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In vitro assessment of the toxicity of pesticides on ammonia-oxidizing microorganisms

Eleftheria Bachtsevani

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**CENTRALE
LYON**

N° d'ordre NNT: 2024ECDL0049

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Ingénierie pour le vivant

Par

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microorganisms**

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Contents

| | |
|---|----|
| Abstract | 9 |
| List of Figures..... | 11 |
| List of Supplementary Tables and Figures | 15 |
| List of abbreviations..... | 16 |
| Chapter 1 | 18 |
| General Introduction..... | 18 |
| 1. Plant protection products..... | 19 |
| 1.1 Definition, history and use over the years | 19 |
| 1.2 Classification | 23 |
| 1.3 Benefits and risks of pesticides..... | 24 |
| 1.4 Environmental fate of pesticides | 25 |
| 1.4.1 Absorption | 26 |
| 1.4.2 Degradation | 26 |
| 1.4.3 Pesticides transportation in soil..... | 27 |
| 1.5 Impact of pesticides on soil microorganisms..... | 27 |
| 1.6 Environmental risk assessment of pesticides | 29 |
| 1.7 New proposed risk assessment scheme of pesticides on soil microorganisms..... | 32 |
| 1.8 Nitrification and nitrifying microorganisms | 34 |
| 1.8.1 Nitrification process | 34 |
| 1.8.3 Ammonia-oxidizing archaea | 38 |
| 1.8.4 Nitrite-oxidizing bacteria | 41 |
| 1.8.5 Complete ammonia-oxidizing bacteria (comammox) | 43 |
| 1.9 Ammonia-oxidizing microorganisms as ideal microbial indicators of pesticides toxicity | 44 |
| 1.10 Aims and objectives | 45 |
| 1.11 References | 46 |
| Chapter 2..... | 60 |
| Single species <i>in vitro</i> assays with nitrifying bacteria and archaea as a first-tier tool for assessing the toxicity of pesticides on soil microorganisms | 60 |
| Abstract | 61 |
| Keywords:..... | 61 |
| 2.1 Introduction | 61 |
| 2.2 Material and Methods..... | 63 |
| 2.2.1 Microbial strains..... | 63 |
| 2.2.5 Calculation of toxicity endpoint values | 65 |

| | |
|---|-----|
| 2.2.6 Data analysis | 69 |
| 2.3 Results | 69 |
| 2.3.1 Impact of pesticides on the activity of AOA strains | 69 |
| 2.3.1.1. Insecticides..... | 69 |
| 2.3.1.2. Herbicides | 69 |
| 2.3.1.3. Fungicides | 69 |
| 2.3.1.4. EC ₅₀ values for AOA..... | 69 |
| 2.3.2. Impact of pesticides on the activity of AOB strains | 70 |
| 2.3.2.1. Insecticides..... | 70 |
| 2.3.2.2. Herbicides | 70 |
| 2.3.2.3. Fungicides | 71 |
| 2.3.2.4. EC ₅₀ values for AOB..... | 71 |
| 2.3.3 Impact of pesticides on the activity of NOB strains | 73 |
| 2.3.4 Sensitivity of AOA, AOB and NOB to each pesticide category | 74 |
| 2.4 Discussion | 76 |
| 2.5 Conclusion | 79 |
| 2.6 Acknowledgments..... | 79 |
| 2.8 References | 80 |
| Chapter 3 | 87 |
| Assessment of pesticide toxicity on sensitive soil nitrifiers: validation of single-species <i>in vitro</i> bioassay..... | 87 |
| Abstract | 88 |
| Keywords:..... | 88 |
| 3.1 Introduction | 88 |
| 3.2 Material and Methods..... | 90 |
| 3.2.1 Microbial strains..... | 90 |
| 3.2.2 Pesticides | 90 |
| 3.2.3 Single species <i>in vitro</i> assays | 91 |
| 3.2.4 Nitrite measurements | 92 |
| 3.2.5 Calculation of toxicity endpoint values | 92 |
| 3.2.6 Data analysis | 92 |
| 3.3 Results | 98 |
| 3.3.1 Impact of pesticides on the activity of <i>Nitrosotalea sinensis</i> | 98 |
| 3.3.2. Impact of pesticides on the activity of <i>Nitrosospira briensis</i> | 98 |
| 3.3.3. Impact of pesticides on the activity of <i>Ca. Nitrobacter laanbroekii</i> NHB1..... | 109 |
| 3.3.4 Sensitivity of the different tested strains to each pesticide category | 109 |

| | |
|---|-----|
| 3.4 Discussion | 115 |
| 3.4 Conclusion | 118 |
| 3.6 Acknowledgments..... | 118 |
| 3.7 References | 119 |
| Chapter 4 | 124 |
| Assessment of pesticide toxicity using the Microbial Assay for Risk Assessment (MARA): A comparative study with soil nitrifying microorganisms..... | 124 |
| Abstract | 125 |
| Keywords..... | 125 |
| 4.1 Introduction | 125 |
| 4.2 Material and Methods..... | 128 |
| 4.2.1 Pesticides | 128 |
| 4.2.2 Preparation of pesticides working solution | 129 |
| 4.2.3 MARA assay | 129 |
| 4.3 Results | 130 |
| 4.3.1 Effects of DMSO on MARA microbial strains | 130 |
| 4.3.2 Toxicity of pesticides on MARA assay..... | 131 |
| 4.3.3 Comparison of pesticides toxicity among MARA microbial strains and nitrifying strains | 133 |
| 4.3.4 Comparison of pesticides toxicity among MARA microbial strains and nitrifying strains..... | 139 |
| 4.4 Discussion | 145 |
| 4.5 Conclusion | 146 |
| 4.5 Acknowledgments..... | 146 |
| 4.6 References | 147 |
| Chapter 5 | 150 |
| Acidotolerant soil nitrite oxidiser ' <i>Candidatus Nitrobacter laanbroekii</i> ' NHB1 alleviates constraints on growth of acidophilic soil ammonia oxidisers | 150 |
| Abstract | 151 |
| Keywords: <i>Nitrobacter</i> , nitrite-oxidising bacteria, ammonia-oxidising archaea, acidophilic, acidic soil, ammonia oxidation, nitrite oxidation, nitrification, cyanas | 151 |
| 5.1 Introduction | 151 |
| 5.2 Material and Methods..... | 152 |
| 5.2.1 Isolation and maintenance of ' <i>Ca. Nitrobacter laanbroekii</i> ' NHB1 in culture | 152 |
| 5.2.2 Ammonia, nitrite and nitrate concentration measurements | 153 |
| 5.2.3 Characterisation of <i>Ca. Nitrobacter laanbroekii</i> NHB1..... | 153 |

| | |
|--|-----|
| 5.2.4 Growth of <i>Nitrosotalea</i> strains in isolation or co-culture with 'Ca. Nitrobacter laanbroekii' NHB1 | 155 |
| 5.2.5 Determination of substrate kinetics..... | 156 |
| 5.2.6 Transmission electron microscopy | 156 |
| 5.2.7 Genome sequencing, assembly and annotation | 156 |
| 5.2.3 Phylogenomic analysis..... | 157 |
| 5.3 Results and Discussion | 157 |
| 5.3.1 Isolation and characterisation of NHB1 | 157 |
| 5.3.2 Influence of NHB1 on acidophilic AOA physiology..... | 161 |
| 5.4 Summary | 166 |
| 5.5 Acknowledgements..... | 166 |
| 5.6 Conflict of interest statement | 166 |
| 5.7 Data availability | 166 |
| 5.8 References | 167 |
| Chapter 6..... | 171 |
| General discussion | 171 |
| 6.1 Overview | 171 |
| 6.2 Development and validation of an <i>in vitro</i> bioassay using nitrifying strains as bioindicators..... | 172 |
| 6.3 Evaluating the limitations of a commercial toxicity kit and the critical role of nitrifiers in pesticides ecotoxicity assessments..... | 174 |
| 6.4 Ecological importance of <i>Ca. Nitrobacter laanbroekii</i> NHB1 in facilitating nitrification in acidic soils | 174 |
| 6.5 Future Perspectives..... | 175 |
| 6.6 Conclusion | 177 |
| 6.7 References | 177 |
| Synthèse en français | 180 |
| Résumé..... | 180 |
| Introduction Générale..... | 182 |
| 1. Produits phytopharmaceutiques..... | 182 |
| 1.1 Définition, histoire et utilisation au fil des ans | 182 |
| 1.2 Classification | 186 |
| 1.3 Avantages et risques des pesticides | 187 |
| 1.4 Le devenir des pesticides dans l'environnement | 189 |
| | 190 |
| 1.4.1 Absorption | 190 |
| 1.4.2 Dégradation | 190 |

| | |
|---|-----|
| 1.4.3 Transport des pesticides dans le sol | 190 |
| 1.5 Impact des pesticides sur les micro-organismes du sol..... | 191 |
| 1.6 Évaluation des risques environnementaux liés aux pesticides | 192 |
| 1.8 Nitrification et micro-organismes nitrifiants | 198 |
| 1.8.1 Processus de nitrification..... | 198 |
| 1.8.2 Bactéries oxydant l'ammoniac | 200 |
| 1.8.3 Archées oxydant l'ammoniac..... | 202 |
| 1.8.4 Bactéries oxydantes les nitrites | 205 |
| 1.8.5 Bactéries oxydantes complètes de l'ammoniac (comammox)..... | 207 |
| 1.9 Les microorganismes oxydant l'ammoniac comme indicateurs microbiens idéaux de la toxicité des pesticides | 208 |
| 1.10 Objectifs..... | 209 |
| 1.11 Références | 210 |
| Discussion Générale..... | 223 |
| 2.1 Vue d'ensemble | 224 |
| 2.2 Développement et validation d'un essai biologique in vitro utilisant des souches nitrifiantes comme bioindicateurs..... | 225 |
| 2.3 Évaluation des limites d'un kit de toxicité commercial et du rôle essentiel des nitrifiants dans les évaluations de l'écotoxicité des pesticides | 226 |
| 2.4 Importance écologique de <i>Ca. Nitrobacter laanbroekii</i> NHB1 pour faciliter la nitrification dans les sols acides | 227 |
| 2.5 Perspectives d'avenir..... | 228 |
| 2.6 Conclusion | 230 |
| 2.7 References | 231 |
| Annex I-Supplemental Material of Chapter 2..... | 233 |
| Single species <i>in vitro</i> assays with nitrifying bacteria and archaea as a first-tier tool for assessing the toxicity of pesticides on soil microorganisms | 233 |
| 1. Material and Methods | 234 |
| 1.1 Culture conditions..... | 234 |
| 1.2 Mode of calculations for pesticides concentration..... | 234 |
| 1.2 Pesticides extraction from liquid cultures..... | 236 |
| 1.3 Recovery tests and validation of the extraction methods | 236 |
| 1.4. Chromatography analyses | 236 |
| 2. Results | 237 |
| 2.1 Stability of pesticides on AOM and NOB cultures..... | 237 |
| 3. References | 239 |

Abstract

The use of pesticides is essential for boosting agricultural yields, but much of these chemicals miss their targets, accumulating in soil and harming ecosystems and microorganisms. Despite the importance of soil microbes in ecosystem health, traditional pesticide risk assessments have not fully addressed the toxicity of pesticides on them. To address this, recent studies proposed a tiered system to assess pesticide effects on the key microbial group of ammonia-oxidizing microorganisms (AOM). The system progresses from *in vitro* toxicity tests (Tier I) to soil microcosms or pot studies (Tier II), and finally to field-scale evaluations (Tier III). The main objectives of this thesis were to i) develop and validate an *in vitro* bioassay using AOM and functionally related NOB as a standard Tier I assay, related to the single-species tests commonly used in aquatic ecotoxicology, ii) compare the toxicity of pesticides on AOM with an existing toxicity assessment assay, the Microbial Assay for Risk Assessment (MARA) kit from NCIMB (Scotland), and iii) characterize the physiology of *Ca. Nitrobacter laanbroekii* NHB1, one of the most sensitive strains identified in this thesis. The toxicity of representative pesticides—covering insecticides, fungicides, and herbicides— was assessed on several ammonia-oxidizing microorganisms (AOM), including ammonia-oxidizing bacteria (AOB), ammonia-oxidizing archaea (AOA), and nitrite-oxidizing bacteria (NOB). Toxicity endpoints (EC_{50}) were determined for each strain-pesticide combination, with *Nitrosotalea sinensis* Nd2 (AOA), *Nitrospira briensis* (AOB), and *Ca. Nitrobacter laanbroekii* NHB1 (NOB) identified as the most sensitive strains and suitable bioindicators for the proposed single species bioassay. Fungicides and insecticides were generally more toxic to AOM than NOB, while herbicides showed variable toxicity across all nitrifier groups. This testing approach proved valuable for Tier I ecotoxicity assessments of pesticides on soil microbial communities. Building on these findings, toxicity of additional pesticides from different categories on the three most sensitive strains was assessed. Using the proposed single-species *in vitro* assay, EC_{50} values confirmed these strains as effective bioindicators for Tier I testing framework. *N. sinensis* was the most sensitive strain, followed by *N. briensis* and *Ca. N. laanbroekii* NHB1, indicating these strains are promising bioindicators for pesticide toxicity in soil. Fungicides were most toxic to *N. sinensis*, causing significant inhibition at agronomical concentration levels. The differential sensitivities of these strains to various pesticide classes highlight the need for a comprehensive approach that captures a broad spectrum of microbial responses. The evaluation of pesticides toxicity using the MARA kit, which uses 11 heterotrophic microbial strains to detect toxicity, demonstrated relatively low sensitivity, with pesticides affecting these strains only at high concentrations, in contrast to AOM which showed much higher sensitivity to pesticides. The physiology of *Ca. Nitrobacter laanbroekii* NHB1, one of the identified bioindicator was also characterised. The strain demonstrated an acidotolerant physiology with optimal growth at pH 6.0 and detectable growth down to pH 3.5. In co-culture, *Ca. N. laanbroekii* NHB1 enhanced the growth of acidophilic ammonia-

oxidizing archaea (AOA), such as *Nitrosotalea devaniterrae* Nd1 and *N. sinensis*, by removing inhibitory nitrite. This cross-feeding mechanism underscores the importance of substrate concentrations in microbial interactions in acidic soils.

