity of total and *hppd* bacterial communities, using 16S rRNA and *hppd* genes as markers (Thiour-Mauprivez et al. 2021), which aligns with the results in **paper II**. In summary, these results and those presented in this thesis indicate low toxicity of herbicides on soil microorganisms, consistent with a recent study (Gaylord et al. 2025).

PAH contamination only affected ammonia oxidation at the highest contamination level. PAO was reduced by 89 % compared to the uncontaminated control at the first sampling time in the sandy soil, and 33 % at the first sampling, and 41 % after 3 months in the loamy soil (**paper III**, Table S5). This decrease at the highest contamination level agreed with a significant decrease in AOA, but not AOB, abundance, reflecting the genetic potential for ammonia oxidation after 16 months in both soils (**paper III**, Table S5). It also coincided with shifts in both AOA and AOB communities (**paper III**, Fig. 2, Table S6) and total prokaryotic communities (Figure 6B) as well as reduced diversity (Shannon) of the total prokaryotic community (Figure 6A). Sverdrup et al. (2002) reported negative effects on nitrification of individual PAH compounds already at soil contents above 20 mg kg^{-1} soil, which is about 7–9 % of the initial levels in **paper III**. Differences within and between studies can be linked to differences in soil properties, specifically characteristics like pH. Under slightly acidic conditions, which applies to the sandy soil in **paper III**, PAH can have stronger adsorption to minerals, leading to differences in exposure, and additionally to lower degradation rates and prolonged exposure (Hu et al. 2025). Indeed, PAH levels were 2.5 times higher in the sandy than in the loamy soil. Over time, PAH degradation or sorption leads to decreased exposure and, therefore, likely effects as well.

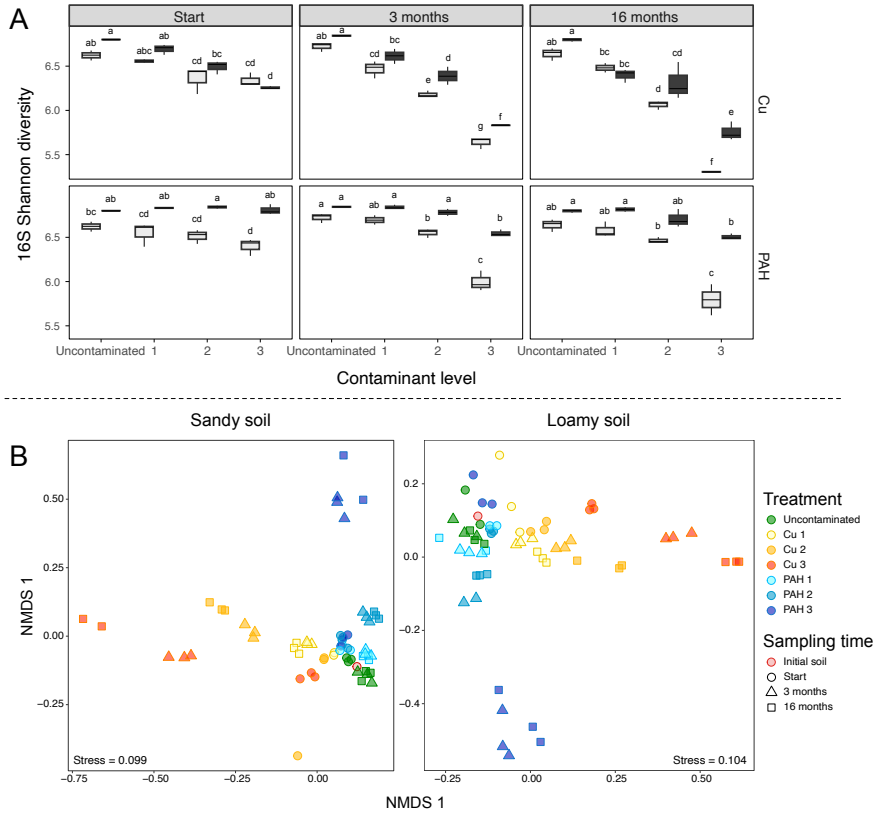


Figure 6. Total prokaryotic community diversity and structure in response to Cu and PAH contamination over 16 months. A) Diversity (Shannon's H′) in soil mesocosms exposed to contamination with Cu and PAH of increasing levels (uncontaminated < 1 < 2 < 3) in sandy (light grey) or loamy soil (dark grey). Letters above boxplots indicate significant differences in mean values per contaminant type within each sampling time across soil types and contamination levels based on Tukey's Honestly Significant Difference tests ($P < 0.05$). B) Differences in the total prokaryotic community structure of sandy and loamy soils in response to Cu and PAH contamination based on non-metric multidimensional scaling (NMDS) of Hellinger distances. Colour denotes treatment, and shape indicates sampling time. Additionally, the red circles indicate initial communities in pristine soils prior to establishment of mesocosms. Results of permutational MANOVAs can be found in **paper III**, table 1 and S6. Figure reproduced from **paper III**.

Inorganic pollutant – Copper

Cu, one of the common heavy metals polluting soils, accumulates and remains stable in soil over long periods. This was reflected in Cu levels at the last timepoint in **paper III**, i.e. 16 months after start of the experiment, where contamination levels still ranged between 71 % and 109 % of the initially targeted levels. Compared to the organic contaminants, Cu contamination exhibited a much stronger effect on soil microorganisms and the soil dependency of Cu effects was not observed to such an extent as for PAH.

PAO consistently declined after 3 and 16 months at both the intermediate and highest Cu levels, independent of soil type (**paper III**, Table S4), confirming earlier observations (He et al. 2018). This was associated with a decrease in AOA abundance. Effects on microbial abundance were particularly strong in the sandy soil, where both total prokaryotic communities and AOA significantly decreased in response to high levels of Cu contamination (**paper III**, Table S4). Diversity and community composition seemed to be more strongly affected in AOB than AOA, especially in the sandy soil (**paper III**, Fig. 2 and Table S6). These findings are consistent with previous studies (Mertens et al. 2010; He et al. 2018; Liao et al. 2019), highlighting the greater overall sensitivity of AOB to Cu contamination in certain soil types. The stronger Cu impact in the sandy, more acidic soil (pH = 5.24) may be explained by the higher solubility of Cu at low pH, leading to increased bioavailability and therefore potential toxicity for microorganisms (Fernández-Calviño & Bååth 2016). Even lower levels of Cu contamination caused detectable changes in the total prokaryotic community diversity and composition in both soils (Figure 6), where communities diverged increasingly from the uncontaminated soil with increasing contamination level and time.

4.2 Multiple stressor effects

The prediction of multiple stressor effects is difficult due to possible synergistic or antagonistic effects. Integrating resistance and resilience to capture effects of contaminants in the context of drying-rewetting in **paper II** and **paper III** revealed some general effects of contamination legacy on microbial responses to drought as a secondary 'pulse' disturbance.

4.2.1 Effects of drought on AOA and AOB in Cu- and PAH-contaminated soils

The strongest modifications of drought effects on the microbial communities were found in **paper III** upon Cu contamination. This phenomenon was visible in both soils, a loamy (pH = 7.99) and a sandy (pH = 5.24) soil, but especially pronounced in the sandy soil. Here, PAO significantly decreased after the second and third drought cycle, while rates did not differ between uncontaminated droughted and control soils (**paper III**, Fig. 3). Although not directly affected by Cu contamination, AOB abundances were influenced by drought in both Cu contaminated soils, but with opposing trends. While they significantly decreased after the first drought in the loamy soil and from then on showed an increasing trend, they decreased after the second drought with a negative trend and no recovery in the sandy soil (**paper III**, Fig. 3). Relative abundances within AOB were affected by drought in both soil types, but especially in Cu-contaminated sandy soil (**paper III**; Fig. S5). Here, changes were in particular found within the Nitrosospira_2a clade, both positively and negatively, with a strict phylogenetic separation (**paper III**; Fig. 6). This division into two sub-clades with decreasing and increasing trends indicated a high level of differentiation within this AOB clade. By contrast, AOA abundances, which were negatively affected by Cu in phase I, were unaffected by drought. There was no strong legacy effect on relative abundances except for low resilience in the Cu-contaminated loamy soil. In fact, increasingly affected relative abundances, while those in the uncontaminated soil recovered until the last time point (**paper III**; Fig. S5). This higher drought sensitivity of AOB compared to AOA was unexpected, as AOB were found to be more resistant to drought in **paper I** and other studies (e.g. Thion & Prosser 2014; Séneca et al. 2020), presumably driven by different preferences in substrate availability (Prosser & Nicol 2012). The reason for stronger drought effects on AOB in combination with Cu contamination is potentially due to different strategies of AOA and AOB to

mitigate Cu toxicity, with implications for their drought tolerance. While genes encoding Cu efflux systems were found in AOB genomes, they appear largely absent in AOA (Shafiee et al. 2021). Instead, AOA have been shown to produce weakly-binding ligands or extracellular polymeric substances to facilitate Cu sequestration (Gorman-Lewis et al. 2019; Dreer et al. 2024), which might provide osmotic protection to some level and thereby enhance the resistance of AOA to drought stress in the presence of Cu. This mechanism could be a form of stress priming (Andrade-Linares et al. 2016), where microorganisms adjust and enter a "primed state" in response to a first stressor, the Cu contamination in this case. This adjustment comes with a physiological cost, but can have benefits when a second stress occurs, as the primed state allows for a better response to the second stress.

Even though AOA and AOB communities in both the loamy and the sandy soil were clearly altered upon exposure to the highest levels of both Cu and PAH contamination (see 4.1), drought effects were less modified by PAH as a preceding stressor than those of Cu and only detected in the low-pH sandy soil and mainly on AOB. The previously discussed longer exposure to PAH in the sandy soil due to higher adsorption and lower degradation rates (see 4.1.2) might be the reason for differences in PAH legacy between soils (Hu et al. 2025). Although the reasoning about differences in the drought response between AOA and AOB under PAH contamination legacy remains speculative, previous work has shown differences between the mechanisms by which aromatic hydrocarbons inhibit the AMO in the two ammonia oxidising guilds (Wright et al. 2020), which could influence this differentiation.

4.2.2 Effects of drought on total prokaryotic communities in Cu- and PAH-contaminated soils

While ammonia oxidising archaeal and bacterial communities responded differently to drying-rewetting cycles across soils and contamination types, the total prokaryotic communities in **paper III** were overall less resilient. Consistent with effects on AOM, Cu caused stronger modifications of drought responses, while the effect of PAH contamination was especially visible in the sandy soil.

Total prokaryotic communities showed progressively increasing dissimilarity with each drought event in Cu-contaminated soils and did not recover (**paper III**, Fig. 4). This points towards a broadly synergistic

negative impact of Cu on microbial resilience to drought (Vinebrooke et al. 2004), irrespective of soil type. Nevertheless, the underlying mechanisms appear to vary between the two soils or their respective microbial communities. This was indicated by a trend of increasing total abundance across successive drought cycles in the loamy soil (**paper III**, Fig. 3), while abundances in the sandy soil slightly declined over time with no recovery. This decline in response to drought may reflect a physiological shift towards stress survival strategies, such as the reallocation of cellular resources at the expense of growth (Schimel et al. 2007) or resource limitations (Fierer & Schimel 2002). An almost 60 times lower shoot biomass in the Cu-contaminated sandy compared to the respective loamy soil in phase I align with a potential resource limitation in the Cu-contaminated sandy soil and subsequent alteration of the drought response. Conversely, cumulative increases in bacterial abundance have previously been linked to the selection of more drought-resilient communities capable of rapid recolonisation upon rewetting (Hicks et al. 2022).

Differential abundance analysis of the dominant OTUs revealed soil- and contaminant-dependent effects on drought responses (**paper III**, Fig. 5), for example, in the phylum Actinomycetota. Increases in relative abundance in both soils and especially under Cu contamination align with multiple studies indicating tolerance to both increased Cu levels and drought of this phylum (Lejon et al. 2008; Barnard et al. 2013; Li et al. 2015; Cordero et al. 2023). Another example of contaminant-mediated alterations of drought effects are relative abundances of Acidobacteriota. In the loamy soil, the majority of affected OTUs within the Acidobacteriota was found under Cu contamination, with a majority decreasing and belonging to the order Vicinamibacterales (**paper III**, Fig. 5B). There are several studies showing general sensitivity to Cu contamination stress in the phylum Acidobacteriota (e.g. Li et al. 2015; Lv et al. 2023), which does not completely align with the greater legacy of Cu contamination on Acidobacteriota OTUs in the high pH loamy soil, where Cu exposure was assumed to lower in comparison to the sandy soil due to lower solubility at higher pH (Fernández-Calviño & Bååth 2016). This potentially indicates differentiation at a finer phylogenetic scale. Additionally, context-dependency as observed here may also be the reason for no or even opposing effects of Cu contamination on Acidobacteriota in other studies (de Boer et al. 2012; Nunes et al. 2016). In the PAH-contaminated sandy soil, the orders Terriglobales and Bryobacterales

increased upon drought, especially after multiple drought cycles, while they showed less consistent response in uncontaminated or Cu-contaminated soil (**paper III**, Fig. 5A). Higher PAH availability after drying-rewetting, as observed by Wang et al. (2018), might be the reason for the increase, especially as Acidobacteriota can degrade PAH compounds at low pH, which reportedly led to an increase in their abundance (Song et al. 2016). Apart from this, modifications of drought response connected to PAH contamination were limited. Considering that PAH contamination of low levels did not affect communities in phase I, and that PAH levels had substantially decreased before the set-up of phase II especially in the loamy soil (**paper III**, Table S3), it might be that the contamination effect was rather transient and that this partly explains the limited degree of drought modification especially in the loamy soil.

4.2.3 Effects of drought on herbicide-contaminated soils

Herbicides tested in **paper II** had minimal effects on nitrifier abundance and activity, as discussed previously (see 4.1.2). In comparison, drought effects were stronger and were, to a degree, dependent on the herbicide treatments in phase I. Metribuzin treatment affected the response of AOM and NOB to drought, with all but the AOB increasing during drying and decreasing after rewetting (**paper II**, Fig. 5). Although not significant, the same trends were observed for the tembotrione-treated soil. As in phase I, nitrate levels increased over time, but the increase was smaller in drought-treated soils compared to the moist controls (**paper II**, Fig. 4). Again, effects were stronger in the metribuzin-treated than the tembotrione-treated soil. Importantly, differences in nitrate content between soils subject to drying-rewetting and soils kept moist were larger in the uncontaminated control than in the herbicide-treated soils. Theoretically, lower nitrate pools could be explained by higher nitrate consumption in the uncontaminated control soil by nitrate-reducing processes, or by lower nitrification due to decreased oxygen levels caused by the release of carbon compounds fuelling heterotrophic respiration, but both scenarios are unlikely. Rather, the mitigation effect can be explained by species co-tolerance (Vinebrooke et al. 2004), where exposure to herbicides might have led to distinct changes in the microbial community composition or activity, but also to the acquisition or expression of traits that improve their ability to cope with drying-rewetting stress. Such changes are, however, not detectable by measuring nitrate pools

or the abundance of AOM and NOB based on DNA. Community analysis could reveal the cause for this difference, as for instance in **paper III** phase I, ammonia oxidation was only affected at higher contamination levels of PAH, while the community composition shifted already at lower levels – indicating an earlier 'silent' effect.

5. Synthesis and perspective

Soil is a vital resource and soil microorganisms play a crucial role for its functioning. This thesis aimed to evaluate the impact of different anthropogenic stressors on microbial communities in agricultural soils, with a special focus on those involved in nitrification. In a series of experiments, soil was exposed to organic and inorganic contaminants as well as drying-rewetting cycles, in order to improve our understanding of the consequences of single and combined exposure to stressors for the resistance and resilience of the nitrifying and total prokaryotic microbial communities. This knowledge is needed to comprehend and predict how climate change and contamination affect soil functioning, including N fluxes in soil. It is also valuable in the frame of soil ecotoxicology research and environmental risk assessment, aiding to fill gaps in our understanding of toxicity mechanisms and context-dependency in soil and to select tools for a comprehensive and standardised risk assessment framework.

The results presented in this thesis show distinct effects of organic and inorganic contaminants on soil microbial communities. Among the organic pollutants, herbicides did not affect the abundance of AOM and NOB nor caused changes in the soil's N pools (**paper II**), indicating low microbial toxicity of these compounds. However, potential "hidden" effects in the community composition might have been missed by focusing solely on abundances. For a more complete assessment, it would therefore be valuable to conduct an experiment using soils with contrasting properties and analyse the community composition and structure of the different guilds.

Negative effects of PAH on nitrifiers were only detected at the highest level of contamination, causing a reduction of PAO and altering the community composition of AOA and AOB (**paper III**). However, decreases in diversity and shifts in the total prokaryotic community composition were apparent already at lower contamination levels and effects increased over time, indicating low resistance to PAH. In comparison with the organic contaminants assessed in this thesis, the consistent decreases in PAO and the strong shifts in community composition of AOA, AOB and total prokaryotic communities across soils at both intermediate and high contamination levels indicate high toxicity of Cu and low resistance among microbial communities (**paper III**). Also here, differences to uncontaminated soils increased over time. Differences in the microbial communities' responses to

Cu between soils were likely connected to pH, as effects were stronger in the soil with lower pH, where the bioavailability of Cu was higher. Results from **paper III** indicate dose-dependent effects for PAH and, to a lesser extent, Cu, with a critical threshold between intermediate and high contamination levels. Regardless of the initial contamination level, PAH content was substantially reduced after 16 months, but with lower reductions in the more acidic soil. Consequently, it might be beneficial for the remediation of PAH-contaminated sites to increase the pH of the soil. Cu toxicity was higher, with stronger effects on nitrification and microbial community composition also at low levels. Effects were stronger in low pH soil, but in contrast to PAH, the soil would not necessarily benefit from pH adjustment, as higher pH increases the stability of Cu in soil.

Drought as a single stressor was investigated in detail in **paper I**, where it was demonstrated that drying temporarily decelerates PAO and affects AOA community composition, indicating low resistance and an important role of AOA in N-poor soils, while AOB and both NIB and NIS were resistant. Subsequent rewetting restored PAO, indicating high resilience, but also caused long-lasting changes in NIS community composition, suggesting both low resistance and resilience to rewetting in this guild. This could either mean that they need more time to recover than the time frame used in the experiment or that the community shifted to a new stable state, better adapted to drought. The latter would have consequences for the response of NIS communities to an additional drought and for the stability of nitrification activity. To assess this further, it would be interesting to conduct a drought experiment including multiple drought cycles with sampling at every dry and rewetted time point, allowing for the assessment of a drought legacy and rewetting legacy in detail over time. Network analysis showed that drying and rewetting affected the co-occurrence patterns of the guilds AOM and NOB involved in shared nitrification. This indicates a potential decoupling in the metabolic interaction between AOM and NOB, which could result in asynchrony in nitrite production and nitrite oxidation. In addition to the methods applied in **paper I**, it would be advantageous to also measure nitrite. If nitrite accumulates during drought and/or rewetting, this would further indicate a decoupling between AOM and NOB. An enhanced understanding of the factors influencing N fluctuations and the stability of microbial communities is highly valuable to inform models predicting, for instance, nitrate leaching or emissions of the highly potent greenhouse gas N_2O.

Results of **paper I–III** also underline that effects of drying and rewetting on soil microbial communities and microbial activity are context-dependent and influenced by soil type and previous exposure to stress. In line with the single stressor experiments indicating higher microbial toxicity of inorganic vs organic contaminants, herbicides and PAH exhibited only minimal to moderate modifications of drought effects, whereas Cu contamination strongly affected drought responses. The multiple stressor scenarios in **paper II-III** focused on stressors applied sequentially, with contamination in phase I and drought in phase II. To get a broader understanding of the effect of multiple stressors on soil microbial communities and because stress effects also depend on the state of a system or community (Calderón et al. 2018), we need to investigate the relative importance of the nature vs the order in which stressors are applied. Likewise, future studies should also assess the effects of stressors applied simultaneously or within short time intervals, giving little room for recovery, two scenarios that are likely to become more common with the increasing frequency of extreme weather events due to climate change. These experiments should ideally be performed across different levels of ecological complexity.

The consideration of a higher number of stressors is crucial for increased realism (Rillig et al. 2023), even though one quickly faces the 'combinatorial explosion problem' with increasing experimental factors. However, computational tools show promising results to simplify experimental setups. For example, based on data from pairwise tests, the effect of a compound mixture with more than two compounds on *E. coli* was predicted, including synergistic and antagonistic effects, with encouraging success (Katzir et al. 2019). Creating stressor combinations by random selection from a pool of individual treatments or stressors, meaning not a full factorial design, is another way to conduct meaningful but feasible multiple stressor experiments and has been done previously (Alsterberg et al. 2017; Rillig et al. 2019).

From a risk assessment perspective, the overall results suggest that the incorporation of both functional measurements and community analysis into the assessment of toxicity or other disturbances on soil microorganisms would be highly valuable. For this, effects on PAO, as an estimation of a functional trait, seem to be rather consistent according to results in **paper I** and **paper III,** to provide insights into soil N fluxes. Community analysis of the functional guilds connected to the measured function, AOA and AOB,

could provide a direct link between the functional and genetic potential upon stress. However, it became evident in **paper III** that community measures of the total prokaryotic community showed higher sensitivity and much more consistent shifts upon contamination and subsequent drought compared to AOA and AOB communities (Figure 6B; **paper III,** Fig. 2; **paper III**, Fig. S4). Functional traits with low redundancy like ammonia oxidation should still be considered valuable indicators for ERA, but at the phylogenetic level, the assessment of a broader community like the 16S rRNA gene-based total prokaryotic community might offer higher depth and sensitivity. Future research should determine if such shifts are linked to functional changes. A remaining challenge for the utilisation of community analysis methods for ERA is the definition of thresholds in community change – how much is too much? And is any change bad? For this, deeper investigations of the severity of a stressor that causes abrupt shifts in microbial communities would be valuable (Clements & Rohr 2009). Additionally, variables that allow for the application of a threshold are needed. However, community composition is often analysed and visualised using ordination techniques, which are helpful to grasp variation or underlying patterns, but are not suitable if a threshold needs to be applied. Measures like the Hellinger similarity used in **paper III** simplify community data by further breaking down the similarity between communities to a measure between 0 and 1. While ecological implications need to be discussed, this method would at least technically be applicable. Furthermore, methods need to be standardised in a regulatory context to allow comparability and ensure the legal recognition of results. Calls for standardisation of methods targeting soil organisms – including microorganisms – and their functions and diversity exist (e.g. Philippot et al. 2012; Griffiths et al. 2018). A progression in this field is needed to allow adequate monitoring and especially the prevention of further contamination of soils, as it is also emphasised in a recent call of the European Environment Agency (2024).

Improvements in ERA procedures should be complemented with the development of less harmful compounds or alternative systems to control weeds and other crop pests and pathogens. Examples for products that often exhibit lower persistency and less effects on non-target organisms are biopesticides, which are products with pesticidal activity that are nature-identical or derived from natural materials, or biological control agents, which can, for instance, be fungal species used to control other – pathogenic

– fungal species. Another option is the development of synthetic compounds with high specificity and an absolute minimum of non-target effects. For this, data on toxicity effects on soil microorganisms are useful to feed into emerging tools that can predict pesticides' transformation products as well as the toxicity of pesticides and their transformation products, for example, on nitrifiers (Zhang & Fenner 2023; Zhang et al. 2025). This could allow for the prediction of optimised compounds. Nevertheless, effects on nitrifiers are not the only effects in soil that should be analysed. For other organisms like fungi, for instance, arbuscular mycorrhizal fungi, a similar line of research exists (Malfatti et al. 2021). Additionally, microbial interactions and potential indirect effects (Meyer et al. 2024), trophic levels, predator-prey interactions with protists, or the impact of nematodes should be considered.

The results presented in this thesis enhance our understanding of the effects of individual and multiple stressors on soil microorganisms, their functioning and N dynamics in soil. This ultimately contributes to a scientific base for the implementation of a regulatory framework informing decision-making processes for the purpose of soil conservation.

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Popular science summary

Soil provides the base for global food production. In the soil, we find myriads of organisms including microorganisms like bacteria and archaea. They perform various processes, for example cycling of plant nutrients. The major nutrient nitrogen comes in many forms, and microorganisms are involved in most of the transformations between forms in what is called the nitrogen cycle.

Nitrification is a process in which microorganisms transform ammonia to nitrite and then further to nitrate. This is a key process influencing the fate of nitrogen in soil. While ammonium is relatively stable in soil, nitrate is easily washed out with rain, which leads to groundwater contamination and eutrophication of rivers and lakes. Nitrification is commonly shared between different microorganisms: ammonia oxidising archaea (AOA), ammonia oxidising bacteria (AOB), and different types of nitrite oxidisers, in soil primarily within the genera *Nitrobacter* (NIB) and *Nitrospira* (NIS). Ammonia oxidisers and nitrite oxidisers interact with each other due to their production or consumption of nitrite. Nevertheless, AOA and AOB as well as NIB and NIS can exhibit quite different environmental preferences.

Human activity is putting soil systems increasingly under pressure. Climate change is causing more frequent and severe droughts, whereas agricultural and industrial activity introduce pollutants, such as pesticides, polycyclic aromatic hydrocarbons (PAH), and heavy metals. These so-called stressors can affect soil microorganisms and processes, including nitrification. Yet, not much is known about the specific mechanisms and consequences, especially when there are multiple stressors. Such knowledge is however highly valuable to be able to better understand and predict consequences of human activity on soil microorganisms and nitrogen cycling. This could inform better soil management.

In this thesis, drought and contamination were studied both as isolated stressors and when occurring sequentially. For this, a series of controlled laboratory and outdoor experiments with agricultural soils were used. As pollutants, different herbicides, PAH, or the heavy metal copper were included. Effects on the soil microbial communities and their activity was assessed in terms of resistance and resilience. Resistance is defined as the degree to which a community remains unchanged after a disturbance, and resilience as its capacity to recover and return to the original state.

Three different herbicides applied individually had no measurable effects on the abundance of ammonia- and nitrite-oxidising communities present in the soil or on nitrogen cycling. This suggests the herbicides had low microbial toxicity. PAH on the other hand altered nitrification activity and composition of the AOA, but only at the highest contamination level. However, the overall soil microbial community was affected already at lower levels. Of all contaminants tested, copper had the most pronounced impact. It caused significant shifts in the composition and abundance of the soil microbial communities, including those involved in nitrification. Copper contamination also strongly reduced ammonia oxidation activity.

Results from the experiments with drought as a single stressor showed that drying temporarily slowed down ammonia oxidation activity and altered the AOA community, indicating low resistance. By contrast, AOB, NIB, and NIS showed resistance and remained largely unaffected. After rewetting of the soil, the nitrification process re-established, which suggest high resilience. However, rewetting caused changes in the NIS community and they did not recover during the remaining four weeks of the experiment. This shows that different groups involved in nitrification react differently to drought and rewetting, with differences in both resistance and resilience. This likely caused the modified mutual relationships between ammonia and nitrite oxidisers, which could lead to a destabilised nitrification process. When drought was applied to contaminated soils, effects on the communities performing nitrification varied depending on the contaminant. There were stronger drought effects when soil was contaminated with copper than PAH and only marginal effects were observed in soils with herbicides.

This thesis highlights how different types of stressors and whether they are considered individually or in combination can affect microbial communities in general and microbial groups performing specific functions in soil. The findings underline the need to consider multiple stressor scenarios in soil research to better understand the consequences of climate change and human activities on the functioning of soil ecosystems.

Populärvetenskaplig sammanfattning

Marken utgör basen för den global livsmedelsproduktionen. I jorden hittar vi myriader av organismer, inklusive mikroorganismer som bakterier och arkéer. De utför olika processer, till exempel omsättning av växtnäringsämnen. Det viktiga näringsämnet kväve förekommer i många former, och mikroorganismer är involverade i de flesta av omvandlingarna mellan dessa former i det som kallas kvävets kretslopp.

Nitrifikation är en process där mikroorganismer omvandlar ammoniak till nitrit och sedan vidare till nitrat. Processen påverkar vad som händer med kvävet i marken. Medan ammonium är relativt stabilt i jord, sköljs nitrat lätt ut med regn vilket leder till förorening av grundvatten och övergödning av floder och sjöar. Nitrifikationsprocessen delas ofta mellan olika mikroorganismer: ammoniakoxiderande arkéer (AOA), ammoniakoxiderande bakterier (AOB) och olika typer av nitritoxiderande bakterier, i jorden främst inom släktena Nitrobacter (NIB) och Nitrospira (NIS). Ammoniak- och nitritoxiderare samspelar med varandra genom produktion och konsumtion av nitrit. Samtidigt uppvisar AOA och AOB såväl som NIB och NIS olika preferenser för ett antal miljöfaktorer.

Mänsklig aktivitet sätter allt större press på marksystemen. Klimatförändringarna orsakar mer frekventa och svårare perioder av torka, medan jordbruk och industri bidrar till föroreningar som till exempel bekämpningsmedel, polycykliska aromatiska kolväten (PAH) och tungmetaller. Dessa så kallade stressfaktorer kan påverka mikroorganismer och processer, inklusive nitrifikation. Ändå vet vi lite om specifika mekanismer och konsekvenser, särskilt när det finns flera stressfaktorer. Sådan kunskap är värdefull för att förstå och förutsäga konsekvenser av mänsklig aktivitet på mikroorganismer och kväveomsättning, vilket kan bidra till bättre markförvaltning.

I denna avhandling studerades torka och föroreningar både som isolerade stressfaktorer och när de förekommer tillsammans efter varandra. För detta användes en serie kontrollerade laboratorie- och utomhusexperiment med jordbruksjord och som föroreningar inkluderades olika herbicider, PAH och tungmetallen koppar. Effekter på markens mikrobiella samhällen och deras aktivitet bedömdes i termer av resistens och resiliens. Resistens definieras som i vilken grad ett samhälle kan stå emot en störning och förblir oförändrat,

och resiliens som dess förmåga att återhämta sig och återgå till det ursprungliga tillståndet.

Tre olika herbicider som tillsattes individuellt visade inga mätbara effekter på antalet ammoniak- och nitritoxiderande mikroorganismer i jorden eller på kväveomsättning. Detta tyder på att herbiciderna hade låg mikrobiell toxicitet. PAH däremot förändrade nitrifikationsaktiviteten och sammansättningen av AOA, men endast vid den högsta föroreningsnivån. Emellertid påverkades det mikrobiella samhället generellt redan vid lägre nivåer. Av alla de föroreningar som studerades hade koppar den mest uttalade effekten. Koppar orsakade betydande förändringar i sammansättningen av det mikrobiella samhället och antalet mikroorganismer, inklusive de som är involverade i nitrifikation. Koppar minskade även nitrifikationsaktiviteten kraftigt.