List of Figures

Chapter 1

Figure 1.1. (a) Regional proportion of pesticide use in agricultural practice globally and (b) total pesticides quantities (tonnes) used in agriculture the past 29 years. Data source: Food and Agriculture Organization of the United Nations (FAO).....20

Figure 1.2. (a) Proportion of the main categories of pesticides used in agricultural practice in Europe and (b) total pesticides quantities (tonnes) per category used in agriculture the past 29 years in Europe. Data source: Food and Agriculture Organization of the United Nations (FAO).....21

Figure 1.3. Behavior of pesticides in the environment.....25

Figure 1.4. Proposed three-tier risk assessment scheme for assessing the toxicity of pesticides on soil microorganisms (Karpouzas et al., 2022). Ammonia-oxidizing microorganisms, AOM; arbuscular mycorrhizal fungi, AMF.....33

Figure 1.5. Chemolithoautotrophic nitrification, reactions and microbial groups performing each step of the process.....35

Figure 1.6. Ammonia oxidation by ammonia-oxidizing bacteria (AOB) (modified from Stein et al., 2019).....37

Figure 1.7. Ammonia oxidation by ammonia-oxidizing archaea (AOA). Panel (a) and (b) show the proposed pathways for hydroxylamine oxidation (Stein et al., 2019).....40

Figure 1.8. The mechanism of oxidation of nitrite to nitrate by the (A) cytoplasmic and (B) periplasmic nitrite oxidoreductase (NXR) (Daims et al., 2016).....42

Chapter 2

Figure 2.1. Mean EC_{50} values (μ M) of pesticide inhibitory concentrations on AOA strains, *N. sinensis*, *Ca. N. franklandianus* and *N. viennensis*.....69

Figure 2.2. Mean EC_{50} values (μ M) of pesticide inhibitory concentrations on AOB strains *N. briensis*, *N. ureae*, *N. europaea*, *N. communis*, *N. multiformis* and *N. oligotropha*.71

Figure 2.3. Mean EC_{50} values (μ M) of pesticide inhibitory concentrations on NOB strains *Nitrobacter* sp. NHB1, *N. winogradskyi* and *N. defluvii*.73

Figure 2.4. Comparison of (a) the toxicity of different pesticide categories (insecticides, fungicides, herbicides) to AOA, AOB, and NOB, and (b) sensitivity of AOA, AOB, and NOB to the different pesticide groups (insecticides, fungicides, herbicides) based on the mean EC₅₀ values calculated for each pesticide category.....74

Chapter 3

Figure 3.1. The effect of different concentrations of pesticides on nitrite production by the AOA strain *Nitrosotalea sinensis*.....98

Figure 3.2. Mean EC₅₀ values (μM) of pesticide inhibitory concentrations on the AOA strain *Nitrosotalea sinensis*.....102

Figure 3.3. The effect of different concentrations of pesticides on nitrite production by the AOB strain *Nitrosospira briensis*.103

Figure 3.4. Mean EC₅₀ values (μM) of pesticide inhibitory concentrations on the AOB strain *Nitrosospira briensis*. Standard error of mean values (denoted by ±) are given in parentheses. Asterisks denote the maximum tested concentration (**).107

Figure 3.5. The effect of different concentrations of pesticides on nitrite consumption by the NOB, *Ca. Nitrobacter laanbroekii* NHB1.109

Figure 3.6. Mean EC₅₀ values (μM) of pesticide inhibitory concentrations on NOB strain *Ca. Nitrobacter laanbroekii* NHB1. Standard error of mean values (denoted by ±) are given in parentheses.113

Figure 3.7. Comparison of the toxicity of different pesticide categories (insecticides, fungicides, herbicides) to *Nitrosotalea sinensis*, *Nitrosospira briensis* and *Ca. Nitrobacter laanbroekii* NHB1 based on the mean EC₅₀ values calculated for each pesticide category.114

Chapter 4

Figure 4.1. Impact of different concentration of dimethyl sulfoxide (DMSO) on the growth of the MARA strains.....130

Figure 4.2. Comparison of the sensitivity of MARA strains, ammonia oxidizing archaea (AOA) and bacteria (AOB) and nitrite oxidizing bacteria (NOB), using calculated MTC

and EC₅₀ values, respectively for (a) insecticides, (b) herbicides, and (c) fungicides.....139

Chapter 5

Figure 5.1. Stoichiometric relationship of NO₂⁻ consumption and NO₃⁻ production during growth of '*Ca. Nitrobacter laanbroekii*' NHB1.153

Figure 5.2. An example of assessing μ_{max} of '*Ca. Nitrobacter laanbroekii*' NHB1 when grown at pH 4.5 with an initial 100 μ M NO₂⁻ concentration.153

Figure 5.3. Examples of plots used for calculating maximum specific growth rates of *Nitrosotalea devaniterrae* Nd1 and *Nitrosotalea sinensis* Nd2 (assessed via NO₂⁻ production) or in co-culture with '*Ca. Nitrobacter laanbroekii*' NHB1 (assessed via NO₃⁻ production) in medium supplied with 500 μ M NH₄⁺.154

Figure 5.4. Characterisation of the isolated strain '*Ca. Nitrobacter laanbroekii*' NHB1.158

Figure 5.5. Apparent half-saturation ($K_{m(app)}$) and maximum oxidation rates (V_{max}) for NO₂⁻ calculated using oxygen microrespirometry (MR) after fitting data of oxidation rate vs NO₂⁻ concentration to the Michaelis–Menten model with 95% confidence intervals (C.I.) plotted.159

Figure 5.6. Influence of '*Ca. Nitrobacter laanbroekii*' NHB1 on growth characteristics of acidophilic AOA *Nitrosotalea devaniterrae* Nd1 and *Nitrosotalea sinensis* Nd2 in batch culture.161

Figure 5.7. Inhibition of acidophilic AOA in 'freshwater medium' batch cultures (pH 6.0) amended with 0.5 mM cyanate.163

Figure 5.8. Production of NH₄⁺ from abiotic decomposition of cyanate in pH 5.2 'freshwater medium' amended with 0.05 mM or 0.5 mM after two days incubation at 25°C.....164

Figure 5.9. Inhibition of acidophilic AOA in 'freshwater medium' batch cultures (pH 6.0) amended with 0.5 mM cyanate.164

List of Tables

Chapter 1

Table 1.1. Classification of pesticides according to their target22

Table 1.2. The main organic groups of pesticides.....22

Chapter 2

Table 2.1. Chemical classification, mode of action and potential inhibition characteristics of pesticides and selected transformation products used in this study.....65

Chapter 3

Table 3.1. Chemical classification, mode of action and potential inhibition characteristics of pesticides and selected transformation products used in this study.....93

Table 3.2. Concentration levels used in the *in vitro* bioassays for each pesticide tested. The concentration of pesticides expected to be found in the soil solution upon application of the recommended agronomic dose are indicated in bold.....96

Chapter 4

Table 4.1. Pesticides and concentrations assessed with the MARA kit.114

Table 4.2. Microorganisms that are used in the MARA assay.....128

Table 4.3. Mean MTC values (μM) of the tested pesticides for each strain of the MARA kit. Standard errors of the mean values are given in brackets. The double asterisk denote the maximum tested concentration.....131

Table 4.4. Mean EC_{50} values (μM) of the tested insecticides for each strain of the MARA kit. Standard errors of the mean values are given in brackets. The double asterisk denote the maximum tested concentration.....133

Table 4.5. Mean EC_{50} values (μM) of the tested herbicides for each strain of the MARA kit. Standard errors of the mean values are given in brackets. The double asterisk denote the maximum tested concentration.....134

Table 4.6. Mean EC_{50} values (μM) of the tested fungicides for each strain of the MARA kit. Standard errors of the mean values are given in brackets. The double asterisk denote the maximum tested concentration.....136

List of Supplementary Tables and Figures

| | |
|---|-----|
| Table S1. The concentration levels used in the in vitro bioassays per pesticide (and transformation product) tested. | 240 |
| Table S2. Recovery levels obtained for the extraction of each of the pesticides tested in the different growth media used (1583 medium; FW: MES buffered freshwater medium). | 241 |
| Table S3. The chromatographic conditions used for the analysis of the residues of the tested pesticides..... | 243 |
| Supplementary Figure S1. The effect of different concentrations of pesticides on nitrite production by the AOA <i>N. sinensis</i> , <i>Ca. N. franklandianus</i> and <i>N. viennensis</i> | 244 |
| Supplementary Figure S2. The effect of different concentrations of pesticides on nitrite production by the AOB <i>N. europaea</i> , <i>N. communis</i> , <i>N. multiformis</i> , <i>N. briensis</i> , <i>N. ureae</i> and <i>N. oligotropha</i> | 247 |
| Supplementary Figure S3. The effect of different concentrations of pesticides on nitrite consumption by the NOB <i>Ca. Nitrobacter laanbroekii</i> NHB1, <i>N. winogradskyi</i> and <i>N. defluvii</i> | 252 |
| Supplementary Figure S4. The degradation of insecticide TCP applied over a range of concentrations in the liquid cultures of the AOA <i>N. sinensis</i> , <i>N. viennensis</i> and <i>Ca. N. franklandianus</i> , the AOB <i>N. europaea</i> , <i>N. multiformis</i> and <i>N. communis</i> , and the NOB <i>Ca. Nitrobacter laanbroekii</i> NHB1 and <i>N. winogradskyi</i> and in abiotic non-inoculated controls (at one selected concentration), determined at least at two time points. | 255 |
| Supplementary Figure S5. The degradation of herbicides (a) metsulfuron-methyl and (b) clethodim applied over a range of concentrations in the liquid cultures of the AOA <i>N. sinensis</i> , <i>N. viennensis</i> and <i>Ca. N. franklandianus</i> , the AOB <i>N. europaea</i> , <i>N. multiformis</i> and <i>N. communis</i> , and the NOB <i>Ca. Nitrobacter laanbroekii</i> NHB1 and <i>N. winogradskyi</i> and in abiotic non-inoculated controls (at one selected concentration), determined at least at two time points. | 256 |
| Supplementary Figure S6. The degradation of fungicides (a) pyraclostrobin, (b) etridiazole, (c) hymexazol and (d) 3,5- DCA applied over a range of concentrations in the liquid cultures of the AOA <i>N. sinensis</i> , <i>N. viennensis</i> and <i>Ca. N. franklandianus</i> , the AOB <i>N. europaea</i> , <i>N. multiformis</i> and <i>N. communis</i> , and the NOB <i>Ca. Nitrobacter laanbroekii</i> NHB1 and <i>N. winogradskyi</i> and in abiotic non-inoculated controls (at one selected concentration), determined at least at two time points. | 258 |

List of abbreviations

FAO: Food and Agriculture Organization of the United Nations

WHO: World Health Organization

PPP: Plant protection product

EU: European Union

OECD: Organisation for Economic Co-operation and Development

EFSA: European Food Safety Authority

ISO: International Standard Organization

AOM: ammonia-oxidizing microorganisms

AMF: arbuscular mycorrhizal fungi

TP: transformation product

NH₃ : ammonia

NO₂⁻ : nitrite

NO₃⁻: nitrate

NH₂OH : hydroxylamine

AMO: ammonia monooxygenase

NO: nitric oxide

AOB: ammonia-oxidizing bacteria

AOA: ammonia-oxidizing archaea

NOB: nitrite-oxidizing bacteria

Comammox: complete ammonia oxidizers

HAO: hydroxylamine hydrogenase

Cu: copper

NXR: nitrite oxidoreductase

e⁻: electron

HNO₂: nitrous acid

NirK: nitrite oxidoreductase

EC₅₀: effective concentration 50%

TPC: 3,5,6-trichloro-2-pyridinol

3,5-DCA: 3,5-dichloroaniline

AMPA: aminomethylphosphonic acid

MARA: Microbial Assay for Risk Assessment

Chapter 1

General Introduction

1. Plant protection products

1.1 Definition, history and use over the years

Pesticides constitute an integral part of modern agriculture and one of the most important factors leading to increased crop yields. According to the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) (2014) a pesticide is “*any substance, or mixture of substances of chemical or biological ingredients intended for repelling, destroying, or controlling any pest, or regulating plant growth*”. Pesticides encompass two main categories: (i) plant protection products (PPPs), controlled by Regulation (EC) No 1107/2009 (“Regulation (EC) No 1107/2009 of the European Parliament and of the Council as regards uniform principles for evaluation and authorisation of plant protection products,” 2011), which aims to enhance agricultural yield and quality and (ii) biocides, governed by Directive 98/8/EC, utilized for managing harmful organisms in non-agricultural contexts. Hereafter, ‘pesticide’ in this thesis refers to PPPs, and these terms will be utilized interchangeably.