Resultaten från experimenten med torka som enskild stressfaktor visade att torka tillfälligt bromsade nitrifikationsaktiviteten och förändrade AOA-samhället, vilket indikerar låg resistens. Däremot uppvisade AOB, NIB och NIS resistens då de i stort sett var opåverkade. Efter återvätning av jorden återgick nitrifikationsaktiviteten, vilket tyder på hög resiliens. Återvätning orsakade dock förändringar i NIS-samhället och de återhämtade sig inte under de återstående fyra veckorna av experimentet. Detta visar att grupper involverade i nitrifikationsprocessen reagerar olika på torka och återvätning, med skillnader i resistens och resiliens. Detta var troligtvis orsak till de förändringar som detekterades i det ömsesidiga förhållandet mellan ammoniak- och nitritoxiderande mikroorganismer, vilket skulle kunna leda till en destabiliserad nitrifikationsprocess. När förorenade jordar utsattes för torka varierade effekterna på mikroorganismsamhällen involverade i nitrifikation beroende på typ av förorening. Det fanns starkare torkeffekter när jorden var förorenad med koppar än PAH och endast marginella effekter syntes i jordar med herbicider.

Denna avhandling belyser hur olika typer av stressfaktorer och om de studeras individuellt eller i kombination kan påverka mikrobiella samhällen i allmänhet och mikrobiella grupper som utför specifika funktioner i jorden. Resultaten understryker behovet av att beakta scenarier med flera stressfaktorer för att bättre förstå konsekvenserna av klimatförändringar och mänskliga aktivitet på markekosystemens funktion.

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Doing a PhD on multiple stressors turned out to be more literal than I had anticipated – while in my experiments, I tested stress effects on soil microbes, I was simultaneously exposed to my own blend of stressors. Fortunately, unlike some of my microbial communities, I showed reasonable resilience. I owe that to the incredible support I received along the way.

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Resistance and resilience of co-occurring nitrifying microbial guilds to drying-rewetting stress in soil

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ABSTRACT

Nitrification, the oxidation of ammonia via nitrite to nitrate, contributes to nitrogen losses in agricultural soils. When nitrification is a two-step process, it depends on the successful metabolic interaction between ammonia oxidising archaea (AOA) and bacteria (AOB), and nitrite oxidising bacteria primarily within *Nitrobacter* (NIB) and *Nitrospira* (NIS). However, consequences of dry spells caused by climate change on the composition and co-associations of these microbial guilds and the fate of nitrogen remain unclear. Here we subject four distinct soils to either one long or two shorter drought periods (7–11 % water holding capacity) followed by rewetting in a microcosm experiment to evaluate the hypothesis that drying-rewetting stress triggers distinct responses in the functional guilds due to differences in environmental preferences and adaptation strategies. While AOB were highly resistant, AOA were the most sensitive to drying among the four guilds and decreased in relative abundance. This coincided with reduced ammonia oxidation rates in three soils by on average 27 % compared to the control. However, we observed almost full recovery of AOA one week after rewetting. NIS, but not NIB, were strongly affected by rewetting with no recovery during the experiment, showing shifts in community composition and relative abundance with up to 30 % affected ASVs. Network analysis revealed that drying-rewetting affected co-occurrences between ammonia and nitrite oxidisers in a soil-dependant manner, possibly indicating a destabilisation of their metabolic interaction. Overall, this study emphasises the importance to consider weather extremes like drought on soil nitrifier community dynamics and the fate of nitrogen in soils.

1. Introduction

Microorganisms play a crucial role in nitrogen (N) transformations, thereby controlling the bioavailability of soil N and in which form it is present (Kuypers et al., 2018). Nitrification, the process in which ammonia is oxidised to nitrate, contributes both directly and indirectly to N loss by driving nitrate leaching and fuelling denitrification. The latter leads to gaseous N loss, including emissions of the greenhouse gas nitrous oxide. Ammonia is either oxidised to nitrate by complete ammonia oxidisers ('comammox' bacteria) or to nitrite by ammonia-oxidising archaea (AOA) and bacteria (AOB) and then to nitrate by nitrite oxidising bacteria within several genera, primarily *Nitrobacter* (NIB) and *Nitrospira* (NIS). Thus, the two-step nitrification process depends on the successful interaction between two functional guilds. AOA and AOB abundance are generally positively correlated to NIS and NIB, respectively (Placella and Firestone, 2013; Simonin et al., 2015; Stempfhuber et al., 2017), and the associations between specific lineages shape the spatial distribution of nitrifying communities in soil (Jones and Hallin, 2019). These associations are largely explained by differences in niche preferences, e.g. capacity to use different substrates, substrate affinity, pH, and osmotic stress tolerance (Wessén et al., 2011; Nowka et al., 2015; Han et al., 2017; Saghaï et al., 2021; Qin et al., 2024). Several studies indicate that AOA and NIS have an advantage at lower nutrient content than AOB and NIB when grouped as functional guilds, but this is not necessarily the case at the population level (e.g. Simonin et al., 2015; Wertz et al., 2012). Moreover, there is evidence that ammonia and nitrite oxidation may become decoupled under conditions of environmental stress such as drying-rewetting events, leading to nitrite accumulation (Gelfand and Yakir, 2008). This shows that the assembly of nitrifier communities not only depends on physicochemical

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conditions in the soil but also on the stability of associations between different nitrifying guilds, and that responses of such nitrifier assemblages to climate-change induced weather extremes may have implications for soil N fluxes.

The frequency and duration of dry and wet spells are increasing due to climate change and subject soil microorganisms to severe stress (Schimel, 2018). As soil becomes drier, diffusion rates decrease, restricting the availability of resources, and osmotic stress increases. By contrast, rewetting causes a rapid drop in osmolality and an increase in the soil content of carbon compounds, ammonium, and nitrate (Birch, 1958). These conditions can change the composition and activity of microbial communities (Barnard et al., 2013; Priemé and Christensen, 2001), with impacts on the cycling of nutrients in soils (Gordon et al., 2008; Zhang et al., 2020). Previous work has shown that drying-rewetting favours AOB over AOA, possibly because of more efficient adaptation to osmotic pressure during drought and higher ammonium availability following the nutrient flush caused by rewetting (Thion and Prosser, 2014; Kaurin et al., 2018; Zhang et al., 2024; Bintarti et al., 2025), but little is known about the effect of drying-rewetting on nitrite oxidisers (Séneca et al., 2020). Nevertheless, re-occurring drying/rewetting events may have other effects as the resistance (the degree to which a community remains unchanged after a disturbance) and resilience (the capacity to return to the original state or to an alternative stable state) of microbial communities to a contemporary disturbance is influenced by previous disturbance events (Shade et al., 2012; Fuchslueger et al., 2016; Canarini et al., 2021). Understanding the impacts of drying-rewetting cycles on nitrifying guilds and possible implications for nitrogen cycling in agricultural soils is particularly important, as drought has been identified as the main threat to global crop yields (Lesk et al., 2016).

Here, we determined the effects of drying-rewetting cycles on the community composition and co-occurrence of ammonia and nitrite oxidising guilds driven by the cooperation between these guilds, as well as on the ammonia oxidation rates in four contrasting soil types (Table 1). To do this, we set up a microcosm experiment where soils were subjected to either one long drought period or two shorter drought periods with a rewetting event after each drought followed by a final recovery period (Fig. 1). We hypothesised that different nitrifying guilds will display distinct responses to drying-rewetting stress with higher substrate concentrations favouring AOB and NIB over AOA and NIS, respectively, due to differences in environmental preferences. As this will result in shifts in community composition, we further hypothesise that these shifts will modify co-associations between lineages of different nitrifying guilds. As an overall consequence, we expect to see changes in nitrification activity.

Table 1
Geographic origin and properties of the soils.

	Soil B	Soil E	Soil U	Soil S
Origin	Bretenière, France	Ekhaga, Sweden	Ulleråker, Sweden	Schnega, Germany
Coordinates	47.234715, 5.109561	59.830742, 17.808193	59.824883, 17.648267	52.904663, 10.831922
Soil type (USDA)	Silty clay	Silty clay loam	Clay loam	Loamy sand
Soil texture	54 % clay 42 % silt 4 % sand	37 % clay 57 % silt 6 % sand	37 % clay 37 % silt 27 % sand	0 % clay 25 % silt 75 % sand
pH (H_2O)	8.12	6.53	8.01	5.39
Tot-C (%)	2.91	3.80	1.90	1.10
Org-C (%)	2.56	3.78	1.87	1.10
Tot-N (%)	0.24	0.40	0.17	0.09

2. Material and methods

2.1. Soil sampling

Soil samples (5–20 cm depth) were collected in June 2021 in four agricultural fields in France (Bretenière; 47°14′05.0″N, 5°06′34.4″E), Sweden (Ekhaga; 59°49′50.7″N, 17°48′29.5″E, and Ulleråker; 59°49′29.6″N, 17°38′53.8″E), and Germany (Schnega; 52°54′16.8″N, 10°49′54.9″E). The soils were homogenized, sieved (2 mm ∅) and stored at −20 °C until the start of the experiment. Soil water content was estimated in duplicates as the difference in weight before and after drying ~5 g soil at 105 °C for 24 h. The maximum water holding capacity (WHC) was estimated as the gravimetric water content of the soil after overnight soaking in water and draining for 5 h. Soil properties, including pH (in water), total carbon (C_{tot}), organic carbon (C_{org}) and total nitrogen (N_{tot}) were determined at the Soil and Plant Laboratory (SLU, Uppsala, Sweden) and soil texture (PARIO method) at the Soil Physics Laboratory (SLU, Uppsala, Sweden; Table 1). Soils will be referred to as soil B (Bretenière), soil E (Ekhaga), soil U (Ulleråker), and soil S (Schnega).

2.2. Experimental design

The microcosm experiment included the four soils B, E, U and S subjected to three treatments: one drying-rewetting cycle, two drying-rewetting cycles, and a control kept at 45–50 % WHC (Fig. 1). In total, 120 microcosms were included to allow destructive sampling of all treatments in triplicate at three timepoints ('drying' on day 42, 'rewetting' on day 49, and 'recovery' on day 77) in addition to triplicate sampling of each soil at day 0. The experiment was set up in a climate chamber with 20 °C, 60 % relative humidity, and continuous darkness throughout the entire experiment.

When establishing the experiment, soils were first thawed at 4 °C for one day followed by 1 day at room temperature. Glass pots with an inner diameter of 12.5 cm were filled with 200 g fresh weight (FW) soil, corresponding to a dry weight (DW) of 138 g (soil B), 138 g (soil E), 149 g (soil U), and 172 g (soil S). The microcosms were covered with sterile cotton cloth and aluminium foil to reduce evaporation while allowing soil aeration. After 7 days of acclimatisation at 45–50 % WHC, soils were either kept at the same conditions or subjected to one long drought period (35 days) or two shorter drought periods (14 days followed by rewetting and 7 days of recovery at 45–50 % WHC in between), with both drought treatments followed by a recovery period of 28 days weeks at 45–50 % WHC (Fig. 1). The microcosms were weighed every second day to monitor WHC and adjust the water content in the control soils. Both watering of the control soils and rewetting after drought were done by carefully pipetting water on the surface of the soil to avoid physical disturbance. The aluminium foil was removed from microcosms undergoing the drought treatment, causing a reduction of the soil moisture to 7 (soil U and S) and 11 % WHC (soil B and E) within 14 days (Fig. 1). At each of the three sampling days (day 42, 49, and 77), the soil from each microcosm was homogenized and stored at −20 °C until further analysis of inorganic N (ammonium and nitrate, section 2.3), potential ammonia oxidation activity (section 2.4), and analysis of the functional guilds (section 2.5).

2.3. Measurement of soil ammonium and nitrate

To determine soil ammonium and nitrate content, soil was extracted with 2 M potassium chloride (1:5 ratio) in 50 mL Falcon tubes and incubated on a horizontal shaker for 1 h at 300 rpm. After centrifuging (5 min, 3500 *g*), the supernatant was filtered through Munktell 00H filter paper (Ahlstrom, Helsinki, Finland) and stored at 4 °C until analysis (max. 4 days). The concentration of ammonium and nitrate was measured on a segmented flow analyser (AutoAnalyzer 500, SEAL Analytical, Inc., Mequon, Wisconsin, US). The ammonium content

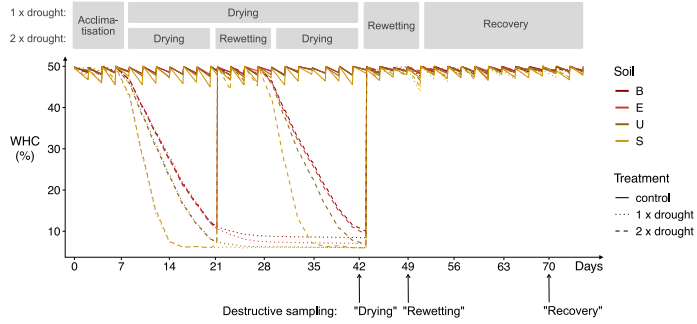

Fig. 1. Experimental set-up and water holding capacity (WHC) in the four soils subjected to either one long (1 x drought) or two shorter droughts (2 x drought) over the course of the experiment. Soil moisture was monitored every two days and adjusted to 45–50 % WHC when necessary. The droughts were imposed after an acclimatisation period of 7 days. Individual microcosms were destructively sampled at the end of the drought treatments (day 42), after rewetting (day 49), and after the recovery phase (day 77) as indicated by arrows. Colours represent different soils and line types indicate treatment.

before subjecting the soils to drought ranged from 0.78 ± 0.25 to 1.33 ± 0.39 mg N kg^{-1} DW soil, whereas the nitrate content ranged from 10.24 ± 10.44 to 32.22 ± 13.19 mg N kg^{-1} DW soil.

2.4. Potential ammonia oxidation assays

Potential ammonia oxidation rates, hereafter ammonia oxidation rates, were measured following the ISO 15685 protocol (2012) with some modifications and with three different sources of ammonium. Soil was thawed at 4–7 °C two days prior to the assay. Soil was mixed in a 1:4 (w:v) ratio with an unbuffered solution containing 1 mM sodium chlorate to inhibit nitrite oxidation (Xu et al., 2010), and either with 198 mg/L diammonium sulphate as per the ISO protocol or with an equivalent amount of nitrogen in the form of urea or yeast extract. These substrates were chosen to account for possible differences in substrate affinities and preferences between and within AOA and AOB (Levičnik-Höfferle et al., 2012; Qin et al., 2024), with the assumption that ammonia concentrations would be lower with urea and yeast extract due to their need to be mineralized to ammonium prior to oxidation. Soil slurries were incubated in loosely capped bottles on an orbital shaker (210 rpm, 25 °C). After 2 h and 8 h, 1 mL of soil slurry was removed and mixed with 1 mL 4 M potassium chloride to stop ammonia oxidation. These times were selected based on preliminary trials showing linearity of ammonia oxidation across all four soils. After centrifugation (2 min, 3000 *g*), nitrite content was measured colorimetrically (Griess test), using a microplate reader (SpectraMax Plus 384, Molecular Devices, LLC, California, US).

2.5. DNA extraction and libraries for sequencing of amoA and nxrB

DNA was extracted from 0.4 g FW soil using the NucleoSpin Soil kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. DNA quality was validated by agarose gel electrophoresis and measurements on a NanoDrop™ (Thermo Fisher Scientific, Waltham, Massachusetts, US), before quantification with a Qubit® fluorometer (Thermo Fisher Scientific). Sequencing libraries were prepared for AOA, AOB, NIB and NIS by using guild specific *amoA* (encoding the ammonia monooxygenase) and *nxrB* (encoding the nitrite oxidoreductase) primers, following a two-step PCR procedure. The first PCR was done in duplicates in 25 μL reaction volume, using 0.5 μM of the respective primers, 1 × Phusion PCR Mastermix (Thermo Fisher Scientific), 1 μg μL^{-1} bovine serum albumin and 5 ng template DNA for AOA, 15 ng for AOB and NIS, and 25 ng for NIB. Primer sequences and thermal cycling conditions are found in Table S1. PCR product size was verified by gel electrophoresis and the duplicates were pooled before purification with Sera-Mag beads (Merck KGaA, Darmstadt, Germany). The second PCR was done in a single 30 μL reaction using the same concentrations of Mastermix and bovine serum albumin as in the first PCR, 0.2 μM primers with Nextera adaptor and index sequences, and 4 μL purified PCR product as template. PCR products were verified and purified as described above and quantified using a Qubit® fluorometer. Two libraries were created by pooling equimolar amounts of *amoA* and *nxrB* amplicons, respectively. After a final quality control using a Bio-Analyzer (Agilent, Santa Clara, CA, US), sequencing was performed by SciLifeLab in Uppsala on an Illumina MiSeq instrument using the 2 x 300 bp chemistry.

2.6. Sequence analyses

Sequence analysis was performed using the R software, version 4.1.1 (R Core Team, 2021). Demultiplexed *amoA* and *nxrB* gene amplicons were processed using the 'dada2' package, version 1.16.0 (Callahan et al., 2016) to infer amplicon sequence variants (ASVs). Forward and reverse reads of all four genes were truncated with the 'filterAndTrim' command using default settings, except for maxEE = c(2,2), based on quality score (AOA *amoA*: 248, 200; AOB *amoA*: 275, 240; NIB *nxrB*: 270, 190; NIS *nxrB*: 290, 220). Forward and reverse sequences were either concatenated (AOA *amoA*) or merged (AOB *amoA* and NIS and NIB *nxrB*). Chimeras ('removeBimeraDenovo' function, 'consensus' method) and singletons were discarded. To identify non-specific ASVs, the representative sequence of each ASV was translated into amino acids ('esl-translate' command implemented in EASEL, version 0.48) and aligned to the corresponding reference alignment using the 'hmmalign' command in HMMER, version 3.3.2 (Eddy, 2011). After back translation to nucleotide sequences in ARB, version 7.0.1 (Ludwig et al., 2004), query ASV sequences were placed on the corresponding reference phylogeny using EPA-NG (Barbera et al., 2019) and sequences falling in the outgroup were discarded. For reference phylogenies, we used published databases for AOA (Alves et al., 2018) and AOB (Jones and Hallin, 2019) and updated the phylogenies for *nxrB* from Jones and Hallin (2019), following the approach described in Saghaï et al. (2023). The resulting ASV tables were rarefied using the 'vegan' package version 2.6–4 (Oksanen et al., 2013).

2.7. Statistical analyses

Statistical analyses were performed using the R Software, version 4.3.3. Based on the rarefied ASV tables for each community, Pielou's evenness was computed in the 'vegan' package, and Faith's phylogenetic diversity (PD) (Faith, 1992) was obtained using the phylogenetic

placements and the 'fdp' command within Guppy, version 1.1 (Matsen and Gallagher, 2011). Rarefied ASV tables were filtered per gene before performing β-diversity analyses to remove low abundant ASVs (i.e. those with abundance <0.001 % in the overall dataset and present in less than 15 % of the samples; Table S3). Zero counts were replaced by Bayesian multiplicative replacement using the 'zCompositions' package, version 1.4.0-1 (Martín-Fernández et al., 2015) and ASV tables were centred log-ratio transformed, to account for the compositionality of the dataset (Gloor et al., 2017). Differences in community composition and structure were visualized with principal component analysis (PCA) using the rda function in 'vegan'. The homogeneity of dispersion between groups was tested using the betadisp function in 'vegan' and their significance assessed using a permutation test. Permutational multivariate analyses of variance (PERMANOVA) were conducted to assess treatment and timepoint effects on community composition using the adonis function in 'vegan'.

Substrate, soil, and timepoint effect on ammonia oxidation rates was assessed using analysis of variance (ANOVA). Variables not following a normal error distribution were transformed by Box-Cox transformation before analysis. For the percent change between control and treatment, the combined standard deviation *SD* of both groups was calculated by error propagation (Taylor, 2022) as follows:

$$SD_{percentchange} = \left|\frac{100}{control}\right| * \sqrt{SD^2_{treatment} + \left(\frac{treatment}{control}\right)^2 SD^2_{control}}$$

The standard error and 95 % confidence interval *CI* were calculated with $n = 6$ data points and a confidence level of $\alpha = 0.05$ using the *t*-distribution. Confidence intervals that did not span over zero were considered significant percentage changes of treatment from control.

2.8. Differential abundance and network analysis

Differential abundance and network analysis were performed using the R Software, version 4.3.3. On non-rarefied ASV tables, a filter was applied to reduce sparsity in the data causing a risk for spurious correlations. Per gene and soil, ASVs with an abundance below 0.001 % and a presence below 45 % were removed (Table S2).

The effect of drying-rewetting cycles on relative abundance of ASVs was estimated per soil and timepoint using a generalised linear mixed model, computed with the glmer function in the 'lme4' package, version 1.1–35.2 (Bates et al., 2015). Generalised linear mixed models allow to infer linear regressions from Poisson distributed count data and support the inclusion of fixed effects (treatment) and random effects (sample ID). Differences in sequencing depth were accounted for by adding an "offset" factor, the $\log_2$ of the read sum per sample. Following Huet et al. (2023), we considered that an ASV of abundance *Y*, in any *k* replicates of any *i* treatment follows a Poisson law of parameter Λ as $Y \sim P(\Lambda)$:

$$log(\Lambda_{ik}) = o_{ik} + \mu + \alpha_i + Z_{ik1 \le j \le 3} idd\ N(0, \sigma^2)$$

where $i = \{1, \ldots, 3\}$ represents the treatments, $k = \{1, \ldots, 3\}$ the replicates, *o* the "offset", α.

The treatment effect, and *Z* the random sampling effect modelling data overdispersion. Multiple pairwise comparisons between treatments were performed with a post-hoc Tukey test using the emmeans function of the 'emmeans' package, version 1.10.0 (Lenth, 2024). After p-value adjustment using the false discovery rate method (Benjamini and Hochberg, 1995), ASVs with $p \le 0.01$ were considered significantly affected.

For the network analyses, only soil B and U could be used due to seven missing samples in soil E (n = 24) and soil S (n = 23). The missing samples cause incomplete replication of the functional guilds in the data set which would result in an incorrect introduction of zero counts that pose a risk for spurious correlations in the networks. For soil B and U (n = 27), networks were inferred using a Poisson log-normal model with a latent Gaussian layer and an observed Poisson layer (Chiquet et al., 2019, 2021), developed to handle sparse count data, using the 'PLNmodels' package, version 1.2.0 (Ibid.). Differences in sequencing depth were accounted for by adding the $\log_2$ of the read sum as an "offset" factor per sample. The most robust network per model was selected using a Stability Approach to Regularization Selection (Liu et al., 2010). Due to the experimental set up, treatment and timepoint could not be separated since treatment phases covaried with timepoints. Per soil, two networks M0 and M1 were constructed. The M0 model was constructed without covariates, which means that all possible effects are included. For the M1 model, treatment and timepoint were added as covariates, which causes the removal of the effect of treatment and timepoint from the generated network. Thus, edges in the resulting M1 network were the ones not affected by the covariates. By subtracting these nodes and edges (i.e. nodes and edges of the M1 network) from the M0 network, only nodes and edges related to treatment and timepoint should remain.

3. Results

3.1. Potential ammonia oxidation rates

The capacity for ammonia oxidation differed between soils, as shown by the rates detected in the control (Table S3), where the highest potential was observed in soil E with values up to 0.67 mg NO_2–N g^{-1} DW soil h^{-1}, followed by soil B with 0.59, soil U with 0.35, and soil S with 0.04 mg NO_2–N g^{-1} DW soil h^{-1} ($F_{(3,\ 72)} = 2986.67$, $p < 0.001$, Table S4). The timepoint had a small significant effect ($F_{(2,\ 72)} = 4.9$, $p < 0.01$), whereas the type of substrate provided during the assay did not ($F_{(2,\ 72)} = 1.17$, $p > 0.05$). The single, long drought treatment resulted in significantly lower ammonia oxidation rates compared to the control in soil B, E, and U with all three substrates, as indicated by a 95 % confidence interval, whereas the rates in soil S were unaffected by the drying-rewetting treatments (Fig. 2). A significant negative effect of the two shorter drying-rewetting cycles was only observed in soil B with yeast extract as substrate. After rewetting, the rates did not differ from the control except for a small increase in soil B (with ammonia) and a decrease in soil E (with yeast extract). After the recovery period, the rates were significantly higher in the drought treated soil U.

3.2. Inorganic N

In line with the observed effects on ammonia oxidation rates, there was a tendency for lower ammonium and nitrate content during drying, with significant effects in soil B, E, and U (Fig. S1). However, there were small but consistent differences between the two drought treatments during drying. The nitrate content was higher in relation to the control with two drying-rewetting cycles compared to the treatment with one cycle, whereas ammonium content showed the opposite pattern (Fig. S1). Thus, nitrate content and ammonia oxidation rates were positively correlated in all soils (Spearman's $\rho = 0.31$–0.42, $p < 0.01$; Fig. 2 and Fig. S1) except in soil S, which had very low ammonia oxidation activity. Correlations between ammonia oxidation rates and ammonium content were only significant in soil B (Spearman's $\rho = 0.41$, $p < 0.001$).

3.3. Diversity and composition of nitrifier communities

Phylogenetic diversity varied between the four guilds, with AOA having the highest and NIB the lowest PD in all soils, whereas evenness was similar in all soils and guilds, except for the lower evenness of NIB (Table S3). Drying-rewetting did not affect the evenness or PD of ammonia oxidisers or nitrite oxidisers in any of the soils (Fig. S2 and S3). By contrast, the treatments affected the community composition of the guilds across all soils, with NIS communities displaying the strongest shifts in *β*-diversity (Fig. 3; Table S5). During drying, communities in the single drought treatment were similar to the control communities, but

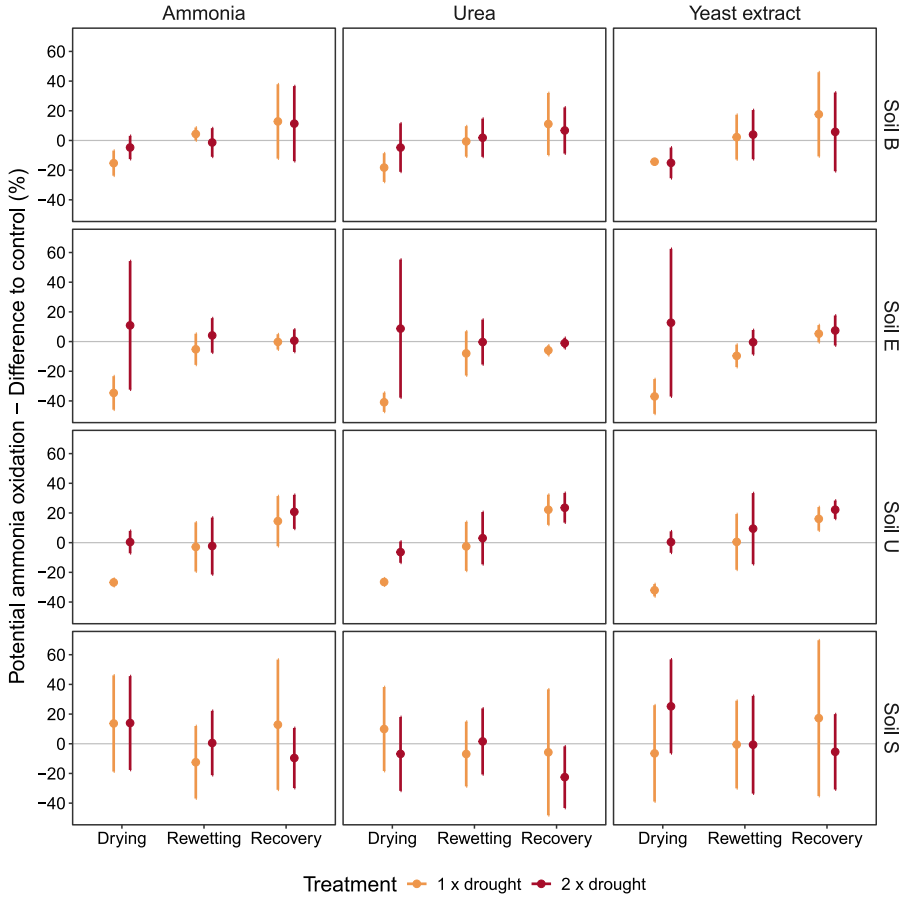

Fig. 2. Potential ammonia oxidation rates with three different nitrogen sources in the four soils. Activity was determined at the end of the two drought treatments (day 42), after rewetting (day 49), and after the recovery phase (day 77) and is presented as percent change in comparison to the control. Bars represent 95 % confidence intervals (hidden behind symbol in some cases). When confidence intervals did not span over zero, the percentage change of treatment from control was considered significant. Colours indicate drought treatments.

differed after rewetting in soil B, E, and U. Communities in soils subject two drying-rewetting cycles did not show this pattern. In two of the soils in which NIS was strongly affected (soil B and U), the AOA community composition or its dispersion was also significantly affected by time and treatment (Fig. 3; Table S5), with communities sampled during drying being most dissimilar to the communities in the control. AOB and NIB communities were unaffected by drying and rewetting (Fig. S5).

In line with the β-diversity patterns, differential abundance analysis showed that the relative abundance of ASVs was most affected in NIS communities (Fig. 4). Effects of drying and rewetting on NIS were detected in all soils and were particularly strong in soil U, which displayed both the highest proportion of positively affected ASVs, ranging from 7.35 to 29.72 %, and the highest proportion of negatively affected ASVs, ranging from 2.45 to 19.23 %. The lowest proportion of combined positively and negatively affected ASVs in soil U was observed in the single drought treatment during drying, and the highest in the double drought treatment after the recovery period. For all soils, large differences in relative abundance of NIS ASVs between control and droughted soil remained after the recovery period. The relative abundance of AOA ASVs was mainly affected by drought and there were only small differences between control and droughted soils after rewetting and at the end of the recovery period. Only in soil S the relative abundance was most impacted after rewetting. Less than 5 % of AOB and NIB ASVs were affected at any of the time points.