The history of pesticide use is dated back to the beginning of agriculture itself. Their first use was recorded about 4500 years ago, when Sumerians used sulfur compounds as insecticides in order to control insects and mites (Unsworth, 2010). Also, Greeks and Romans used oil and ash sulfur to control various pests while mercury and arsenic compounds were applied in China for body lice approximately 3200 years ago (Hansen, 2013). In ancient times, before the advent of the chemical industry, people relied solely on natural resources for their products. A typical example is Pyrethrum, an insecticide derived from the dried flowers of chrysanthemum (*Cineraria folium*). This natural insecticide has been utilized for over two millennia, highlighting its longstanding effectiveness in pest control. This is the first of three phases in the history of pesticide use. The second period was between 1870 and 1945 and characterized by the use of inorganic synthetic compounds. In Sweden, copper and sulfur compounds were first applied for controlling fungal diseases of fruits and potatoes crops at the beginning of the 19th Century (Bernardes et al., 2015) and are still used in agricultural practice (Sheail, 1991). Following World War II (1945), the third period of pesticides use began (Unsworth, 2010) with a pivotal shift in pest management occurring with the emergence of synthetic pesticides like dichlorodiphenyltrichloroethane (DDT), captan, parathion, and β -hexachlorocyclohexane (BHC). These compounds facilitated widespread pest control, increased food production, and reduced insect-borne diseases. However, many drawbacks were associated with their use, including non-selectivity, high application rates, and toxicity. In the subsequent decades, significant pesticide development occurred. In the 1970s and 1980s, the application of more targeted pesticides like glyphosate and various organophosphate insecticides and fungicides, such as triazoles,

imidazoles, pyrimidines, and dicarboxamides was established. In the 1990s, a shift towards more selective compounds with improved environmental and toxicological profiles occurred, necessitating lower application volumes. This trend continues today, with ongoing research focusing on developing pesticides with enhanced selectivity, improved resistance management, and enhanced safety for both users and the environment to meet global demand (Unsworth, 2010).

Pesticides play a significant role in modern agriculture, controlling various threats such as weeds and insects, and bacteria and fungi that cause crop disease. However, their widespread use leads to potential harm to human health and the environment. Pesticides can contaminate soil, water sources, and non-target organisms, resulting in a decline of biodiversity and posing risks to ecosystems (Bernardes et al., 2015). Monitoring the use and trade of pesticides is essential for evaluating the sustainability of agricultural practices. The FAOSTAT Pesticides Use database provides valuable information on pesticide usage, including data on active ingredients, major pesticides categories, and relevant indicators such as usage per hectare of cropland, per capita, and per agricultural production value. This data aids in assessing the impact of pesticides on agriculture and the environment, guiding efforts towards more sustainable farming practices (*Pesticides use and trade 1990–2021*, 2023).

In 2021, global pesticide usage reached 3.4 million tons, marking a 4% increase from 2020 and an 11% surge over the past decade. Notably, at the continental scale, America (both South and North America) has maintained its position as the foremost user of pesticides since the mid-1990s, consuming approximately 1.12 million tons and representing 43% of global usage of PPPs annually, followed by Asia (33%), and Europe (18%) (Figure 1.1 a). The latter witnessed a moderate rise in pesticide application from 468 kt in 2020 to 505 kt in 2021, representing a 4% increase (Figure 1.1 b). Africa and Oceania exhibit the lowest rates of pesticide usage at 4% and 2% respectively. Africa experienced a small increase from 203 kt in 2020 to 204 kt in 2021, while Oceania's pesticide usage remained stable at 72 kt (Figure 1.1) (*Pesticides use and trade 1990–2021*, 2023).

Among European countries, Spain and France emerged as the largest consumers of pesticides, with 76 and 70 kt respectively in 2021, placing them among the top-ranking countries globally in terms of pesticide usage (*Pesticides use and trade 1990–2021*, 2023). Fungicides and bacteriocides constitute the majority, comprising 51% of pesticide usage in Europe, followed by herbicides at 36%, while insecticides make up the remaining 13% (Figure 1.2 a). This distribution likely reflects Europe's stringent regulatory measures governing pesticide usage. Interestingly, the quantities of herbicides and insecticides have remained relatively stable over the past decade, remaining around 11 kt and 5 kt respectively, while fungicides and bacteriocides amount to approximately 15 kt (Figure 1.2 b) (*Pesticides use and trade 1990–2021*, 2023).

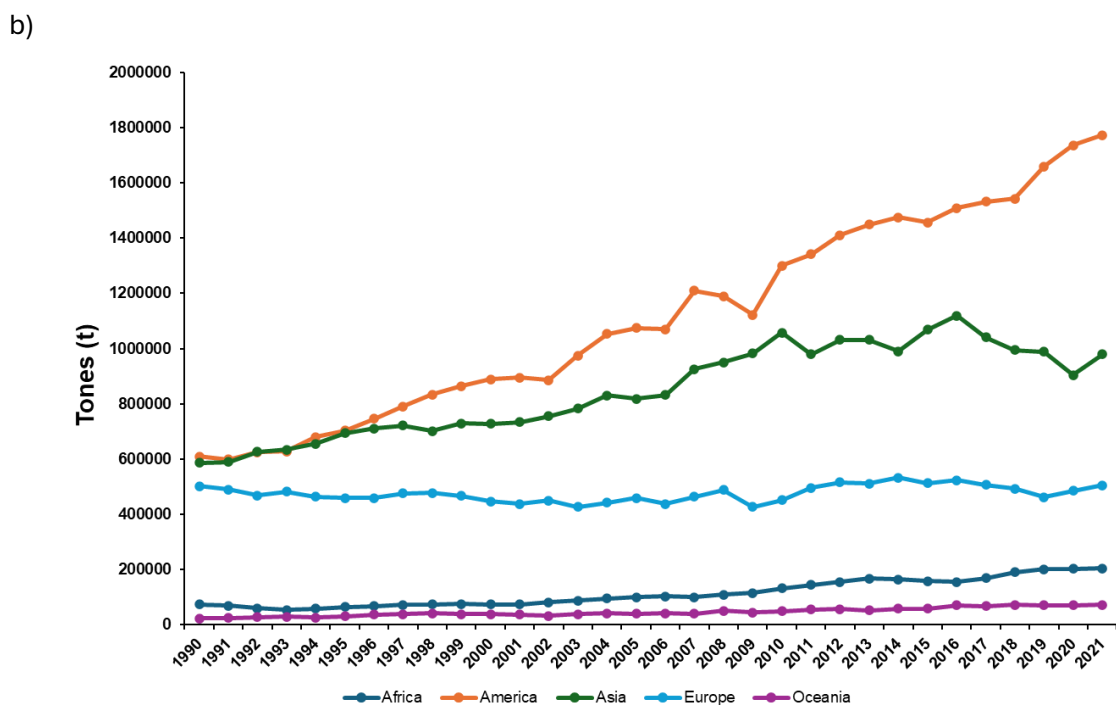
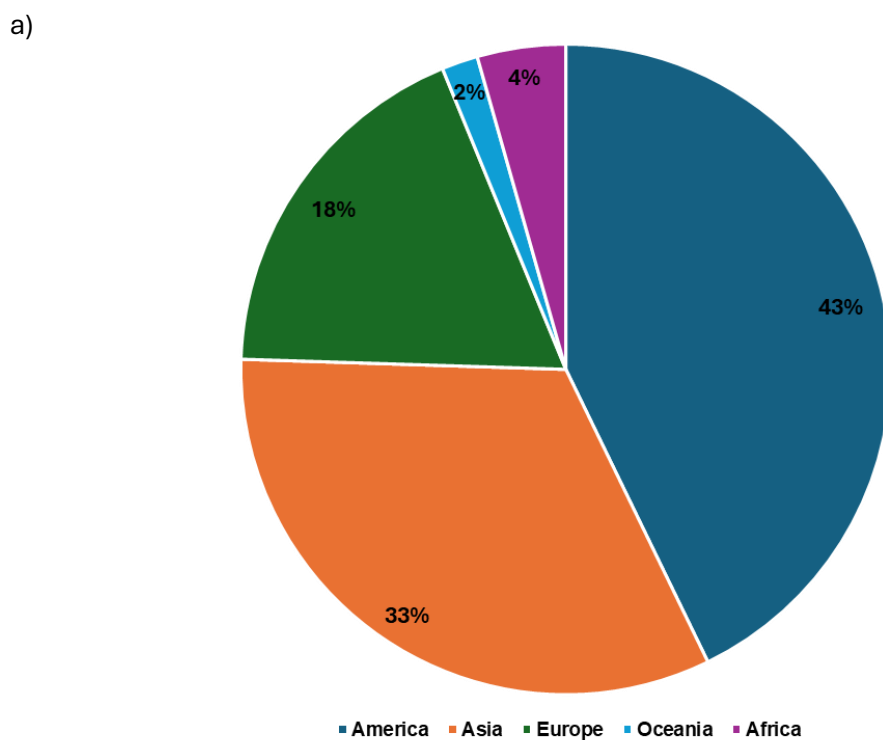
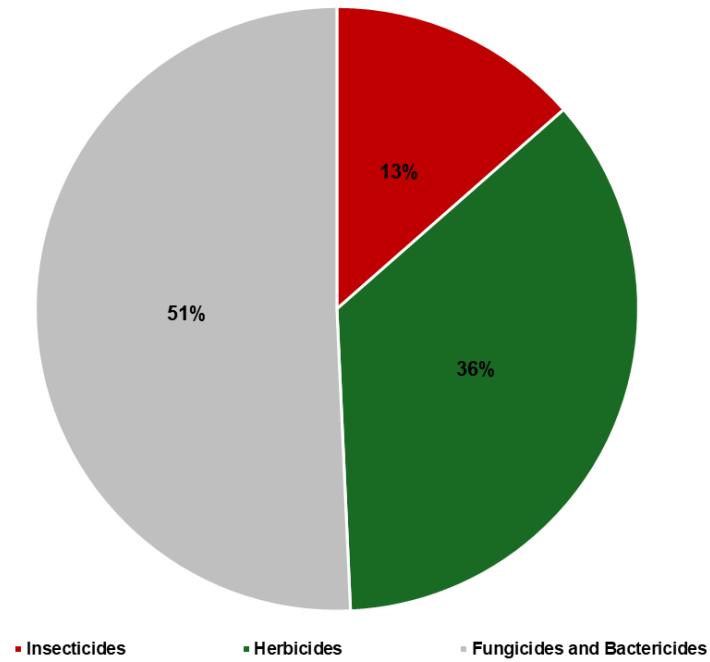


Figure 2.1. (a) Regional proportion of pesticides in agricultural practice globally and (b) total pesticides quantities (tonnes) used in agriculture the past 29 years. Data source: Food and Agriculture Organization of the United Nations (FAO).

a)



b)

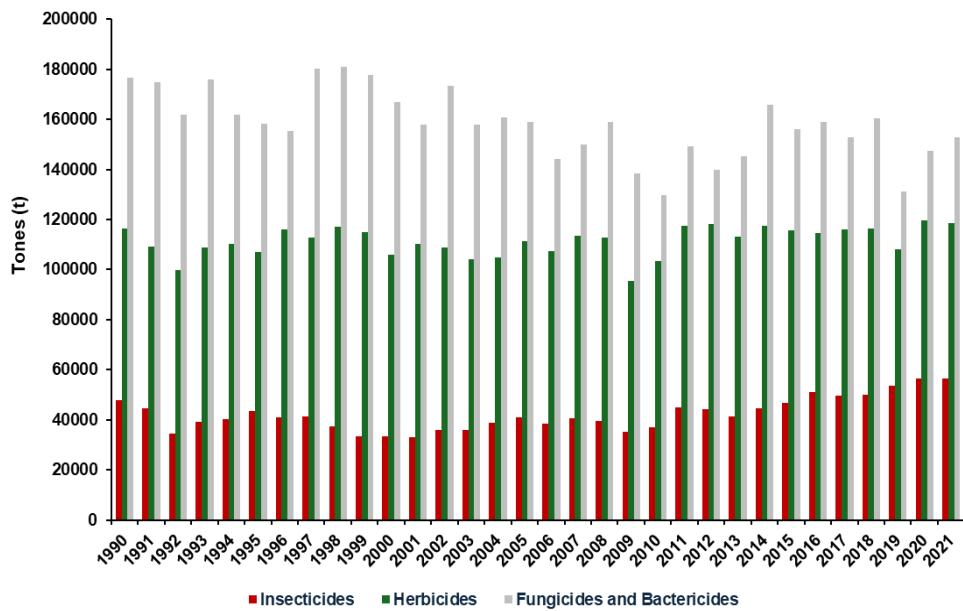


Figure 1.2. (a) Proportion of the main categories of pesticides in agricultural practice in Europe and (b) total pesticides quantities (tonnes) per category used in agriculture the past 29 years in Europe. Data source: Food and Agriculture Organization of the United Nations (FAO).

1.2 Classification

Pesticides can be classified in different ways. The most commonly used approaches are classification based on the mode of action, targeted organisms or chemical structure. In classification by mode of action/entry, pesticides are grouped based on how they come in contact or enter the target organism (Zacharia & Tano, 2011) and are classified as non-systemic (external contact) and systemic (penetration) pesticides. Non-systemic pesticides do not appreciably penetrate tissues and consequently are not transported within tissues and are effective at the point of contact with the target pest. On the other hand, systemic pesticides penetrate tissues and move towards a target area to bring about the desired effect. Classification by pest categorizes pesticides at the high taxonomic ranks of the target (Table 1.1) (Tudi et al., 2021). In chemical classification, pesticides are grouped according to the chemical nature of the active ingredient, establishing a correlation between structure, activity, toxicity, and degradation mechanisms. Classification based on chemical structures involves both organic and inorganic pesticides. Inorganic pesticides includes copper sulfate, ferrous sulfate, copper, lime and sulfur (Hassaan & El Nemr, 2020). The most important groups of organic pesticides are listed in Table 1.2 (Kaur et al., 2019).