3.4. Network analysis

Associations between ammonia and nitrite oxidisers examined by network analysis in soil B and U were dominated by positive associations (Fig. S6) and showed similar patterns across soils when edge numbers were adjusted to the average number of ASVs within each guild in the networks with and without covariates (Fig. 5A). When considering the overall networks, patterns of associations within ammonia or nitrite oxidisers differed more between soils and likely indicate shared niche (Fig. S7). Based on Fig. S7, 20 % of all edges and 4.4 % of all nodes were specifically related to drying-rewetting in soil B, whereas in soil U it was

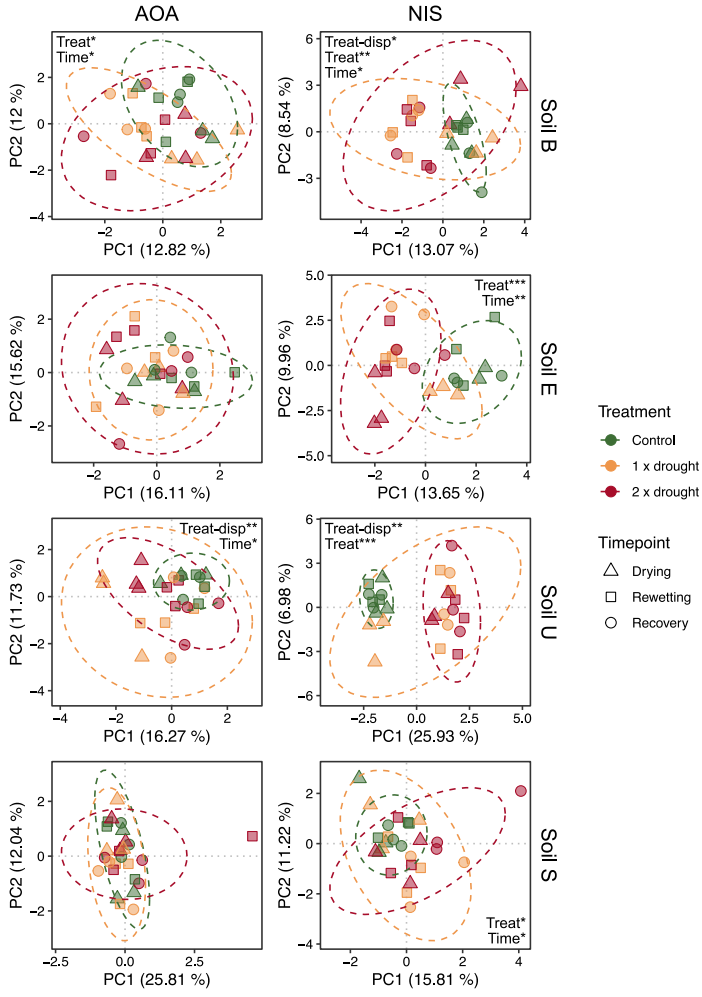

Fig. 3. Principal component analysis (PCA) of ammonia oxidising archaeal (AOA; left panels) and nitrite oxidising bacterial *Nitrospira* (NIS; right panels) communities in the four soils. The composition of the communities was determined at the end of the two drought treatments (day 42), after rewetting (day 49), and after the recovery phase (day 77). Significant differences in community structure and beta-dispersal ('-disp') across treatments and timepoints are indicated inside the plot ($p < 0.05$*, $p < 0.01$**, $p < 0.001$***). Colours represent treatments, shapes time points, and dashed ellipses 95 % confidence intervals of the respective treatment.

38 % of all edges and 8.3 % of all nodes. A major difference between the soils was that treatment-affected edges indicating associations between ammonia and nitrite oxidisers were dominated by AOA and NIS in soil B and by AOB and NIS in soil U (Fig. 5B and C). The taxa involved differed substantially between soils for ammonia oxidisers, whereas the nitrite oxidiser clades largely overlapped (Fig. 5B and C).

4. Discussion

Drought significantly reduced both the ammonia oxidation rates and soil nitrate content, indicating a negative impact on both ammonia and nitrite oxidation, i.e. an overall lower nitrification activity. The effect was stronger in soils subjected to a single, long drought compared to two shorter drought periods. This either suggests that the effect of a long drought on nitrifiers is more severe or that previous exposure to stress, here the first drying-rewetting event, was enough to increase the resistance of these communities to future stress. It is possible that the first drying-rewetting cycle shifted the microbial communities towards an alternative state that was better at coping with additional drying-rewetting events. This aligns with the ecological theory on 'catastrophic shifts in ecosystems' (Scheffer et al., 2001). Ammonia oxidation activity in soil B, E and U was also resilient as there was no difference between soils subjected to drought and control soils seven days after rewetting. Soil U, however, showed higher activity in droughted soils after the recovery period, possibly triggered by increased nutrient availability after rewetting. By contrast, soil S was unaffected,

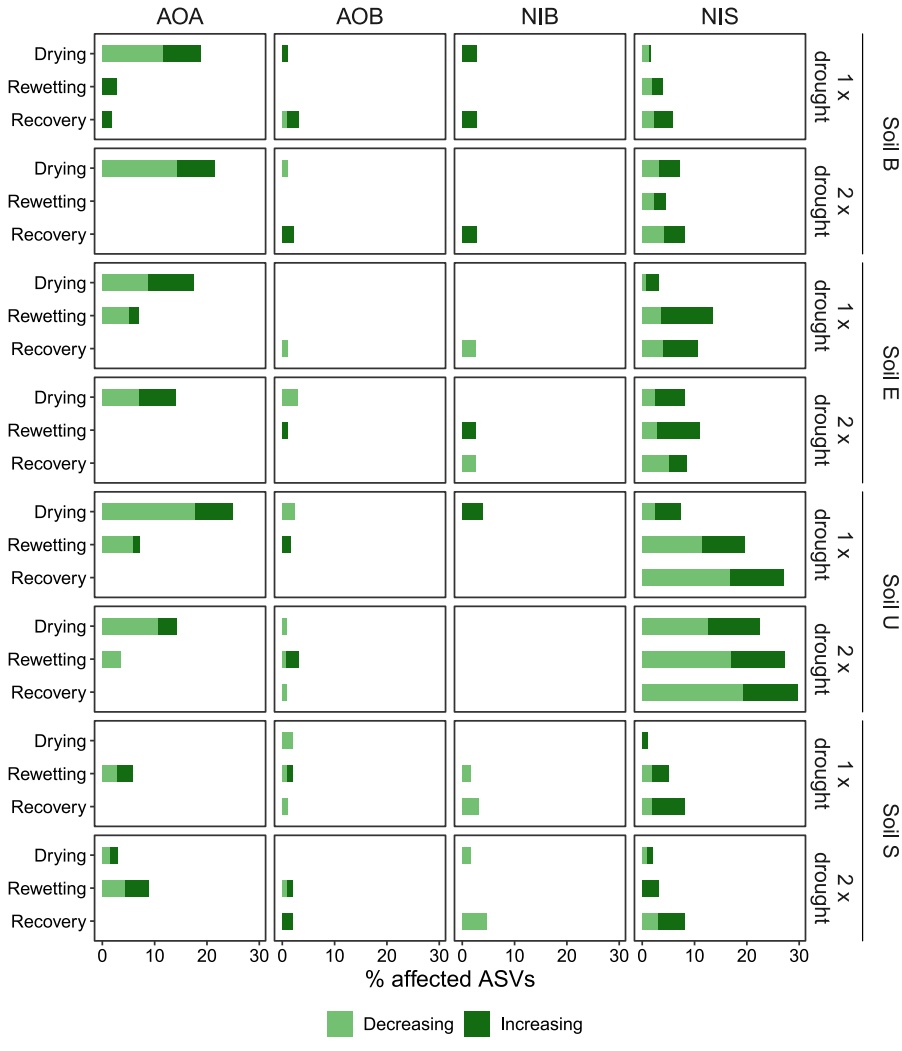

Fig. 4. Percentage of ASVs significantly decreasing or increasing after one long or two shorter droughts in comparison to the control in the four functional groups and across the four soils. Differential abundances were determined at the end of the drought treatments (day 42), after rewetting (day 49) and after the recovery phase (day 77). AOA: ammonia oxidising archaea; AOB: ammonia oxidising bacteria; NIB: *Nitrobacter* type nitrite oxidisers; NIS: *Nitrospira* type nitrite oxidisers.

potentially because sandy soils may be subjected to more regular moisture fluctuations and thus harbour communities better acclimated to drying-rewetting stress than those found in more clayey soils (Peralta et al., 2013; Placella and Firestone, 2013). However, it is more likely that the communities did not respond due to inactivity, as the lower total N and ammonium levels combined with low pH suggest that the ammonia oxidisers are more substrate limited in soil S compared to the other soils. This was supported by the low ammonia oxidation rates observed in the control treatment in soil S. The treatment effects on ammonia oxidation were consistent across substrates of varying complexity (ammonium < urea < yeast extract) and nature (organic vs inorganic) that were used to account for differences in substrate preferences and affinities between and within AOA and AOB (Levičnik-Höfferle et al., 2012; Qin et al., 2024). Since we do not know which community members were active during the assay, we cannot exclude known biases associated with this assay, where the choice of substrate can promote or inhibit specific AOA or AOB clades (Hazard et al., 2021). Likewise, this assay does not enable to estimate the contribution of autotrophic versus heterotrophic nitrification (Gao et al., 2023) to the observed ammonia oxidation rates or increased in nitrate pools.

The decrease in ammonia oxidation rates during drought coincided with changes in the relative abundance within AOA but not within AOB, indicating an important role of AOA for ammonia oxidation in the

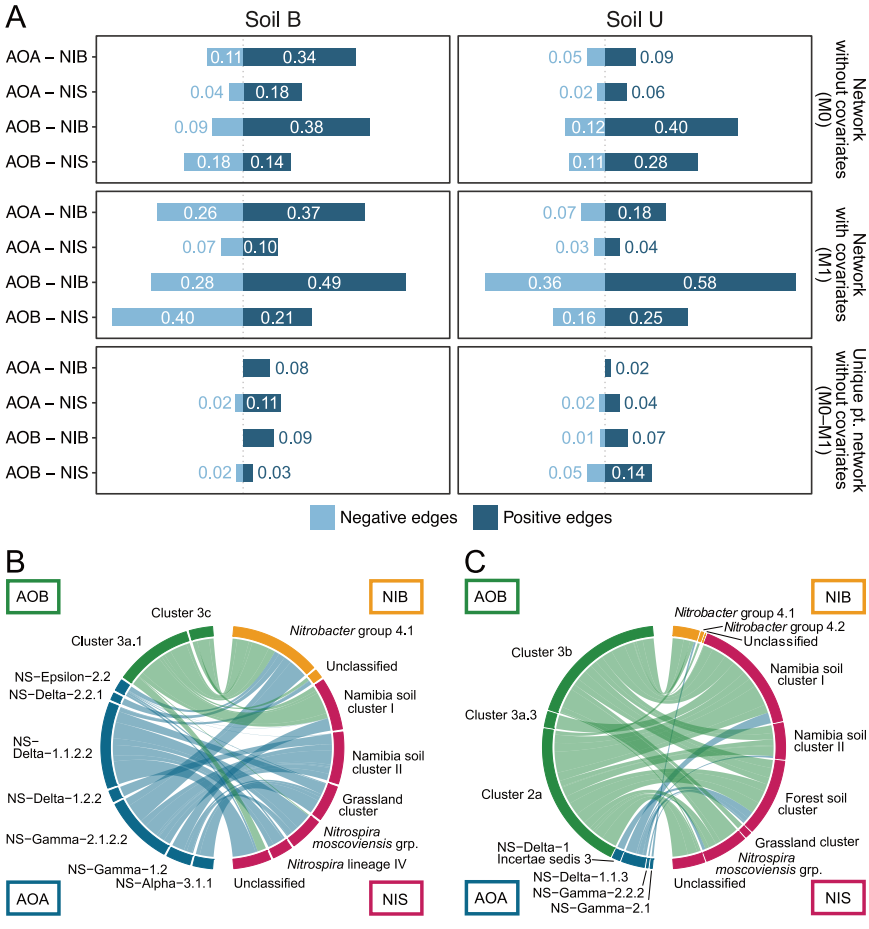

Fig. 5. Network analyses of ammonia and nitrite oxidising microorganisms in soil B and U. **A)** Proportion of negative and positive edges per node in the networks. Upper panels: networks built without covariates (M0). Middle panels: networks where the effects of treatment and timepoint on nodes and edges were removed by including treatment and timepoint as covariates (M1). Lower panels: networks containing only the nodes and edges affected by treatment and timepoint, obtained by subtracting the nodes and edges not affected by the covariates from the network without covariates (M0–M1). Edges per node were calculated by dividing the edge count by the average number of ASVs of each functional group to which the two connected nodes belong (AOA: ammonia oxidising archaea; AOB: ammonia oxidising bacteria; NIB: *Nitrobacter* type nitrite oxidisers; NIS: *Nitrospira* type nitrite oxidisers). Colours represent negative and positive edges. **B, C)** Chord diagrams showing unique (M0–M1) positive network edges between clades of ammonia oxidisers (AOA and AOB) and nitrite oxidisers (NIB and NIS), i.e. edges affected by treatment and timepoint, in soil B (**B**, 42 edges) and soil U (**C**, 43 edges). Edge width denotes edge degree and colours in the ring denote the functional guild.

affected soils. This is supported by observations that ammonia oxidation in soils most often seems to be driven by AOA rather than AOB, which are typically favoured when N levels are higher (e.g. Sterngren et al., 2015; Verhamme et al., 2011), in combination with the low ammonium levels in the soils used in this study. This would imply a general deceleration of N cycling during times of drought, which should be more pronounced in soils where AOA drive ammonia oxidation. The changes in AOA but not AOB community composition also indicate a higher resistance to drought stress among AOB, which aligns with work showing higher sensitivity to osmotic stress of AOA compared to AOB in pure culture (Bello et al., 2019), as well as higher sensitivity to drought as shown in both microcosm (Thion and Prosser, 2014; Bello et al., 2019) and field experiments (Fuchslueger et al., 2014; Séneca et al., 2020; Bintarti et al., 2025). Differential abundance analysis further indicated that although the relative abundance of the majority of affected AOA decreased, a substantial fraction also increased, especially in soil E. This illustrates the large variation in niche preferences that exists within this guild (Saghaï et al., 2021; Wright and Lehtovirta-Morley, 2023; Qin et al., 2024). Notably, effects on the relative abundance of AOA between the droughted and the control soils started to decrease after rewetting and were minimal or absent after the recovery period, indicating high resilience among AOA, with the exception of AOA communities in soil S.

Despite the decrease in ammonia oxidation rates and soil nitrate content during drought, both NIS and NIB community composition were little affected by drought. Possible strategies include mixotrophic

growth (e.g. Daims et al., 2001; Starkenburg et al., 2008), dormancy (Roszak and Colwell, 1987) and adaptation to osmotic pressure, although osmoadaptation does not appear to be widespread among soil NOB (Wu et al., 2024). Instead, NIS communities were significantly affected by rewetting and did not recover within the time frame of the experiment. This coincided with a decrease in soil nitrate content, indicating a decreased nitrification activity in soils B and E. Rewetting events are characterised by a nutrient flush in combination with re-established diffusion (Birch, 1958; Moyano et al., 2013), offering favourable substrate conditions for ammonia oxidation and subsequently increasing resource levels for nitrite oxidisers. At the same time, soil rewetting rapidly changes the osmotic pressure and can limit oxygen diffusion, which would supress ammonia oxidation. Effects of rewetting differed between the two drought treatments, with the largest shift in community composition observed after rewetting of the single drought treatment. In contrast to AOA, NIS communities did not appear to be resilient as the relative abundance of 5–30 % of the ASVs still differed from that of the control at the end of the recovery period, except in soil E. In fact, the fraction of affected NIS ASVs had increased after the recovery period. This challenges the assumption that NIS in comparison to NIB prefer low nitrite conditions (Wertz et al., 2012; Nowka et al., 2015; Simonin et al., 2015) and are more sensitive to changes in osmotic pressure (Li et al., 2021). Instead, these findings confirm niche differentiation at fine phylogenetic scale within this poorly characterized group (Maixner et al., 2006; Gruber-Dorninger et al., 2015; Jones and Hallin, 2019).

In addition to canonical nitrite oxidisers, NIS can also include comammox bacteria that can perform both ammonia and nitrite oxidation, and it is not possible to disentangle these two groups based on *Nitrospira*-type *nxrB* gene sequences (Daims et al., 2016), meaning that comammox sequences could be present in our NIS dataset. However, quantitative analysis in agricultural soil have shown that comammox bacteria have a lower abundance than AOA and AOB (Bintarti et al., 2025) and comammox specific *amoA* genes are typically found at lower abundance compared to *nxrB*, ranging from about three times (Wang et al., 2023) over ten times (Li et al., 2020; Xu et al., 2020) to more than one hundred times lower abundance (Li et al., 2024). Moreover, the total abundance of nitrite-oxidising communities has been shown to be comparable to that of AOA and AOB (Jones and Hallin, 2019). Altogether, these findings suggest comammox constitute a minor fraction of the NIS communities in agricultural soils.

The network analysis of nitrifying communities in soil B and U revealed that positive edges, i.e. co-occurrences between ammonia oxidisers and nitrite oxidisers, generally dominated over negative edges. Further, co-occurrences were more affected by drying-rewetting than negative associations. Co-occurrences could signal shared niche, but since canonical ammonia and nitrite oxidisers are involved in mutualistic relationships, our results could also indicate that drying-rewetting can destabilise associations between these functional guilds. Such decoupling between ammonia and nitrite oxidation may affect N fluxes

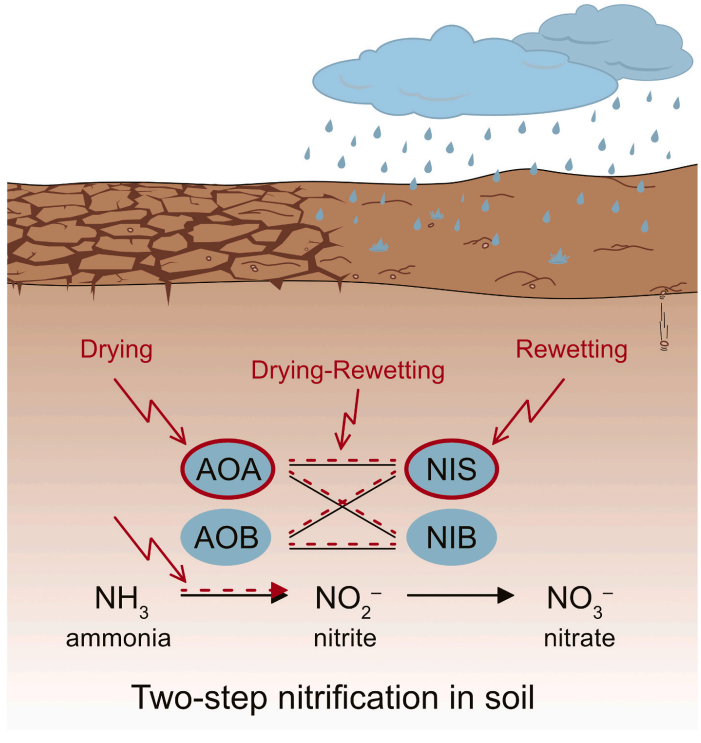

Fig. 6. Conceptual model of observed effects of drying-rewetting stress on nitrifier community composition, ammonia oxidation rates and co-associations between ammonia oxidisers and nitrite oxidisers. Black lines indicate processes and co-associations under control conditions (45–50 % water holding capacity), whereas dashed red lines indicate reduced process rates and weakened co-associations. Red elbow arrows indicate significant effects of drying and/or rewetting on community composition, ammonia oxidation rates or co-associations. AOA: ammonia oxidising archaea; AOB: ammonia oxidising bacteria; NIB: *Nitrobacter* type nitrite oxidisers; NIS: *Nitrospira* type nitrite oxidisers.

in soil and result in accumulation of nitrite (Gelfand and Yakir, 2008). Contrary to previous work suggesting that NIB tend be more often associated with AOB and NIS with AOA (Simonin et al., 2015; Stempfhuber et al., 2017; Jones and Hallin, 2019), we found that NIS ASVs co-occurred with both AOB and AOA ASVs in a soil-dependent manner, even after normalizing the number of edges for each functional group and soil. This likely reflects that NIS is a highly diverse functional group that can fill multiple nonoverlapping niches (Daims et al., 2016). NIB displayed similar patterns, although to a smaller extent due to the lower phylogenetic diversity in this group.

5. Conclusions

Our results show that drought has a strong but short-term impact on ammonia oxidation rates and the relative abundance of AOA ASVs, indicating low resistance but high resilience in this group (Fig. 6). This led to a temporary decelerated nitrogen turnover in dry soil. Effects of rewetting on NIS were more pronounced, as shown by shifts in community composition and in the relative abundance of individual ASVs, without recovery by the end of the experiment, indicating low resilience. By contrast, AOB and NIB communities seemed largely unaffected by drying-rewetting events. Nevertheless, drying-rewetting events affected the co-occurrences of ammonia and nitrite oxidisers, possibly leading to a destabilisation of metabolic interactions among the functional guilds completing nitrification. This study helps to understand the impact of weather extremes on soil nitrifiers and calls for further investigation of the effects of climate change related impacts on soil nitrifier community dynamics and the fate of N in soils.

CRediT authorship contribution statement

Laura J. Müller: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Mara Alicke:** Investigation, Formal analysis. **Sana Romdhane:** Supervision, Formal analysis. **Grace Pold:** Writing – review & editing, Supervision. **Christopher M. Jones:** Writing – review & editing, Supervision. **Aurélien Saghaï:** Writing – review & editing, Writing – original draft, Supervision. **Sara Hallin:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Data availability

Data from this experiment is shared in the supplementary material and amplicon sequencing data has been deposited in the NCBI Sequence Read Archive under the BioProject accession number PRJNA1208997.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2025.109846.

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– SUPPLEMENTARY INFORMATION –

Resistance and resilience of co-occurring nitrifying microbial guilds to drying-rewetting stress in soil

Laura J. Müller, Mara Alicke, Sana Romdhane, Grace Pold, Christopher M. Jones, Aurélien Saghaï, Sara Hallin

Content

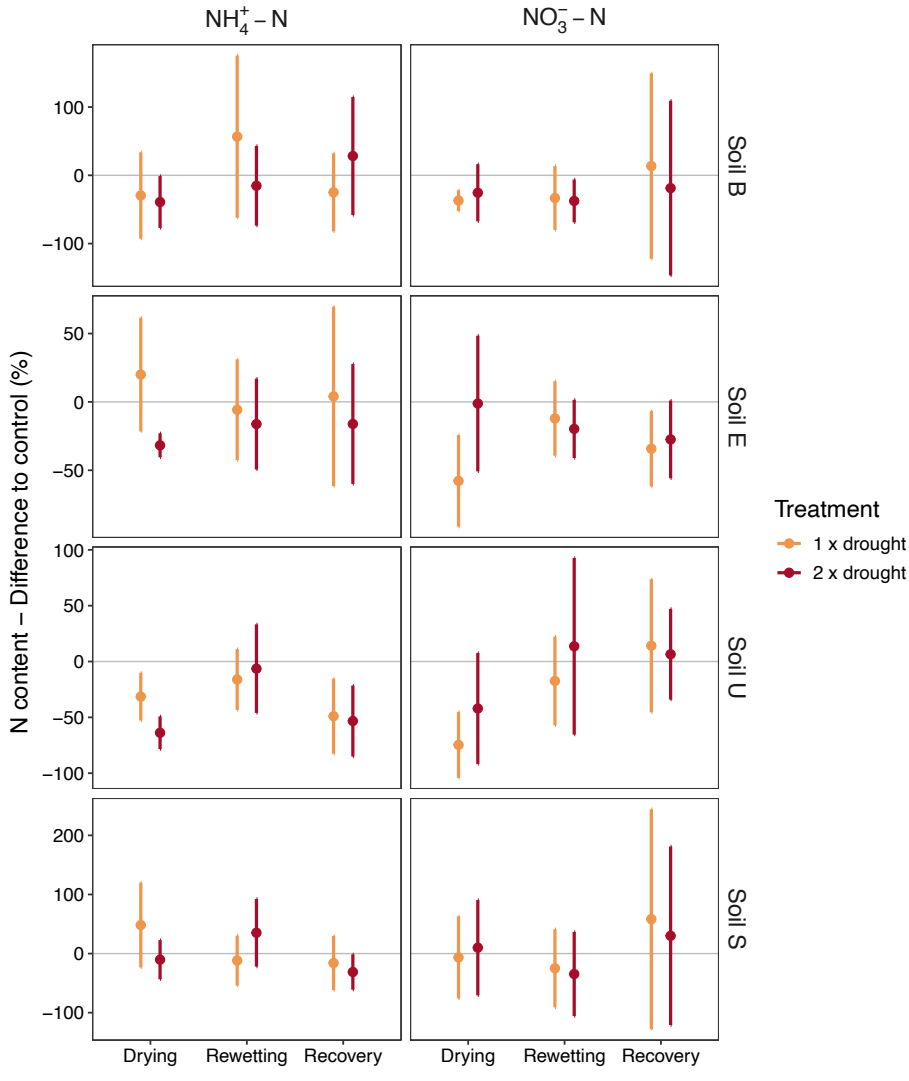

Fig. S1. Pools of soil ammonium (NH_4^+) and nitrate (NO_3^-) in the four soils when subject to one long or two shorter droughts, presented as percent change in comparison to the control at the end of the drought treatments/during drying (day 42), after rewetting (day 49) and after the recovery phase (day 77). Bars represent 95 % confidence intervals and when confidence intervals did not span over zero, the percentage change of treatment from control was considered significant. Colours indicate drought treatments.

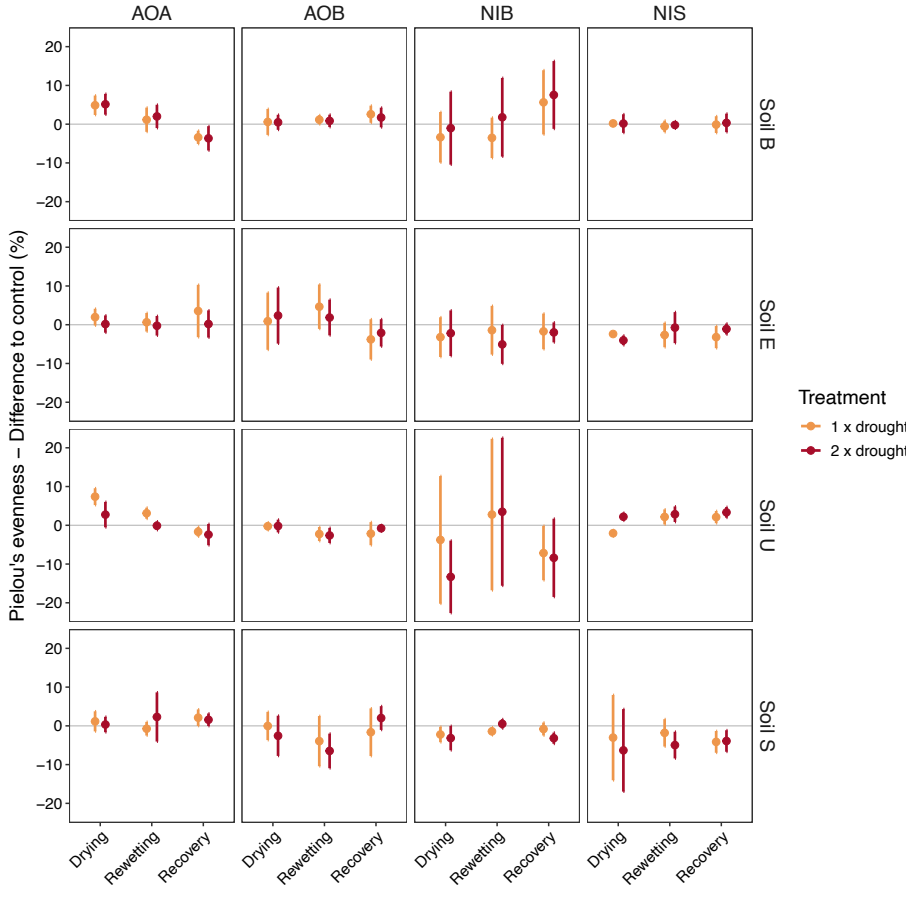

Fig. S2. Pielou's evenness of archaeal *amoA* (AOA), bacterial *amoA* (AOB), *Nitrobacter* type *nxrB* (NIB), and *Nitrospira* type *nxrB* (NIS) communities in the four soils, presented as percent change in comparison to the control at the end of the drought treatments/during drying (day 42), after rewetting (day 49) and after the recovery phase (day 77). Bars represent 95 % confidence intervals and when confidence intervals did not span over zero, the percentage change of treatment from control was considered significant. Colours indicate drought treatments.

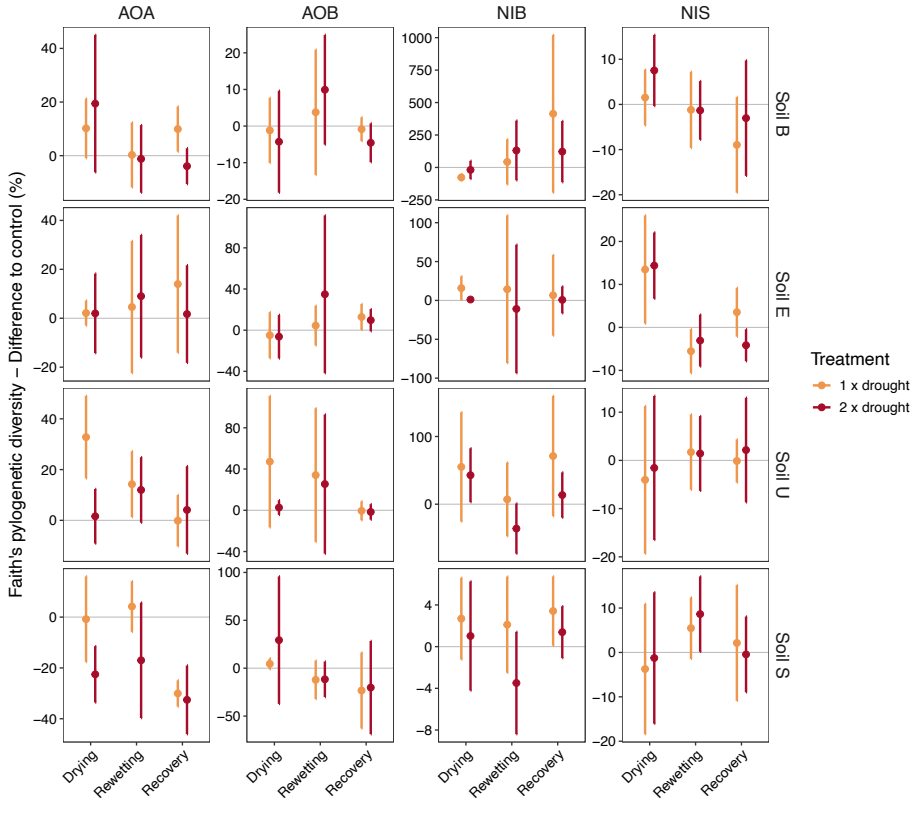

Fig. S3. Faith's phylogenetic diversity of archaeal *amoA* (AOA), bacterial *amoA* (AOB), *Nitrobacter* type *nxrB* (NIB), and *Nitrospira* type *nxrB* (NIS) communities in the four soils, presented as percent change in comparison to the control at the end of the drought treatments/during drying (day 42), after rewetting (day 49) and after the recovery phase (day 77). Bars represent 95 % confidence intervals and when confidence intervals did not span over zero, the percentage change of treatment from control was considered significant. Colours indicate drought treatments.