Table 1.1 Classification of pesticides according to their target.

| Pesticides name | Target pest |
|------------------------|--------------------|
| Algicides | Algae |
| Bactericides | Bacteria |
| Fungicide | Fungi |
| Insecticide | Insects |
| Herbicide | Plants |
| Nematicides | Nematodes |
| Virucides | Viruses |

Table 1.2. The main organic groups of pesticides.

| Group | Target pest |
|---------------------------|---------------------------|
| Organophosphates | Insects, Nematodes |
| Carbamates | Insects, Nematodes, Fungi |
| Pyrethroids | Insects |
| Organochlorines | Insects |
| Neonicotinoids | Insects |
| Strobilurins | Fungi |
| Benzimidazoles | Fungi |
| Dicarboxamides | Fungi |
| Thiocarbamates | Fungi, Plants |
| Anilinopyrimidines | Fungi |

Table 1.2. Continued

| Group | Target pest |
|--------------------------------|--------------------|
| Triazoles, Imidazoles | Fungi |
| Phenoxy-alkanoics | Plants |
| Triazines | Plants |
| Substituted Phenylureas | Plants |
| Sulfonylureas | Plants |
| Triketones | Plants |
| Diphenylethers | Plants |
| Chloroacetamides | Plants |

1.3 Benefits and risks of pesticides

The utilization of pesticides is widely recognized as an integral part of modern agricultural practices to reduce agricultural losses from pests and increase crop yields. This is achieved through effectively reducing weed populations, thereby mitigating their competition with crops for vital nutrients, combatting diseases primarily caused by plant pathogenic fungi and bacteria, and curbing insect pests, which in tandem with pathogens, pose substantial threats to agricultural productivity and can lead to significant economic losses due to diminished harvests or post-harvest losses (Abubakar et al., 2020). Boosting agricultural yields is important to accommodate the increasing needs of our ever-expanding global population and the utilization of PPPs has increased yields and accessibility of nutritious foods such as fruits and vegetables. Consequently, PPPs significantly mitigate hunger and ensure a plentiful supply of high-quality sustenance (Tudi et al., 2021), improved nutrition and offer a higher standard of living.

Pesticides are also used for controlling human and animal disease vectors and nuisance organisms; controlling pests that carry diseases affecting humans and animals is crucial for preventing the spread of serious illnesses like malaria, sleeping sickness, and river blindness. By managing these disease vectors, not only do we alleviate suffering and reduce deaths, but we also reduce the risk of diseases spreading across borders. Additionally, in livestock, effective pest control leads to increased revenue from healthier animals and decreased expenses on veterinary care and medication (Cooper & Dobson, 2007).

Despite these positive outcomes, the use of pesticides can also have a deleterious impact on the environment as well as on the health of the population. The use of these chemical compounds in agricultural practices leads to the contamination of various environmental niches and agricultural products, in particular fresh fruit and vegetables (Farahy et al., 2021). Soil, which sustains agricultural productivity, receives the highest levels of residues (Abubakar et al., 2020). Their negative effects stem from the chemical structure and essential physicochemical properties of the soil, which allow them to spread uncontrolled in terrestrial and aquatic ecosystems (Carvalho,

2017). The result is deterioration of soil quality and subsequent accumulation of chemicals in groundwater and drinking water supplies. There is therefore an urgent need for sustainable agricultural practices that can mitigate these negative impacts on ecosystems and on human well-being.

Numerous studies have highlighted the impact of PPPs on non-target organisms, spanning a diverse array of species. Non-target organisms are defined as species that are separate from the group of target organisms affected by pesticide application and include plants, algae, birds, fish, beneficial insects, aquatic and soil microorganisms (Iyaniwura, 1991). Of particular concern is the effect of PPPs on soil microorganisms, whose activity is pivotal for maintaining soil health and functionality. Their diminished function due to pesticide exposure can impede organic matter breakdown and compromise the intricate balance of biogeochemical cycles, further exacerbating soil degradation .

Furthermore, pesticide accumulation within ecosystems amplifies ecological concerns. This phenomenon, known as bioaccumulation, entails the progressive buildup of pesticide residues in organisms across various trophic levels. Such accumulation occurs through multiple pathways, including direct uptake from water sources and, more prominently, through the consumption of contaminated plant tissue, sediment, and other members of the food chain (Alexander & Fairbridge, 1999).

1.4 Environmental fate of pesticides

Pesticides are subjected to many processes in the environment after application. Through these processes, pesticides enter into the environment either with a positive (on-target) or negative (non-target) influence. The main processes that determine the environmental fate of pesticides are absorption, degradation (biotic, abiotic), transportation to surface water (runoff, erosion, spray drift) or groundwater (leaching) and air (Figure 1.3) (Tudi et al., 2021).

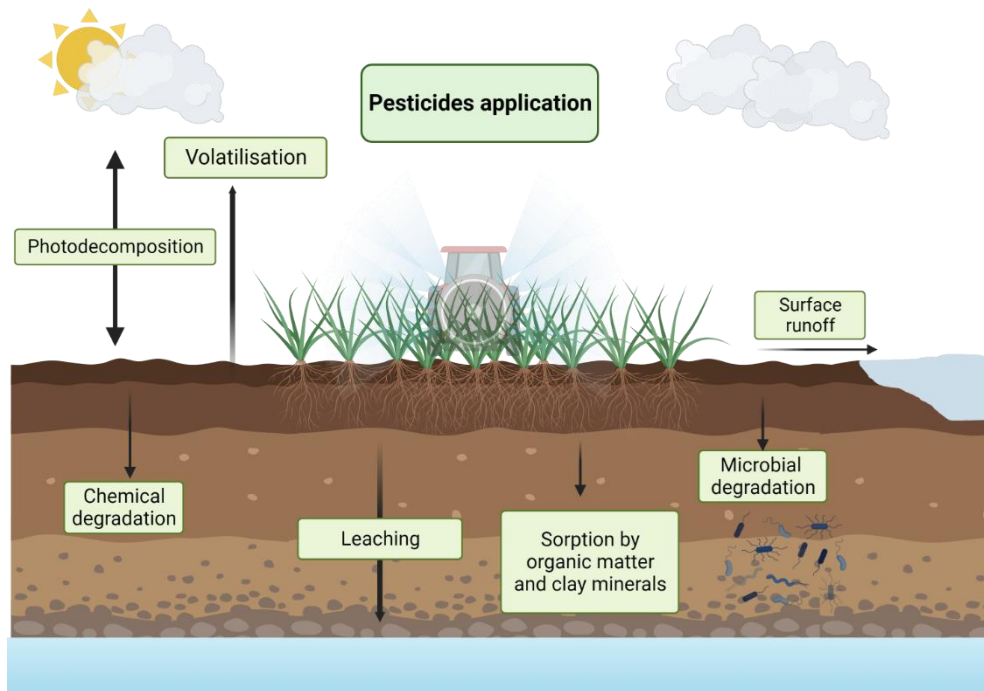


Figure 1.3 Behavior of pesticides in the environment.

1.4.1 Absorption

Absorption is defined as the binding of chemicals to soil particles (Peña et al., 2020). Soil properties such as pH, moisture, salinity, and organic matter significantly influence pesticide absorption, with organic matter being the most crucial factor. Organic matter serves as the primary absorption surface for non-polar compounds and some polar ones (De Wilde et al., 2009; Wauchope et al., 2002). Additionally, the chemical properties of pesticides, particularly hydrophobicity and water solubility, play a critical role in their absorption (Delgado-Moreno et al., 2017).

1.4.2 Degradation

Degradation controls the persistence of pesticides and the yield of different metabolites in soil. Furthermore, degradation data can be used to determine the half-life of pesticides i.e. the time for 50% degradation of the applied pesticide in the environment (Marie et al., 2017). After application pesticides can be degraded abiotically (chemical degradation, photo-degradation) or biotically (microbial degradation) (Abian et al., 1993). Environmental factors and chemical characteristics affect the degradation of pesticides, resulting in the production of new compounds, which can be more toxic than the parent compound (Zhao et al., 2017).

Photo-degradation take place on the surface of soil and water systems, in the air, or on the foliage of treated plants, as it heavily depends on light intensity

(Gavrilescu, 2005). Chemical degradation encompasses a range of reactions, including ionization, hydrolysis, and oxidation-reduction, and typically depends on the pH of the environmental system (Tudi et al., 2021).

Biotic degradation is performed by soil microorganisms which are able to use the compounds as a nutrient source (Han et al., 2013). The ability of microorganisms to degrade agrochemicals is due to their adaptability within environments exposed to pesticides. This adaptability is facilitated by their genetic plasticity and rapid generation time, allowing them to develop pesticides catabolic genes and pathways (Russell et al., 2021; Upadhyay et al., 2019; Zhu et al., 2020). Additionally, microorganisms can acquire these genes through horizontal gene transfer of mobile genetic elements such as plasmids and transposons (French et al., 2020; Rios Miguel et al., 2020). Microbial degradation is influenced by oxygen, temperature, moisture and pH (Singh, 2012; Su et al., 2017; Yue et al., 2017).

1.4.3 Pesticides transportation in soil

Pesticides are transported in the environment through volatilization, leaching, runoff and erosion. The process by which a solid or liquid pesticide is converted to gas with subsequent evaporation to the atmosphere from the soil environment, is called volatilization (Navarro et al., 2013). It is influenced by a range of factors such as vapor pressure, temperature, humidity, air movement, and soil properties such as texture, organic matter, and moisture (Singh, 2012; Tudi et al., 2021). The vertical movement of pesticides through soil results in leaching into groundwater and causes water pollution (Fontana et al., 2010; Singh, 2012). Leaching is influenced by soil properties, pesticides' chemical properties, site conditions and management practices (Gavrilescu, 2005; Peña et al., 2020). The high solubility of pesticides and soil permeability increase the possibility of pesticides leaching from soil (Tudi et al., 2021). Runoff is the movement of pesticides in water over a sloping surface (Das et al., 2020). Pesticides may move with runoff as compounds dissolved in water or attached to soil particles. Runoff can also occur when water is added to a field at a faster rate than it can be absorbed by the surface soil (Singh et al., 2012). Runoff is promoted by over-irrigation, high soil moisture content, slope of the point of introduction and amount and timing of rainfall (Tudi et al., 2021).

1.5 Impact of pesticides on soil microorganisms

Soil is the main sink for pesticides applied in agricultural environments. The degradation of agrochemicals may lead to the production of metabolic compounds which are more persistent and toxic compared to the parent compound (Marie et al., 2017; Tudi et al., 2021). The contamination of soil by pesticides has caused adverse impacts on the quality of food and agricultural sustainability, with a widespread threat to the water and food chain (Tudi et al., 2021).

Soil microorganisms play a critical role in soil health and participate in major biochemical processes such as nitrogen and carbon cycles (Li et al., 2021). Agricultural practices including the application of pesticides influence soil microorganisms (Bedano et al., 2021; Sim et al., 2023). In soil, pesticides interact with soil microorganisms resulting in either pesticides toxicity or increased growth linked to the microbial degradation of pesticides and is influenced by a range of biotic and abiotic factors. The toxicity of pesticides on soil microorganisms is likely due to the high applied level of pesticides that cannot be effectively dissipated, while the growth-linked microbial degradation is driven by specific microorganisms that possess enzymes for the degradation of these compounds (Horemans et al., 2016).

Pesticides can have both short-term or long-term impacts on soil microorganisms, either directly or indirectly, and with positive or negative results, depending on the pesticide type and the specific microorganisms involved. The direct effects include production of specific compounds by soil microorganisms after contact with the pesticides. These compounds can lead to the death of the microorganisms or significantly change their metabolisms. The indirect effects of the pesticides arise from modifications on the microorganisms' metabolism by pesticides (Jeyaseelan et al., 2024).

The interaction between pesticides and soil processes is complex and multifaceted. Several studies have investigated the effects of pesticides on soil microbiota at the laboratory and field scale (Carpio et al., 2020). Substantial evidence indicates that long-term pesticide use can inhibit crucial soil functions and alter metal ion dynamics. Ammonification, a significant process in the nitrogen (N) cycle, can be significantly reduced by approximately 70% to 90%, upon pesticide application for 3 years or more, resulting in a negative impact on plant growth and soil fertility (Rajak et al., 2023). Over time, pesticides can accumulate, leading to long-term soil contamination, which affects soil health and crop productivity. One critical soil process impacted by pesticides is the reduction of ferric ions to ferrous ions. This redox process is essential for various soil biochemical functions and nutrient availability. Furthermore, pesticides possess binding sites that allow them to form complexes with metal ions present in agricultural environments, affecting their availability and mobility. For instance, pesticides such as thiophanate methyl can bind essential micronutrients like iron, copper, and zinc, reducing their availability to plants and potentially leading to deficiencies. The formation of complexes of pesticides with metal ions can also limit their accessibility to soil microorganisms, decreasing their contribution to functional processes and potentially harming land integrity and disrupting the ecosystem (Jeyaseelan et al., 2024; Rajak et al., 2023).

Several studies have also identified negative or positive effects of pesticides on soil microbial communities. Walder et al. (2022) reported an increase in fungal community size in the presence of the pesticides residues while a negative correlation was observed with the abundance of nitrogen-fixing bacteria in several studies,

illustrating a long-term risk to biological nitrogen fixation in agricultural soil (Angelini et al., 2013; Walder et al., 2022; Zhang et al., 2016). Commonly used herbicides such as glyphosate, dichlorprop and napropamide, can inhibit the activity and growth of soil microorganisms (Lu et al., 2018) but also change the soil microbial community and diversity (Cycoń & Piotrowska-Seget, 2015; Tang et al., 2023). Sim et al. demonstrated that azoxystrobin and flutriafol (fungicides), chlorsulfuron (herbicide), and fipronil (insecticide) impacted microorganisms that participate in the N cycle such as *Nitrososphaeraceae* (AOA), *Nitrosospira* (AOB), and *Nitrospira* (NOB), with the effects varying by soil type. Similarly, the application of the herbicide mesulfuron-methyl and the insecticide thiamethoxam was observed to affect soil microorganisms participating in the N cycle and other non-target organisms for 15 days post-application. However, the microbial populations tended to recover after 60 days. The impact of these pesticides on microbial communities is significantly influenced by pesticide dosage and soil type (Cao et al., 2023; Wu et al., 2021).

1.6 Environmental risk assessment of pesticides

Pesticides are significant environmental pollutants and can disperse across various environmental matrices (Carvalho, 2017). The European Union (EU) recognizes the pollution potential of pesticides and governs the use of PPPs via Directive 2009/128/EC, which establishes a framework for the sustainable use of pesticides by mitigating the risks and impacts on human health and the environment (“Directive 2009/128/EC of the European Parliament: establishing a framework for Community action to achieve the sustainable use of pesticides Text with EEA relevance,” 2009). Additionally, EU continuously reviews and re-evaluates active substances to ensure they meet approval criteria for placement on the market as per Regulation 1107/2009 (“Regulation (EC) No 1107/2009 of the European Parliament and of the Council as regards uniform principles for evaluation and authorisation of plant protection products,” 2011) and Regulation 2020/1740 (“Setting out the Provisions Necessary for the Implementation of the Renewal Procedure for Active Substances, as Provided for in Regulation (EC) No 1107/2009 of the European Parliament and of the Council, and Repealing Commission Implementing,” 2020)

The EU has developed a well structure risk assessment scheme which requires that the predicted environmental exposure concentration is lower than the concentration considered safe for non-target organisms (Boivin & Poulsen, 2017). The environmental risk assessment of pesticides is structured around a tier system, which consists of multiple levels that progressively incorporate more complex and realistic scenarios. The set of standardized ecotoxicity tests supporting the risk assessment is used to evaluate the toxicity of pesticides on aquatic organisms and soil fauna, unlike soil microorganisms, whose ecotoxicological response to pesticides has not been set as a priority in the current risk assessment framework. The first step (first tier) consists of standard toxicity tests using one chemical against one species without additional

stressor factors in the laboratory. In the case that the pesticides present higher predicted exposure than the safe concentration, an extension of the standard toxicity tests to more complex scenarios is required (Boivin & Poulsen, 2017; Rohr et al., 2016). All these toxicity tests are known as OECD (Organisation for Economic Co-operation and Development) tests. OECD are standardized guidelines used globally to evaluate the safety and environmental impact of chemicals, including pesticides and are designed to ensure consistency, reliability, and reproducibility of results across different laboratories and countries (“APPENDIX 3. OECD guidelines for the testing of chemicals,” 2016).

The higher tiers in pesticide risk assessment integrate processes and characteristics found in natural ecosystems, such as multi-species semi-field test systems used in the EU and reduce exposure through mitigation measures in risk management (“Regulation (EC) No 1107/2009 : implementing of the European Parliament and of the Council as regards uniform principles for evaluation and authorisation of plant protection products Text with EEA relevance,” 2011). This tiered framework operates on two key assumptions: (i) the first tier provides an overly protective estimation of potential field effects; (ii) the higher tiers offer more ecologically relevant predictions of field effects or concentrations that do not result in unacceptable impacts (Boivin & Poulsen, 2017).

The EFSA provides a comprehensive framework for the risk assessment of pesticides to ensure they do not pose unacceptable risks to the aquatic environment. It aims to evaluate the potential impacts of pesticides on aquatic ecosystems, including water bodies, sediment, and aquatic organisms (EFSA Panel on Plant Protection Products and their Residues (PPR), 2013). The first-tier includes single species tests using three taxonomic groups representing different trophic levels: i. fish (Zebrafish (*Danio rerio*), Fathead Minnow (*Pimephales promela*), and Rainbow Trout (*Oncorhynchus mykiss*); ii. *Daphnia magna*; and iii. a green alga (*Pseudokirchneriella subcapitata*). A series of OECD tests are included in this tier which assesses the acute and prolonged toxicity on fish and their embryos (OECD TG 203, OECD TG 204, OECD TG 236 and OECD TG 210) (OECD, 1984, 2013b, 2013a, 2019), acute immobilization and reproduction on *Daphnia magna* (OECD TG 202, OECD TG 211) (OECD, 2004a, 2012a) and growth inhibition of algae (OECD TG 201) (OECD, 2006). The output of these tests is the LC₅₀ (lethal concentration for 50% of test organisms) or EC₅₀ (effective concentration causing 50% inhibition of test organisms). Tier 1 uses worst-case scenarios and conservative assumptions to ensure that any potential risks are not overlooked, and this includes maximum application rates, conditions that maximize runoff and pesticides entry into surface waters and most sensitive species data available to ensure protection across different aquatic organisms. Based on the calculated endpoints pesticides can illustrate either low risk to aquatic organisms and no further assessment is needed or high risk, necessitating a more refined risk assessment at Tier 2.

In higher-tier assessment using standard tests with multiple species are conducted under water-sediment conditions simulating natural environmental exposure. Tier 2 investigates the initial risk assessment using more realistic and specific data for a better understanding of the toxicity of pesticides. The main aim of this tier is to reduce conservatism and uncertainty by incorporating more detailed information about exposure and effects. Various OECD tests and guidelines are commonly used to generate the necessary data. These tests provide standardized methodologies for assessing the environmental fate of pesticides focusing on adsorption and aerobic/anaerobic transformation in both soil and in aquatic sediments (OECD 106, OECD 307, OECD 308) (OECD, 2000a, 2002a, 2002b). Furthermore, OECD tests for the effect of pesticides on aquatic organisms are used (OECD 210, OECD 211, OECD 201 and OECD 218) (OECD, 2006, 2012a, 2013a, 2023). These tests include toxicity tests on fish at early-life stages, reproduction tests on *Daphnia magna*, growth inhibition test on fresh algae and cyanobacteria and toxicity test on sediments-water chironomid life cycle. Assays for bioaccumulation of pesticides on fish and aerobic mineralization in surface water and sediment system also are used in Tier 2 (OECD 305 and OECD 309) (OECD, 2004b, 2012b). This tier is also characterized using modeling tools like FOCUS models (e.g. FOCUS surface water scenarios (reference)) for more complex scenarios that simulate various agricultural and environmental conditions for prediction of pesticides concentrations in edge-of-field surface waters and calibrate using field data to ensure accuracy and reliability. Higher-tier models are also common for predictions of pesticide movement in surface water and aquatic environments. Likewise, in tier 1, if the endpoints indicate acceptable risk levels, a pesticide is considered safe for use under the assessed conditions. If not, further refinement or higher-tier assessments (Tier 3) may be necessary.

The main aim of Tier 3 is to refine and validate the risk assessment conducted in Tier 2 through more detailed and often site-specific studies. This tier involves more complex and resource-intensive approaches to provide a more accurate characterization of potential environmental risks. The central component in this tier involves field studies with aquatic mesocosms and soil microcosms in order to stimulate natural aquatic ecosystems and assess the effects of pesticides on complex aquatic communities over extended periods (EFSA Panel on Plant Protection Products and their Residues (PPR), 2013) . Another approach consists of Species Sensitivity Distributions (SSD) which are derived from Tier 2 data or additional higher-tier studies that can provide a distribution of sensitivity across various species, helping to refine risk estimates and assess potential impacts on biodiversity. Additionally, site specific modeling is used for environmental monitoring data from specific sites where pesticides are applied, providing real-world exposure concentrations to improve the accuracy of risk assessments. Further investigation into sub-lethal effects on aquatic organisms (including physiological, behavioral, and reproductive endpoints) to understand potential long-term impacts are performed in this tier. Based on the results

of the different tests, a comprehensive integration of all available data (environmental fate, exposure, toxicity) is performed to refine risk estimates and provide a robust characterization of the potential risks posed by pesticides. Tier 3 represents the highest level of complexity in the risk assessment process for PPPs, involving advanced methodologies, detailed studies, and comprehensive data integration. By addressing uncertainties and providing a different understanding of environmental risks, Tier 3 ensures that regulatory decisions are based on robust scientific evidence, aiming to protect aquatic ecosystems while allowing for the safe and sustainable use of pesticides in agriculture (EFSA Panel on Plant Protection Products and their Residues (PPR), 2013).

In contrast to aquatic ecotoxicology and the corresponding bioassays, a well-structured risk assessment scheme is not available for soil microorganisms. Considering the key functional roles of soil microbiota in ecosystem functioning, the European Food Safety Authority (EFSA) identified soil microorganisms as a specific protection goal for environmental risk assessment of pesticides (European Food Safety Authority, 2010). Soil microorganisms participate in nutrient cycling, modulate soil fertility and health, promote plant growth and preserve the balance between biotic and abiotic stressors. Assessment of the soil microbial toxicity of pesticides relies on effect in soil microbial enzymatic activities in terms of N and C transformation. However, these toxicity assessments rely on out-of-date microbial tests of N and C transformation (OECD 216 and 217, respectively) (OECD, 2000b, 2000c). The evolution of soil microbiology has established a range of International Standard Organization (ISO) standards based on soil DNA extraction, phospholipid fatty acid (PLFA) analysis, soil enzymatic activity measurements and determination of microbial abundances via q-PCR. In 2016, the EFSA identified arbuscular mycorrhizal fungi as potential indicators of pesticide soil microbial toxicity based on advanced molecular tools (EFSA Panel on Plant Protection Products and their Residues (PPR) et al., 2017). Recent studies have also pointed to ammonia-oxidizing microbes (AOM) as ideal microbial indicators of agrochemicals' toxicity.

1.7 New proposed risk assessment scheme of pesticides on soil microorganisms

The environmental risk assessment of pesticides uses a tier system that progresses through levels of increasing complexity and realism as described above. However, this tiered approach has not yet been fully applied to soil microorganisms. Initial research provided first experimental guidelines for assessing the toxicity of pesticides on soil microorganisms, suggesting that significant inhibitory effects observed in laboratory settings should prompt further field-scale investigations. In line with this approach, Karpouzas et al. proposed a similar tiered system specifically for assessing the impact of pesticides on soil microbial communities. This system includes

three levels of increasing complexity, focusing on the ecotoxicological responses of key soil functional groups, such as ammonia-oxidizing microorganisms (AOM) and arbuscular mycorrhizal fungi (AMF) (Figure 1.4). Tier I involves *in vitro* screening of pesticides against a diverse range of the most sensitive soil-derived AOM and AMF strains, representing various ecophysiological and phylogenetic groups. If this preliminary assessment suggests potential toxicity effects, the evaluation proceeds to tiers II and III. The second and third tier assess the toxicity of pesticides in laboratory soil microcosms or planted pot studies (for AMF) and field scale assessment under agricultural conditions respectively, using AOM and AMF as bioindicators. If unacceptable levels of toxicity from pesticides on soil microorganisms are observed in laboratory microcosm or pot studies, further evaluation at the field level is conducted. In both tiers advanced biochemical and molecular tools can be used to monitor the activity, abundance and diversity of the selected bioindicators. At each of these experimental scales, the assessment focuses exclusively on natural microbial communities. Regardless of whether the study is conducted in a microcosm, pot, or field setting, it is essential to monitor the dissipation and transformation of the pesticide over time. This allows for the identification of the specific toxicant, distinguishing between the parent compound and its transformation products (TPs).