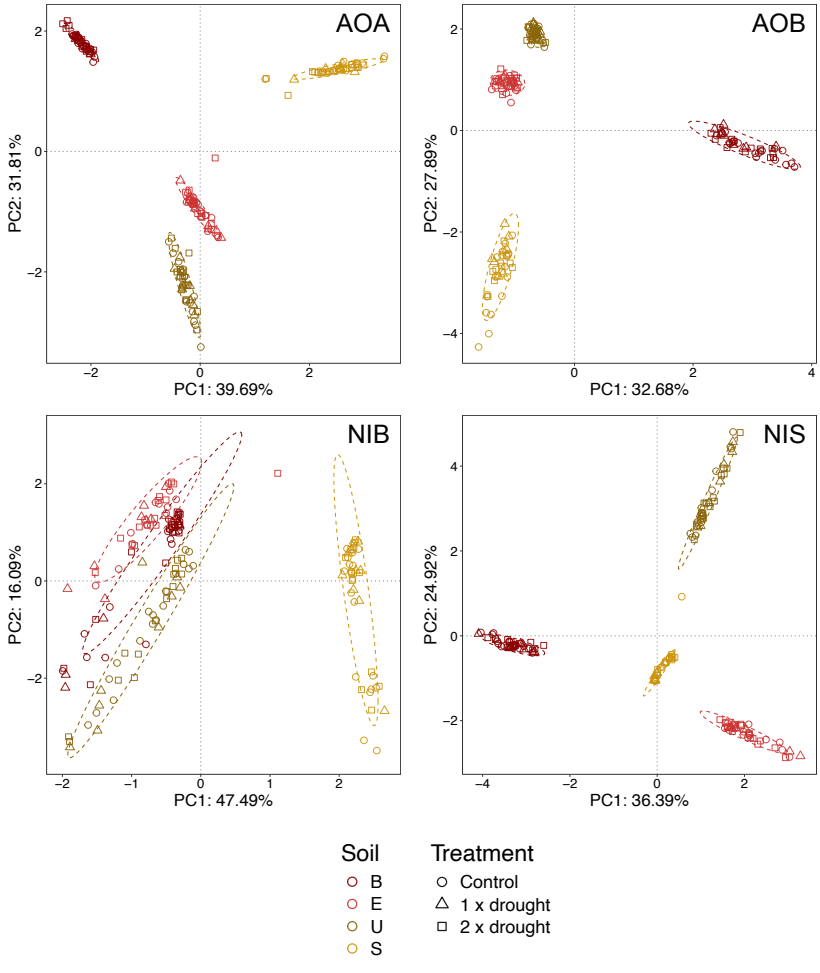

Fig. S4. Principal component analysis (PCA) of archaeal *amoA* (AOA), bacterial *amoA* (AOB), *Nitrobacter* type *nxrB* (NIB), and *Nitrospira* type *nxrB* (NIS) communities in the four soils when subject to one long or two shorter droughts. Communities were assessed at the end of the drought treatments/during drying (day 42), after rewetting (day 49) and after the recovery phase (day 77) but timepoints are not distinguishable in this figure. Dashed ellipses indicate 95 % confidence intervals for each soil. Colours represent soils and shapes drought treatment at all timepoints.

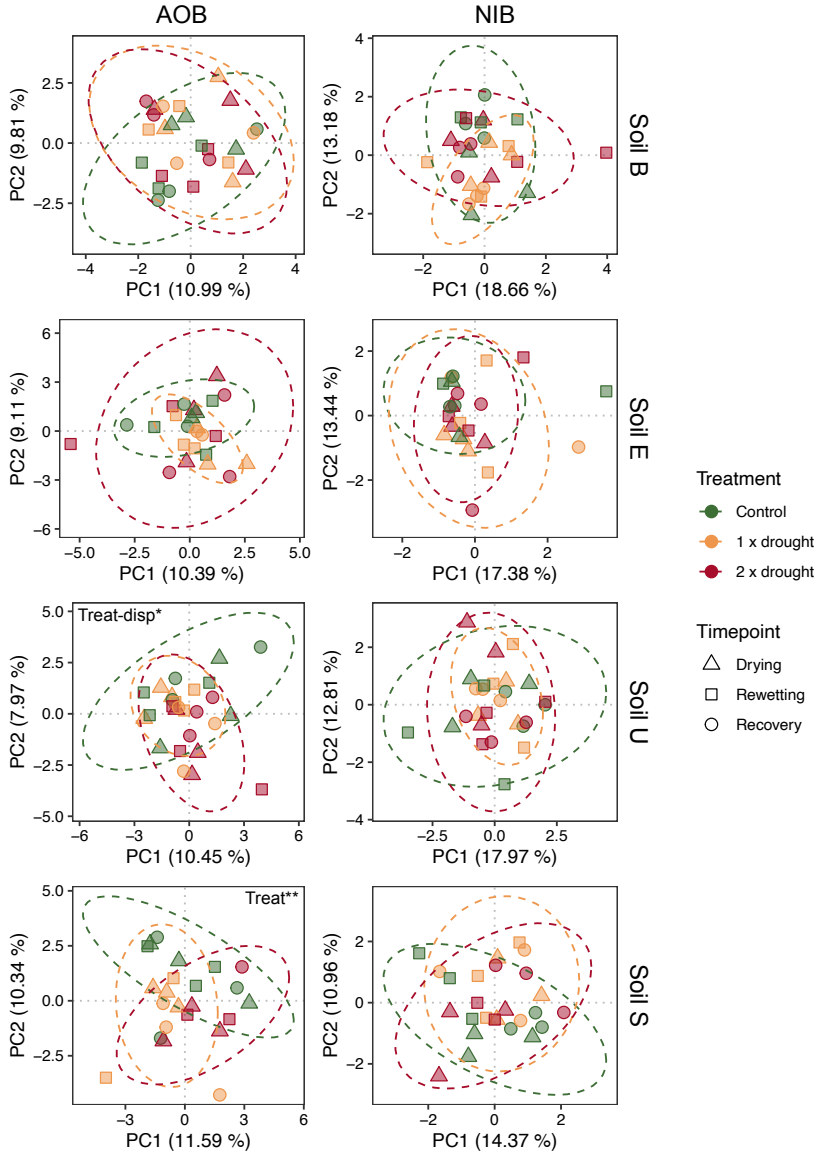

Fig. S5. Principal component analysis (PCA) of ammonia oxidizing bacterial *amoA* (AOA) and *Nitrobacter* type *nxrB* (NIB) communities in the four soils after subjected to one long or two shorter droughts. The communities were analysed at the end of the drought treatments (day 42), after rewetting (day 49), and after the recovery phase (day 77). Significant differences in community structure and β-dispersal ('-disp') across treatments and timepoints are indicated inside the plot ($p < 0.05$*, $p < 0.01$**, $p < 0.001$***). Colours represent treatments, shapes time points, and dashed ellipses 95 % confidence intervals of the respective treatment.

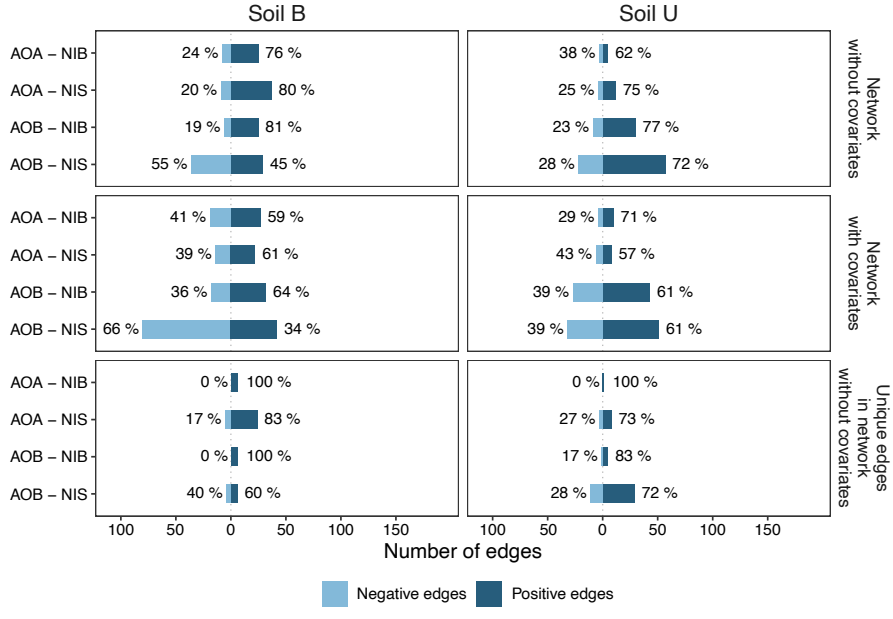

Fig. S6. Negative and positive network edges between ammonia oxidisers (ammonia oxidising archaea/AOA and ammonia oxidising bacteria/AOB) and nitrite oxidisers (*Nitrobacter* type nitrite oxidisers/NIB and *Nitrospira* type nitrite oxidisers/NIS) in networks built without covariates (treatment and timepoint), without covariates, and unique edges in the network without covariates, i.e. edges affected by treatment/timepoint. Numbers indicate the percent positive and negative edges per type of edge, model, and soil.

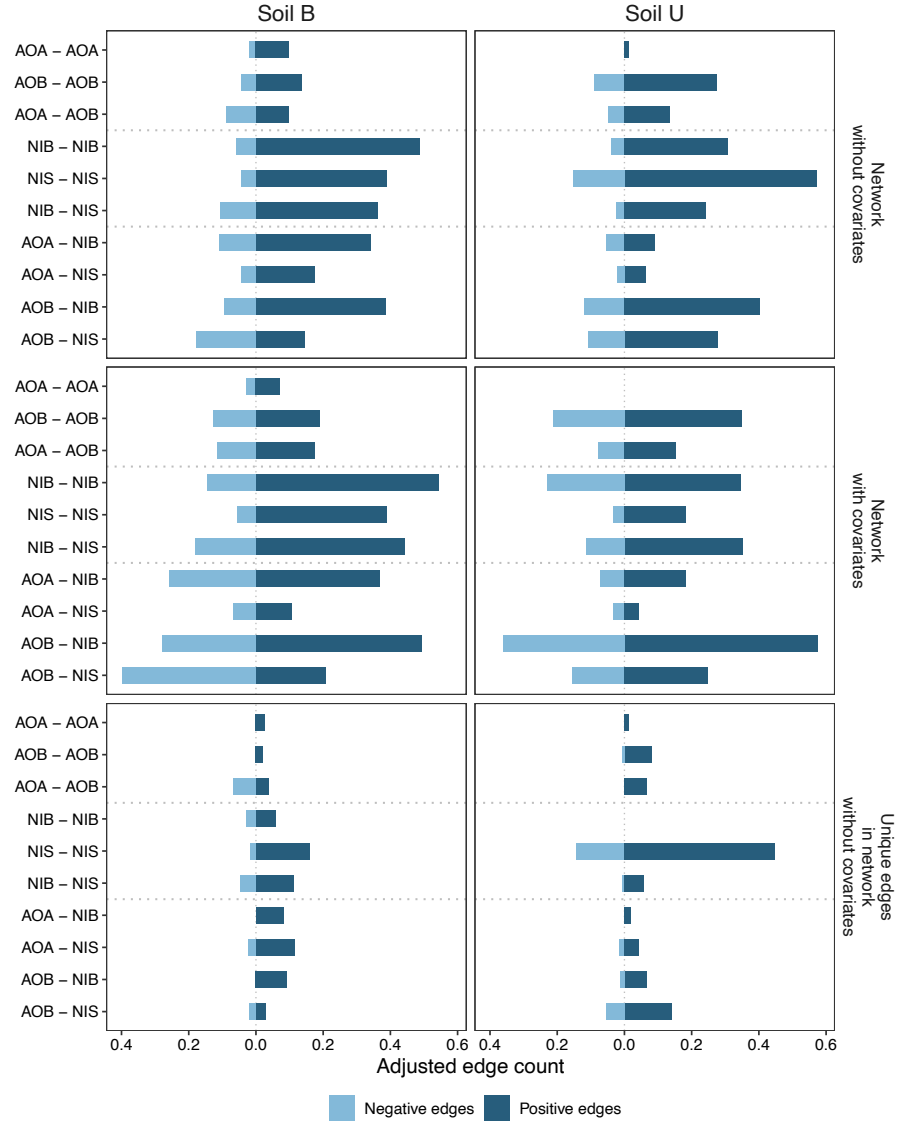

Fig. S7. All combinations of negative and positive network edges per node in networks built without covariates, with covariates (treatment and timepoint), and unique edges in the network without covariates, i.e. edges affected by treatment/timepoint. Edges per node were calculated by dividing the edge count by the average number of ASVs of the functional groups (ammonia oxidising archaea (AOA), ammonia oxidising bacteria (AOB), *Nitrobacter* type nitrite oxidisers (NIB), and *Nitrospira* type nitrite oxidisers (NIS) to which the two connected nodes belong. Colours represent negative/positive edges.

Table S1. Primers and thermal cycling conditions for library preparation for sequencing of *amoA* and *nxrB* genes.

Group	Target (gene)	Primer	Sequence (5'–3')	Reference	Conc.	PCR conditions
AOA	archaeal *amoA*	crenamoA23F	ATG GTC TGG CTW AGA CG	(Tourna et al., 2008)	0.5 µM	98°C 3 min; (98°C 30s, 55°C 30s, 72°C 60s) x 30; 72°C 5 min
		crenamoA616R	GCC ATC CAT CTG TAT GTC CA			
AOB	bacterial *amoA*	AmoA1F	GGG GTT TCT ACT GGT GGT	(Rotthauwe et al., 1997)	0.5 µM	98°C 3 min; (98°C 30s, 55°C 30s, 72°C 45s) x 30; 72°C 10 min
		AmoA2R	CCC CTC KGS AAA GCC TTC TTC			
Nitrobacter NOB	*Nitrobacter* type *nxrB*	nxrB1F	ACG TGG AGA CCA AGC CGG G	(Vanparys et al., 2007)	0.5 µM	98°C 3 min; (98°C 30s, 66°C 30s, 72°C 45s) x 31; 72°C 10 min
		nxrB1R	CCG TGC TGT TGA YCT CGT TGA			
Nitrospira NOB	*Nitrospira* type *nxrB*	nxrB169f	TAC ATG TGG TGG AAC A	(Pester et al., 2014)	0.5 µM	98°C 3 min; (98°C 30s, 56°C 30s, 72°C 45s) x 29; 72°C 10 min
		nxrB638r	CGG TTC TGG TCR ATC A			
All	1st PCR product	Forward tag			0.2 µM	98°C 3 min; (98°C 30 sec, 55°C 30 sec, 72°C 40 sec) x 8; 72°C 5 min
		Reverse				

Table S2. Numbers of sequences and ASVs during pre- and postprocessing.

	Merged reads	Reads after quality filtering[a]	ASVs after rarefaction	Filter 1: raref. ASVs[b]	Filter 2: non-raref. ASVs[c]
AOA	4,413,689	3,092,108	816	233	263
AOB	5,219,882	3,771,264	1,928	277	327
NIB	6,878,348	6,556,409	685	111	127
NIS	7,307,591	3,636,164	3,766	674	801

[a] Removal of chimeras and singletons

[b] ASVs removed with abundance < 0.001 % and presence < 15 %

[c] ASVs removed with abundance < 0.001 % and presence < 45 %

Table S3. Potential ammonia oxidation (PAO), NH_4^+-N, and NO_3^--N, and phylogenetic diversity and Pielou's evenness of archaeal *amoA* (AOA), bacterial *amoA* (AOB), *Nitrobacter* type *nxrB* (NIB), and *Nitrospira* type *nxrB* (NIS) communities in the control and two drought treatments at the end of the drought treatments (day 42), after rewetting (day 49), and after the recovery phase (day 77) in four different soils (mean ± SD; continued on next page).

						Phylogenetic diversity				Evenness			
soil	time-point	treatment	PAO (mg NO_2^--N g^{-1} DW)	NH_4^+-N (mg kg^{-1} DW)	NO_3^--N (mg kg^{-1} DW)	AOA	AOB	NIB	NIS	AOA	AOB	NIB	NIS
B (Bretenière, France)	drying	control	0.53 ± 0.04	1.92 ± 0.96	27.43 ± 6.22	3.56 ± 0.19	1.75 ± 0.11	0.19 ± 0.09	1.53 ± 0.05	0.7 ± 0.01	0.83 ± 0.01	0.46 ± 0.03	0.86 ± 0
		1xDrought	0.45 ± 0.04	1.35 ± 0.93	17.28 ± 0.61	3.92 ± 0.31	1.73 ± 0.1	0.04 ± 0.04	1.55 ± 0.07	0.74 ± 0.01	0.83 ± 0.02	0.45 ± 0.01	0.86 ± 0
		2xDrought	0.49 ± 0.04	1.17 ± 0.37	20.42 ± 9.9	4.25 ± 0.84	1.68 ± 0.21	0.15 ± 0.1	1.64 ± 0.1	0.74 ± 0.01	0.83 ± 0.01	0.46 ± 0.03	0.86 ± 0.02
	rewetting	control	0.59 ± 0.06	1.54 ± 0.91	36.92 ± 5.24	4.04 ± 0.4	1.61 ± 0.19	0.11 ± 0.1	1.53 ± 0.09	0.7 ± 0.02	0.83 ± 0	0.44 ± 0.01	0.85 ± 0
		1xDrought	0.6 ± 0.03	2.41 ± 0.98	24.65 ± 16.05	4.05 ± 0.23	1.67 ± 0.17	0.15 ± 0.1	1.52 ± 0.09	0.71 ± 0.01	0.84 ± 0.01	0.43 ± 0.02	0.85 ± 0.01
		2xDrought	0.59 ± 0.05	1.3 ± 0.36	23.06 ± 10.54	3.99 ± 0.28	1.76 ± 0.08	0.24 ± 0.03	1.51 ± 0.04	0.71 ± 0.01	0.83 ± 0.01	0.45 ± 0.04	0.85 ± 0.01
	recovery	control	0.51 ± 0.09	1.81 ± 0.85	31.78 ± 30.81	3.66 ± 0.15	1.73 ± 0.05	0.04 ± 0.04	1.53 ± 0.15	0.7 ± 0.01	0.82 ± 0.02	0.41 ± 0.03	0.86 ± 0.02
		1xDrought	0.58 ± 0.07	1.36 ± 0.75	36.11 ± 21.66	4.03 ± 0.24	1.72 ± 0.02	0.21 ± 0.11	1.4 ± 0.08	0.68 ± 0.01	0.84 ± 0.01	0.43 ± 0.01	0.86 ± 0
		2xDrought	0.55 ± 0.05	2.32 ± 1.01	25.82 ± 29.59	3.52 ± 0.18	1.65 ± 0.07	0.09 ± 0	1.49 ± 0.12	0.68 ± 0.02	0.83 ± 0.01	0.44 ± 0.01	0.87 ± 0.01
E (Ekhaga, Sweden)	drying	control	0.62 ± 0.06	0.89 ± 0.02	27.58 ± 7.21	2.69 ± 0.01	0.75 ± 0.11	0.26 ± 0	0.98 ± 0.04	0.66 ± 0.01	0.64 ± 0.03	0.56 ± 0.02	0.82 ± 0
		1xDrought	0.38 ± 0.05	1.07 ± 0.35	11.64 ± 8.26	2.74 ± 0.13	0.71 ± 0.08	0.31 ± 0.03	1.12 ± 0.09	0.67 ± 0.01	0.65 ± 0.02	0.54 ± 0.01	0.8 ± 0
		2xDrought	0.68 ± 0.24	0.61 ± 0.07	27.25 ± 10.92	2.74 ± 0.42	0.7 ± 0.07	0.27 ± 0	1.12 ± 0.04	0.66 ± 0.01	0.66 ± 0.02	0.55 ± 0.01	0.79 ± 0.01
	rewetting	control	0.67 ± 0.08	1 ± 0.35	31.46 ± 5.63	2.54 ± 0.54	0.76 ± 0.05	0.25 ± 0.17	1.09 ± 0.03	0.66 ± 0.01	0.63 ± 0.02	0.57 ± 0.02	0.8 ± 0.02
		1xDrought	0.61 ± 0.04	0.94 ± 0.12	27.65 ± 6.49	2.65 ± 0.32	0.8 ± 0.13	0.29 ± 0.02	1.03 ± 0.04	0.66 ± 0.01	0.66 ± 0.03	0.56 ± 0.02	0.78 ± 0.01
		2xDrought	0.67 ± 0.04	0.84 ± 0.12	25.24 ± 4.5	2.76 ± 0.12	1.03 ± 0.55	0.22 ± 0.08	1.06 ± 0.05	0.66 ± 0.01	0.64 ± 0.02	0.54 ± 0.02	0.8 ± 0.02
	recovery	control	0.64 ± 0.04	1.4 ± 0.57	49.2 ± 7.06	2.64 ± 0.43	0.65 ± 0.03	0.28 ± 0.04	1.04 ± 0.03	0.65 ± 0.02	0.67 ± 0	0.56 ± 0.01	0.81 ± 0.01
		1xDrought	0.64 ± 0.05	1.46 ± 0.65	32.34 ± 12.06	3 ± 0.51	0.74 ± 0.07	0.29 ± 0.11	1.07 ± 0.05	0.67 ± 0.04	0.65 ± 0.03	0.55 ± 0.02	0.79 ± 0.02
		2xDrought	0.65 ± 0.06	1.18 ± 0.34	35.69 ± 12.3	2.68 ± 0.24	0.72 ± 0.06	0.28 ± 0.02	0.99 ± 0.02	0.65 ± 0.01	0.66 ± 0.02	0.55 ± 0.01	0.8 ± 0.01

soil	time-point	treatment	PAO (mg NO_2^--N g^{-1} DW)	NH_4^+-N (mg kg^{-1} DW)	NO_3^--N (mg kg^{-1} DW)	Phylogenetic diversity				Evenness			
						AOA	AOB	NIB	NIS	AOA	AOB	NIB	NIS
U (Ulleråker, Sweden)	drying	control	0.34 ± 0.02	2.07 ± 0.3	33.16 ± 25.95	2.86 ± 0.24	0.8 ± 0.03	0.15 ± 0.03	1.27 ± 0.18	0.67 ± 0.01	0.75 ± 0.01	0.39 ± 0.04	0.82 ± 0.01
		1xDrought	0.25 ± 0.02	1.42 ± 0.37	8.4 ± 6.63	3.8 ± 0.31	1.18 ± 0.49	0.24 ± 0.11	1.22 ± 0.07	0.72 ± 0.01	0.75 ± 0	0.38 ± 0.05	0.8 ± 0
		2xDrought	0.34 ± 0.03	0.75 ± 0.27	19.21 ± 4.49	2.91 ± 0.17	0.83 ± 0.05	0.22 ± 0.04	1.25 ± 0.03	0.69 ± 0.02	0.75 ± 0.01	0.34 ± 0.01	0.84 ± 0.01
	rewetting	control	0.35 ± 0.03	1.32 ± 0.25	23.16 ± 9	2.94 ± 0.27	0.89 ± 0.08	0.21 ± 0.09	1.21 ± 0.08	0.67 ± 0.01	0.76 ± 0.01	0.32 ± 0.03	0.82 ± 0.01
		1xDrought	0.34 ± 0.05	1.11 ± 0.27	19.12 ± 4.65	3.36 ± 0.19	1.2 ± 0.54	0.22 ± 0.04	1.23 ± 0.03	0.69 ± 0.01	0.74 ± 0.01	0.33 ± 0.05	0.84 ± 0
		2xDrought	0.36 ± 0.05	1.24 ± 0.44	26.32 ± 14.14	3.29 ± 0.19	1.12 ± 0.56	0.13 ± 0.04	1.23 ± 0.03	0.67 ± 0	0.74 ± 0.01	0.34 ± 0.05	0.85 ± 0
	recovery	control	0.31 ± 0.02	1.85 ± 0.99	26.6 ± 9.34	2.79 ± 0.11	0.85 ± 0.04	0.12 ± 0.02	1.23 ± 0.03	0.68 ± 0.01	0.76 ± 0	0.34 ± 0.02	0.82 ± 0.01
		1xDrought	0.36 ± 0.02	0.94 ± 0.3	30.36 ± 10.69	2.79 ± 0.24	0.84 ± 0.06	0.2 ± 0.1	1.22 ± 0.04	0.67 ± 0	0.74 ± 0.02	0.31 ± 0.01	0.84 ± 0.01
		2xDrought	0.37 ± 0.01	0.86 ± 0.31	28.33 ± 2.68	2.91 ± 0.44	0.83 ± 0.04	0.13 ± 0.03	1.25 ± 0.12	0.67 ± 0.02	0.75 ± 0	0.31 ± 0.03	0.85 ± 0
S (Schnega, Germany)	drying	control	0.04 ± 0.01	0.82 ± 0.17	19.98 ± 13.68	4.51 ± 0.5	0.83 ± 0.04	0.5 ± 0.01	1.31 ± 0.16	0.74 ± 0.01	0.83 ± 0.02	0.77 ± 0.01	0.71 ± 0.08
		1xDrought	0.04 ± 0.01	1.22 ± 0.5	18.68 ± 3.33	4.47 ± 0.53	0.86 ± 0.01	0.52 ± 0.02	1.26 ± 0.1	0.75 ± 0.01	0.83 ± 0.02	0.75 ± 0	0.69 ± 0.01
		2xDrought	0.04 ± 0	0.74 ± 0.21	21.97 ± 3.05	3.49 ± 0.28	1.07 ± 0.52	0.51 ± 0.02	1.29 ± 0.09	0.75 ± 0	0.81 ± 0.04	0.74 ± 0.02	0.66 ± 0.01
	rewetting	control	0.04 ± 0.01	1.08 ± 0.36	21.05 ± 11.08	4.54 ± 0.26	0.87 ± 0.14	0.51 ± 0.01	1.19 ± 0.06	0.74 ± 0.01	0.85 ± 0.03	0.76 ± 0.01	0.68 ± 0.02
		1xDrought	0.03 ± 0.01	0.96 ± 0.29	15.82 ± 10.34	4.73 ± 0.34	0.77 ± 0.07	0.53 ± 0.02	1.26 ± 0.04	0.73 ± 0.01	0.82 ± 0.04	0.75 ± 0	0.67 ± 0.01
		2xDrought	0.04 ± 0.01	1.46 ± 0.33	13.77 ± 12.3	3.77 ± 0.96	0.77 ± 0.04	0.5 ± 0.02	1.29 ± 0.07	0.76 ± 0.04	0.79 ± 0.02	0.77 ± 0	0.65 ± 0.01
	recovery	control	0.04 ± 0.01	1.18 ± 0.45	17.56 ± 18.24	4.7 ± 0.22	1.06 ± 0.52	0.5 ± 0.01	1.24 ± 0.07	0.73 ± 0.01	0.82 ± 0.01	0.77 ± 0.01	0.69 ± 0.01
		1xDrought	0.04 ± 0.01	0.99 ± 0.35	27.8 ± 11.68	3.29 ± 0.17	0.81 ± 0.04	0.51 ± 0.01	1.26 ± 0.14	0.75 ± 0.01	0.8 ± 0.05	0.76 ± 0.01	0.66 ± 0.01
		2xDrought	0.04 ± 0.01	0.81 ± 0.13	22.84 ± 8.73	3.17 ± 0.59	0.84 ± 0.02	0.5 ± 0	1.23 ± 0.07	0.74 ± 0.01	0.83 ± 0.02	0.74 ± 0.01	0.66 ± 0.01

Table S4. Effects of substrate (N-source in form of ammonium, urea, or yeast extract), soil, and timepoint on potential ammonia oxidation rates in the control treatment, analysed by three-way ANOVA after Box-Cox transformation ($p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***).

Term	Statistic
Substrate	$F_{(2, 72)} = 1.17$
Soil	**$F_{(3, 72)} = 2986.67$ *** **
Timepoint	**$F_{(2, 72)} = 4.91$ ** **
Substrate:Soil	**$F_{(6, 72)} = 4.55$ *** **
Solution:Timepoint	$F_{(4, 72)} = 0.08$
Soil:Timepoint	**$F_{(6, 72)} = 3.20$ ** **
Solution:Soil:Timepoint	$F_{(12, 72)} = 0.27$

Table S5. Summary of permutational test for homogeneity of dispersion between groups ("Permdisp") and permutational multivariate analyses of variance ("PERMANOVA") to assess treatment and timepoint effects on *β*-diversity (Figs. 2 and S5). Results are shown per guild of ammonia oxidising archaea (AOA), ammonia oxidising bacteria (AOB), *Nitrobacter* type nitrite oxidisers (NIB), and *Nitrospira* type nitrite oxidisers (NIS) and soil.

		Treatment			Timepoint		
		Permdisp	PERMANOVA		Permdisp	PERMANOVA	
Gene	Soil	*p*-value	*p*-value	R^2	*p*-value	*p*-value	R^2
AOA	B	0.767	**0.016** *	0.10917	0.687	**0.029** *	0.1041
	E	0.313	0.13	0.09496	0.566	0.729	0.06734
	U	**0.006** **	0.094	0.09695	0.785	**0.029** *	0.11073
	S	0.375	0.323	0.08243	0.601	0.748	0.06719
AOB	B	0.854	0.073	0.09252	0.606	0.349	0.07957
	E	0.562	0.693	0.07849	0.165	0.547	0.08163
	U	**0.046** *	0.053	0.09118	0.855	0.861	0.06788
	S	0.638	**0.006** **	0.12277	0.634	0.659	0.08538
NIB	B	0.858	0.17	0.09166	0.729	0.761	0.06475
	E	0.95	0.616	0.08142	0.838	0.195	0.10143
	U	0.256	0.91	0.05521	0.278	0.335	0.08316
	S	0.510	0.2	0.09227	0.828	0.128	0.0981
NIS	B	**0.015** *	**0.003** **	0.10891	0.208	**0.02** *	0.09838
	E	0.601	**0.001** ***	0.14854	0.71	**0.009** **	0.10895
	U	**0.003** **	**0.001** ***	0.2208	0.203	0.181	0.0912
	S	0.579	**0.014** *	0.10809	0.371	**0.023** *	0.10319

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Soil microorganisms are essential for ecosystem functioning but are threatened by contamination and climate change. This thesis explored how microorganisms involved in nitrification respond to individual and multiple stressors. While herbicides had little impact, copper and PAH altered microbial communities and impaired ammonia oxidation. Drought destabilised nitrifier interactions, especially after rewetting. Responses varied with soil type and contamination history, highlighting the importance to consider context-dependency and multiple stressors to understand stressor impacts on soil functioning and microbial communities.

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