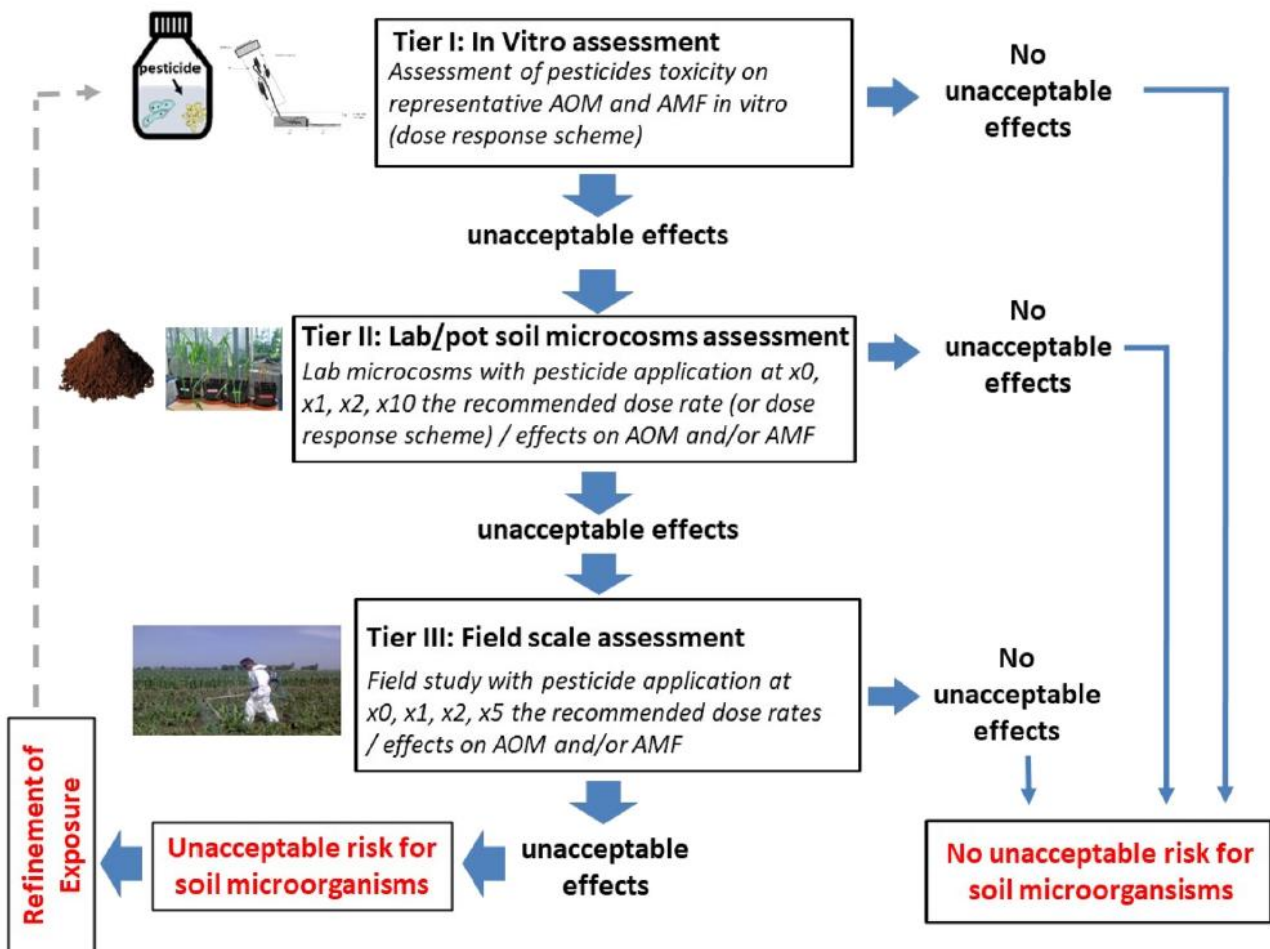


Figure 1.4 Proposed three-tier risk assessment scheme for assessing the toxicity of pesticides on soil microorganisms (Karpouzas et al., 2022). Ammonia-oxidizing microorganisms, AOM; arbuscular mycorrhizal fungi, AMF

1.8 Nitrification and nitrifying microorganisms

1.8.1 Nitrification process

Nitrification plays a vital role in the global nitrogen cycle, converting ammonia (NH_3) into nitrate (NO_3^-) via nitrite (NO_2^-). This process increases nitrogen availability for plants and enhances soil fertility (Schleper & Nicol, 2010). Chemolithoautotrophic nitrification involves microorganisms that generate energy by oxidizing inorganic compounds, specifically ammonia or nitrite, using this energy for their metabolic processes. In soil environments, competition for energy is intense, which often limits microbial activity (Martikainen, 2022). In fertilized agricultural soils, the high availability of ammonia supports high rates of chemolithoautotrophic nitrification activity. However, in acidic, unfertilized soils, this process becomes more challenging, although ammonium and nitrite oxidation can still occur (De Boer & Kowalchuk, 2001; Faeflen et al., 2016, n.d.). The microorganisms and genes involved in nitrification have

been extensively studied, highlighting the economic and environmental significance of this process (You et al., 2009). Nitrification is a modular process which is performed by a range of specialized microbial groups described below (Figure 1.5). Nitrification was considered a two-step process with the oxidation of NH_3 and NO_2^- considered to be performed by two independent functional groups. The first step, which is generally considered the rate-limiting step of nitrification, involves the oxidation of NH_3 to hydroxylamine (NH_2OH) using ammonia monooxygenase (AMO) and then the conversion of the latter to nitric oxide (NO) by hydroxylamine dehydrogenase (HAO), and finally to nitrite (NO_2^-) (Beeckman et al., 2018; Caranto & Lancaster, 2017; James I. Prosser et al., 2020; Stein, 2019). The microorganisms that carry out this step are ammonia-oxidizing bacteria (AOB) (Kowalchuk & Stephen, 2001), and more recently discovered ammonia-oxidizing archaea (AOA) (Könneke et al., 2005). Ammonia monooxygenase (AMO) is present in both microbial groups. This enzyme is a transmembrane protein. The activation of this enzyme require two electrons and copper bound in the AmoB and AmoC subunit (Beeckman et al., 2018; James I. Prosser et al., 2020). AmoA, AmoB and AmoC subunits are conserved in both AOB and AOA (Wright & Lehtovirta-Morley, 2023). The archaeal AMO appears to have three additional subunits, AmoX, AmoY and AmoZ (Hodgskiss et al., 2023) . The second reaction involves the oxidation of NH_2OH to NO, by hydroxylamine hydrogenase (HAO). This enzyme is a multiheme protein and has only been identified in AOB (Arp & Stein, 2003; Caranto & Lancaster, 2017). Several mechanisms have been proposed for the conversion of NO to NO_2^- including (a) abiotic transformation, (b) a nitrite reductase which is encoded by *nirK* as this enzyme can also catalyze the reverse reaction and (c) an uncharacterized nitric oxide reductase with the potential candidate a red Cu protein, nitrosocyanin (Caranto & Lancaster, 2017; James I. Prosser et al., 2020). The second step of nitrification involves the conversion of NO_2^- to NO_3^- by nitrite oxidizing bacteria (NOB) which use nitrite oxidoreductase (NXR) (Beeckman et al., 2018; Coskun et al., 2017). This enzyme is a transmembrane protein that carries three subunits NxrA, NxrB and NxrC. Depending on the location of the substrate-binding subunit NxrA, there are two types of NXR enzyme; periplasmic and cytoplasmic (Daims et al., 2016). Recent studies challenged the theory of obligatory division of metabolic labor in nitrification and reported the discovery of bacteria from the genus *Nitrospira sp.* with the metabolic capacity to perform the complete oxidation of ammonia to nitrate (“complete ammonia oxidizers”, comammox) (Daims et al., 2015; van Kessel et al., 2015).

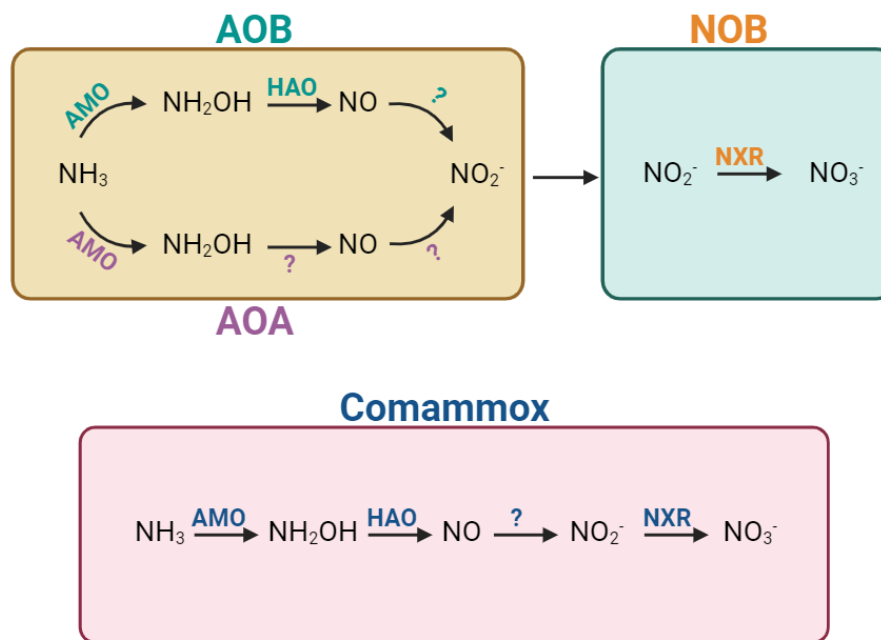


Figure 1.5 Chemolithoautotrophic nitrification, reactions and microbial groups performing each step of the process.

1.8.2 Ammonia-oxidizing bacteria

Chemolithoautotrophic AOB were first isolated in 1890 (Winogradsky, 1890) with cultivated representatives belonging to beta- and gammaproteobacterial lineages. Betaproteobacteria AOB belong to two genera within the same family, *Nitrosomonas* and *Nitrospira* of the *Nitrosomonadaceae*, and gammaproteobacteria AOB belong to the genus *Nitrosococcus*, *Nitrosoglobus* and *Nitrosacidococcus* (Dang et al., 2018; Di et al., 2010; Kowalchuk & Stephen, 2001; Purkhold et al., 2000). In soil, the dominant AOB are *Nitrospira* (Aigle et al., 2019; Purkhold et al., 2000, 2003) while *Nitrosoglobus* and *Nitrosacidococcus* are commonly found in acidic soils (Hayatsu et al., 2017; Picone et al., 2021). *Nitrosomonas* have been found in soil but dominate mainly in engineered ecosystems (Mobarry et al., 1996; A. Schramm et al., 1996) while *Nitrosococcus* are mainly found in marine environments (Campbell et al., 2011).

The membrane structure, size and shape of AOB varies between the different microbial groups involved in nitrification. AOB are rod-shaped (*Nitrosomonas*), spiral-shaped or pleomorphic lobate (*Nitrospira*) and spherical/ellipsoidal (*Nitrosococcus*, *Nitrosoglobus* and *Nitrosacidococcus*). The cell membrane of AOB is characterized by the presence of ether-linked lipids that have fatty acids as side chains. The membrane structure determines the permeability of the cell and consequently its susceptibility to xenobiotic substances such as pesticides (Shen et al., 2013).

AOB are ubiquitously distributed in terrestrial habitats such as agricultural areas, grasslands and forests (Gubry-Rangin et al., 2010) as well as in aquatic habitats (Kowalchuk & Stephen, 2001). Their abundance in soil ranges from about 10^4 - 10^6 AOB cells g^{-1} soil. Environmental factors such as the heavy metal content, the presence or absence of plants, and pH can affect the abundance and activity of AOB (Gubry-Rangin et al., 2010). Previous studies have shown that an increase in soil pH leads to an increase in AOB *amoA* gene transcripts (Nicol et al., 2008). Temperature is another factor that seems to affect the activity of AOB. The optimal temperature for AOB growth is at 25-30°C while decrease in temperature can reduce NO_2^- production (Tourna et al., 2008). Furthermore, temperature is a factor that can cause changes in the structure and diversity of the microbial community of AOB in plant and artificial ecosystems (You et al., 2009).

AOB are characterized as autotrophic, chemolithotrophic microorganisms with a genetic inventory for the oxidation of ammonia to generate energy for the fixation of CO_2 using the Calvin cycle (Kowalchuk & Stephen, 2001). Ammonia (NH_3) is the main substrate of AMO (Suzuki et al., 1974). Nitrogen can enter in the cells by diffusion through the biological membranes in the form of NH_3 or through membrane proteins including ammonia and ammonium (NH_4^+) transporters (Andrade et al., 2005; Ellerbeck et al., 2013; Tobias Wacker et al., 2014). The ammonium transporters or Amt transporters are energy-dependent and present high affinity occurring in the survival of microorganisms under conditions of low pH and limited substrate concentration (Wright & Lehtovirta-Morley, 2023). Rh-type proteins serve as ammonia transporters, functioning as low-affinity channels that allow the bidirectional movement of NH_3 . These proteins are effective at high pH levels and elevated substrate concentrations (Ellerbeck et al., 2013; T. Wacker et al., 2014; Wright & Lehtovirta-Morley, 2023). According to the available AOB genomes, some AOB contain Rh proteins while the absence of recognizable ammonia transporters probably illustrates that they rely on ammonia diffusion (Lehtovirta-Morley, Sayavedra-Soto, et al., 2016a; Wright & Lehtovirta-Morley, 2023).

Ammonia oxidation is a specialized metabolism of AOB, despite the toxic intermediate products, such as NH_2OH , NO and NO_2^- . AOB oxidize NH_3 to NH_2OH using AMO (Beeckman et al., 2018; James I. Prosser et al., 2020; Stein, 2019). This enzyme is a copper-dependent heterotrimeric transmembrane complex, composed of three subunits (AmoA, AmoB and AmoC) with the active site being undefined (Tolar et al., 2017; Wright & Lehtovirta-Morley, 2023). Besides copper, AMO needs O_2 and two electrons (e^-) to oxidize NH_3 to NH_2OH , which is further transformed to NO by hydroxylamine hydrogenase. During this step 3 e^- are produced and the two of them meet the needs of AMO whereas the third one ends up in the electron transport chain (Stein, 2019). The intermediate NO indicates the existence of a nitric oxide oxidoreductase which catalyzes the transformation of NO to NO_2^- . Two different enzymatic complex have been proposed for AOB: (a) nitrosocyanin, encoded by *ncyA* gene and (b) reverse

operating copper-containing nitrite reductase encoded by *nirK* regulated by exposure to high NH_3 . (Caranto & Lancaster, 2017; James I. Prosser et al., 2020; Stein, 2019; Zorz et al., 2018). *NcyA* is the most possible candidate of this step as it is present in all AOB genomes and is a highly expressed gene in contrast with *nirK* which is absent in some AOB and not systematically expressed (Zorz et al., 2018) (Figure 1.6).

Many studies have reported the isolation of AOB strains in the laboratory belonging to *Nitrosomonas* and *Nitrospira* genera and grow at neutral pH and optimal growth temperature of 25-30°C. Recently the isolation of acidophilic AOB strains, which grows at pH 3.5 has been reported (Hayatsu et al., 2017; Picone et al., 2021). Within the studies presented in this thesis, only *Betaproteobacteria* strains were used (*Nitrosomonas europaea* ATCC25978, *Nitrospira multififormis* ATCC25196, *Nitrosomonas communis* DSM 28436, *Nitrospira briensis* C-128, *Nitrosomonas ureae* Nm 10 and *Nitrosomonas oligotropha* Nm 45). All these AOB strains are Gram-negative and are isolated from soil.

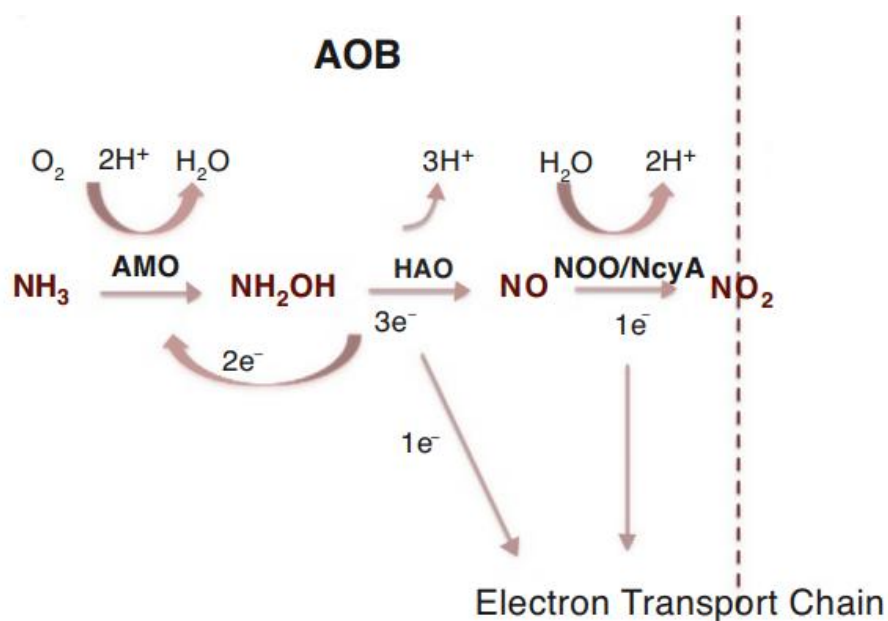


Figure 1.6 Ammonia oxidation by ammonia-oxidizing bacteria (AOB) (modified from Stein et al., 2019).

1.8.3 Ammonia-oxidizing archaea

AOA are the second microbial group which participate in ammonia oxidation through to NO_2^- . In 2005 the first isolation of a marine AOA, *Nitrosopumilus maritimus*, was described (Könneke et al., 2005). AOA are ubiquitous and highly diverse in terrestrial and marine ecosystems (Alves et al., 2018; Gubry-Rangin et al., 2010). They belong to the class *Nitrososphaeria* within the phylum *Nitrososphaerota*

(or *Thermoproteota* in GTDB taxonomy) and is synonymous with the previously used term *Thaumarchaeota*. All known AOA diversity is represented by four phylogenetic lineages: *Nitrososphaerales*, *Nitrosopumilales*, *Nitrosotaleales* and thermophilic *Ca. Nitrosocaldales* (Brochier-Armanet et al., 2008; Kerou et al., 2016; Stieglmeier et al., 2014) with *Nitrososphaerales* and *Nitrosotaleales* being the two dominant lineages in soil ecosystems. AOA dominate ammonia oxidation in acidic soils (approximately 30% of soil globally) with *Nitrosotaleales* being the main microorganisms that oxidize NH_3 at low pH. In acidic soils, this activity was considered paradoxical due to the low availability of NH_3 and the sensitivity of AOM to nitrous acid (HNO_2) and its decomposition products, which are formed from NO_2^- at low pH levels (Braida & Ong, 2000). The subsequent discovery and cultivation of acidophilic and acidotolerant *Nitrosotaleales* explained this paradox to a large extent (Hayatsu et al., 2017; Lehtovirta-Morley et al., 2014; Picone et al., 2021). However, mechanisms such as ureolytic activity (Burton & Prosser, 2001; De Boer et al., 1988), growth within biofilms (Allison & Prosser, 1993), and aggregates (De Boer et al., 1991) also support the growth of neutrophilic nitrifiers in acidic environments.

As with AOB, the membrane structure, size and shape of AOA distinguish them from other microbial groups involved in nitrification. AOA are rod-shaped or spherical-shaped (Hatzenpichler, 2012; Lehtovirta-Morley, Ross, et al., 2016). The cell membrane of AOA is distinguished by the presence of glycerol-ether lipids, where isoprenoid side chains are attached via an ether bond to a glycerol backbone, unlike the fatty acids in AOB. Additionally, these isoprenoid side chains are often linked together, resulting in the formation of a lipid monolayer with C40 chains, rather than the typical bilayer structure seen in other organisms. Consequently, the core, hydrophobic component of archaeal cell membrane lipids is predominantly composed of glycerol dialkyl glycerol tetraethers (Schleper & Nicol, 2010).

AOA are consistently found in greater abundance in both terrestrial and marine ecosystems in comparison to AOB. Soil typically contains more than 10^7 AOA cells g^{-1} soil and they are found in many terrestrial ecosystems such as agricultural areas, grasslands, forests, alpine soils (Gubry-Rangin et al., 2010), but also in many aquatic habitats, and are considered to be the dominant AOM in marine systems (reference). AOAs have also been found in wastewater treatment plants (You et al., 2009). The factors that affect the abundance and function of AOB in different environments also affect AOAs to the same extent (Gubry-Rangin et al., 2010).

AOA are also chemolithoautotrophic microorganisms and utilize ammonia monooxygenase (AMO) to oxidize ammonia for energy requirements. However, they use the 3-hydroxypropionic acid/4-hydroxybutyric acid pathway instead of the Calvin Cycle (Walker et al., 2010) for carbon fixation. As with AOB, NH_3 is the main substrate of AMO (Jung et al., 2022). Ammonia enters archaeal cells by diffusion through the biological membranes. However, due to the different composition of archaeal and bacterial membranes the membrane permeability and the rate at which ammonia can

diffuse is varied. Most AOA strains encode Amt-type transporters for the uptake of NH_4^+ (Lehtovirta-Morley, Sayavedra-Soto, et al., 2016b; Wright & Lehtovirta-Morley, 2023). The transcriptional response of archaeal Amt transporters to varying ammonia concentrations suggests that these transporters function as both high- and low-affinity systems (Nakagawa & Stahl, 2013; Qin et al., 2018; Santoro & Casciotti, 2011).

AOA oxidize NH_3 to NH_2OH using AMO (Beeckman et al., 2018; James I. Prosser et al., 2020; Stein, 2019). This enzyme is similar in AOA and AOB with the main difference being the presence of the three additional subunits, AmoX, AmoY and AmoZ (Hodgskiss et al., 2023). AMO requires oxygen and two e^- to oxidize NH_3 to NH_2OH . The subsequent oxidation of hydroxylamine is still uncharacterized with no candidate enzyme identified. Kozłowski et al. (2016) proposed that a copper-based enzymatic complex co-oxidizes NH_2OH and NO to form two molecules of NO_2^- (with the release of five e^-) with one NO_2^- molecule reduced to NO by nitrite oxidoreductase (NirK) and returned back to the copper-based enzymatic complex (Kozłowski et al., 2016; Stein, 2019). A second pathway has been proposed based on metagenome, transcriptome and metatranscriptome data whereby NirK either oxidizes NH_2OH to NO with the release of 3 e^- or, in a reverse function (rNIR), oxidizes NO to NO_2^- with production of 1 e^- , and a copper-based enzymatic complex that act as either hydroxylamine-oxidoreductase or NO-oxidoreductase, depending on the role of NirK or another Cu-ME capable of these reactions (Carini et al., 2018) (Figure 1.7).

Soil AOA strains can be either acidophilic or neutrophilic, grow in liquid cultures with an optimal growth temperature of 30-40°C. AOA belonging to the *Nitrosotalea* genus grow in media with a pH between 4 to 6 (Lehtovirta-Morley et al., 2014, 2011) and all other strains growing at neutral pH. Within the studies presented in this thesis, acidophilic (*Nitrosotalea sinensis* Nd2) and neutrophilic (*Candidatus Nitrosocosmicus franklandianus* C13, *Nitrososphaera viennensis* EN76) AOA strains were used.

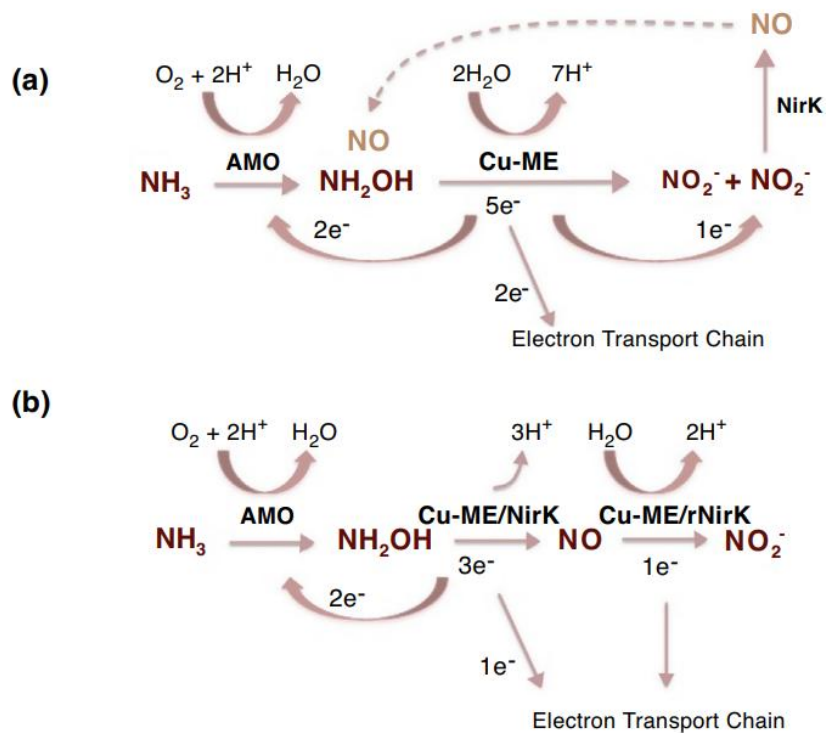


Figure 1.7 Ammonia oxidation by ammonia-oxidizing archaea (AOA). Panel (a) and (b) show the proposed pathways for hydroxylamine oxidation (Stein et al., 2019).

1.8.4 Nitrite-oxidizing bacteria

The second step of the modular nitrification process involves the transformation of NO_2^- to NO_3^- , the end-product of nitrification. This process is performed by nitrite-oxidizing bacteria (NOB) which belong to seven genera in four bacterial phyla. Specifically, *Nitrobacter*, *Nitrococcus* and *Nitrotoga* are placed within the phylum *Proteobacteria*, *Nitrospira* within the phylum *Nitrospirae*, *Nitrospina* and *Candidatus Nitromaritima* within the phylum *Nitrospinaciae*, and *Nitrolancetus* within the *Chloroflexi* (Daims et al., 2016; Liu et al., 2010). In terrestrial ecosystems, *Nitrospira* and *Nitrobacter* are the dominant NOB, with *Nitrobacter* typically being more active and with faster growth compared to *Nitrospira*, under high nitrogen supply (Daims et al., 2016; Nowka, Off, et al., 2015; Xia et al., 2011). As such *Nitrobacter* are considered r-strategists while *Nitrospira* are k-strategists and reach a high abundance under nitrite limitation (Daims et al., 2016; Andreas Schramm et al., 1999). In addition to associations with different phylogenetic lineages, there are structural and biochemical differences among NOB (Daims et al., 2016; Lücker et al., 2010).

All NOB are Gram-negative. *Nitrobacter* strains are rod or pear shaped and possess a characteristic layered intracytoplasmic membrane (Pillay et al., 1989; Jim I. Prosser, 2007). *Nitrospira* strains grow as helical cells or vibrios (Ehrich et al., 1995).

The oxidation of NO_2^- contributes to the energy requirements for NOB and they are considered chemoautolithotrophic (Daims et al., 2016; Lücker et al., 2010). NOB fix CO_2 through the Calvin cycle to meet their carbon requirements (Daims et al., 2016; Lücker et al., 2015, 2010). However, *Nitrospira* are also capable of mixotrophy and can switch between carbon fixation and consumption of organic molecules such as glycerol, pyruvate, or formate, depending on environmental conditions (Koch et al., 2015). Recent studies also indicated that some *Nitrospira* from different terrestrial and aquatic ecosystems can utilize urea as a nutrient source (Koch et al., 2015). Their genomes can encode urease or cyanase, which transform urea or cyanate into CO_2 and ammonia (Fujitani et al., 2014; Koch et al., 2015), facilitating reciprocal cross-feeding ammonia-oxidizing microorganisms and utilization of generated NO_2^- (Daims et al., 2016). Genome analysis also indicated that NOB can switch between nitrite oxidation and alternative metabolisms such as H_2 or formate oxidation (Koch et al., 2014).

NOB catalyze the oxidation of NO_2^- to NO_3^- using nitrite oxidoreductase (NXR). During this step two e^- are transferred into the respiratory chain. This enzyme belongs to the type II DMSO reductase-like family of molybdopterin-binding enzymes (Lücker et al., 2013). NXR is typically associated with the cytoplasmic membrane and likely comprises three subunits: NxrA, NxrB, and NxC (Lücker et al., 2013, 2010). In species such as *Nitrospira*, *Nitrospina*, and '*Candidatus Nitromaritima*,' the substrate-binding subunit NxrA is located in the periplasmic space whereas in *Nitrobacter*, *Nitrococcus*, and *Nitrolancea*, it is found in the cytoplasm (Daims et al., 2016). NOB with cytoplasmic NXR use transporters for transfer of nitrite and nitrate across the cytoplasmic membrane potentially creating a physiological bottleneck depending on the transporter's substrate affinity and turnover rate (Daims et al., 2016) (Figure 1.8).

Pure cultures of *Nitrobacter* strains from soil are available with different representatives growing at acidic or neutral pH (De Boer et al., 1988; Hink et al., 2024; K. D. Kits et al., 2017; Nowka, Daims, et al., 2015) while *Nitrospira* strains have been isolated from waste water treatments (Keuter et al., 2023; Nowka, Off, et al., 2015). Within the studies presented in this thesis, *Candidatus Nitrobacter laanbroekii* NHB1, *Nitrobacter winogradskyi* DSM 10237 and *Nitrospira defluvii* A17 were used. All NOB strains are isolated from soil with the exception of *N. defluvii* which is isolated from activated sludge.

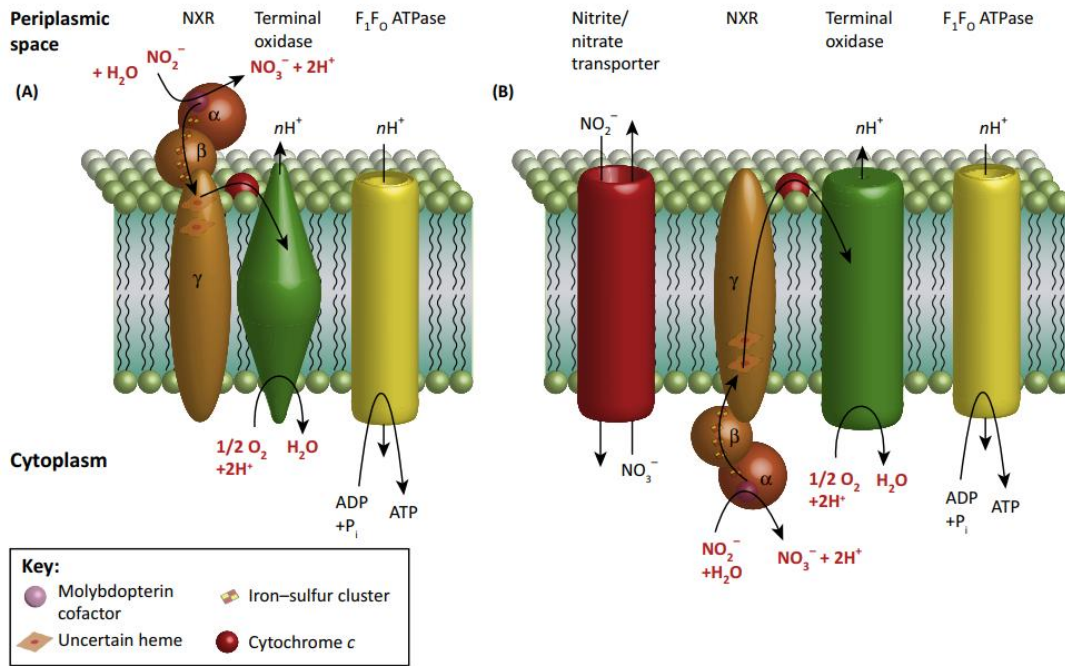


Figure 1.8 The mechanism of oxidation of nitrite to nitrate by the (A) cytoplasmic and (B) periplasmic nitrite oxidoreductase (NXR) (Daims et al., 2016).

1.8.5 Complete ammonia-oxidizing bacteria (comammox)

The dogma of an obligatory division of metabolic labor in nitrification was challenged from 2015 with the cultivation of *Nitrospira* strains capable of oxidizing ammonia through to nitrate in the same cell; *Candidatus Nitrospira inopinata*, *Candidatus Nitrospira nitrificans*, *Candidatus Nitrospira nitrosa* and *Candidatus Nitrospira kreffii* which are capable of performing both steps of nitrification. These microorganisms are called comammox (complete ammonia oxidizers). Comammox bacteria belong to the genus *Nitrospira* which also includes canonical NOB (Daims et al., 2015), and have been detected in a range of natural and engineered environments (Fowler et al., 2018; Palomo et al., 2018; Pjevac et al., 2017; Wang et al., 2019; Zheng et al., 2019). Analysis of *amoA* gene sequences suggest two phylogenetic groups (clade A and B), both of which can be found in soil (Pjevac et al., 2017).

Comammox *Nitrospira* are Gram-negative with a spiral shape (Daims et al., 2015; van Kessel et al., 2015). Comammox have a comparatively high affinity for ammonia substrate, indicating that at low concentrations they gain a competitive advantage over AOB and AOA. Contrastingly, their affinity for nitrite is much lower compare to the canonical NOB (K. Dimitri Kits et al., 2017). The different clades of comammox have different ammonia/ammonium uptake mechanisms. Comammox clade A encode Rh-type transporters with high similarity with the ones in AOB while comammox clade B encode Amt-type transporters (Palomo et al., 2018).

Genomic analyses of cultivated *Nitrospira* strains, have shown the presence of genes necessary for oxidation of NH_3 to NO_3^- . Comparative genomic analysis of comammox *Nitrospira* reveals that their ammonia oxidation genes are more similar to those of AOB than AOA (Daims et al., 2015; Sakoula et al., 2021; van Kessel et al., 2015). Homologues of all *amo* genes are present in comammox, along with AOB-like HAO and c-type cytochromes, which facilitate electron transfer to the quinone pool (Daims et al., 2015). As with other AOM, the enzymatic complex which catalyzes the transformation of NO to NO_2^- is currently uncharacterized. NirK is a possible candidate for this step as comammox genomes lack *ncyA* (Kits et al., 2019). These microorganisms can also use urea as alternative N source as they encode the necessary genes for urea hydrolysis and high affinity of urea transporters (Daims et al., 2015; Sakoula et al., 2021; van Kessel et al., 2015).

The cultivation of comammox is challenging. Thus far, only one comammox strain, *Ca. Nitrospira inopinata*, belonging to clade A has been isolated, derived from a microbial biofilm in a hot water pipe and grows at neutral pH (Daims et al., 2015). Enrichment cultures of clade A comammox, *Ca. Nitrospira nitrificans*, *Ca. Nitrospira nitrosa* and *Ca. Nitrospira kreftii* from aquaculture system have been also reported (Sakoula et al., 2021; van Kessel et al., 2015). Currently, no soil-derived comammox strains are available despite their widespread occurrence and abundance in terrestrial ecosystems.

1.9 Ammonia-oxidizing microorganisms as ideal microbial indicators of pesticides toxicity

Soil microbes and enzyme complexes involved in nitrification have been extensively studied due to the high economic and ecological importance of the process (Brochier-Armanet et al., 2008; Francis et al., 2007; You et al., 2009). Several studies using advanced molecular and biochemical tools assessed the toxicity of pesticides and its metabolic product on AOM in soil (Karas et al., 2015; Papadopoulou et al., 2016; Vasileiadis et al., 2018) and at the *in vitro* level (Papadopoulou et al., 2020; Vasileiadis et al., 2018). Beyond their key ecological role, AOMs have specific traits that make them ideal candidates as bioindicators for toxicity of pesticides: i. they show sensitivity to xenobiotic compounds including pesticides (Wessén & Hallin, 2011), ii. they participate in the rate-limiting step of the N cycle (Beeckman et al., 2018; James I. Prosser & Nicol, 2008), and iii. there is substantial knowledge of their ecology, physiology and diversity with well-established tools to measure their activity and abundance *in vitro* (Lehtovirta-Morley et al., 2013) and in soil (Junier et al., 2010).

The investigation of the impact of pesticides against the NOB group is important to broaden the spectrum of non-target toxicity of pesticides on a microbial group functionally associated with AOM and to predict possible indirect contribution to N_2O emissions. Reduction of the activity of NOB or inhibition after application of

agrochemicals might increase nitrogen loss from disturbed agricultural ecosystems and aggravate the problem of atmospheric pollution (James I. Prosser et al., 2020).

1.10 Aims and objectives

The primary aim of this thesis was the development and standardization of pioneering *in vitro* tests for a new risk assessment scheme to evaluate the toxicity of pesticides on sensitive, phylogenetically and ecophysiologicaly distinct soil AOM. Given the limited number of available cultures of soil-derived AOA and AOB, our main goal was to select AOA, AOB, and NOB that are consistently more sensitive and exhibit clear ecotoxicologically relevant responses to pesticide exposure (Chapter 2).

These tests are intended to serve as a first-tier testing step, analogous to Single Species Tests performed for aquatic organisms in pesticide risk assessment, within a revised tiered risk assessment framework for evaluating the toxicity of pesticides on soil microorganisms. Specifically, the aim was to assay the toxicity of selected pesticides on the activity levels of a range of AOA, AOB, and NOB strains found in soil ecosystems, and calculate toxicity endpoints such as EC₅₀ values. As a proof of concept, a second set of pesticides were used to test the sensitivity of the selected strains, further validating the robustness and reliability of the initial findings (Chapter 3).

Results from Chapter 2 were compared with a commercially available microbial toxicity assessment kit (MARA) provided by the biotechnology company NCIMB to validate and benchmark the findings (Chapter 4). This work could contribute to the development of a fast-track, high-throughput format that would facilitate pesticide toxicity testing practices, providing a practical tool for regulatory agencies and environmental monitoring programs.

In Chapter 5, *Ca. Nitrobacter laanbroekii* NHB1, one of the most sensitive strains studied, was characterized to gain deeper insights into its physiological traits and interactions with acidophilic ammonia-oxidizing archaea (AOA), such as *Nitrosotalea* strains. This chapter highlights pivotal role of *Ca. Nitrobacter laanbroekii* NHB1 in promoting ammonia oxidizer (AO) activity under low pH conditions and emphasizes the importance of mutualistic interactions between nitrite-oxidizing bacteria (NOB) and ammonia-oxidizing microorganisms (AOM) in acidic environments.

1.11 References

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Chapter 2

Single species *in vitro* assays with nitrifying bacteria and archaea as a first-tier tool for assessing the toxicity of pesticides on soil microorganisms

Statement on author contributions

The work presented in Chapter 2 is a manuscript currently under revision for consideration of publication in the journal *Environmental Science and Technology*. This work was carried out in collaboration with the University of Thessaly. The work examining the toxicity of pesticides on two AOA strains (*Nitrosotalea sinensis*, *Nitrososphaera viennensis*), four AOB strains (*Nitrosomonas europaea*, *Nitrosomonas oligotropha*, *Nitrosomonas ureae*, *Nitrospira briensis*), and one NOB strain (*Nitrospira defluvii*) was performed at ECL by E. Bachtsevani, and in parallel, PhD candidate M. Kolovou conducted similar experiments at UTH, testing the toxicity on one AOA strain (*Candidatus Nitrosocosmicus franklandianus*), two AOB strains (*Nitrosomonas communis*, *Nitrospira multiformis*), and two NOB strains (*Nitrobacter winogradskyi*, *Candidatus Nitrobacter laanbroekii* NHB1). The study of pesticide stability in liquid cultures was also performed at UTH. Both Ph.D. candidates collaborated on the analysis of the results. The manuscript was written entirely by E. Bachtsevani, with all authors providing comments only.

Abstract

Single species tests on surrogate organisms from different trophic levels constitute a cornerstone of aquatic and terrestrial ecotoxicology and a major tool for assessing the ecotoxicity of pesticides. However, no corresponding assays are available for soil microorganisms despite their pivotal role in ecosystems. This study aimed to develop and standardize a single species *in vitro* testing system as a Tier I ecotoxicity assay for ammonia-oxidizing microorganisms (AOM), recognized as potential indicators of pesticide toxicity on soil microorganisms. In this context, we assessed the toxicity of ten compounds representing main categories of pesticides (insecticides, fungicides, herbicides) on a range of ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) grown in liquid culture and representing all major lineages of both groups. We extended our testing to functionally associated nitrite-oxidizing bacteria (NOB). Toxicity endpoint values (EC_{50}) were calculated for each strain and pesticide combination. Our assays demonstrated that *Nitrosotalea sinensis* Nd2, *Nitrosospira briensis* and *Ca. Nitrobacter laanbroekii* NHB1 were the most sensitive strains. Fungicides and insecticides were more toxic to AOM than NOB, whereas herbicides had a wide range of toxicity on all tested groups of nitrifiers. Our study introduces a new testing tool for the assessment of the ecotoxicity of pesticides on soil microorganisms that could be readily implemented as a standard tier I type assay in environmental risk analysis.

Keywords: ammonia-oxidizing bacteria, ammonia-oxidizing archaea, nitrite-oxidizing bacteria, single species *in vitro* tests, ecotoxicity, pesticides

2.1 Introduction

Pesticides constitute an integral part of modern agriculture and are heavily used in agroecosystems to ensure high yields and meet growing global food demands (Tudi et al., 2021). While their use has increased by nearly 50% over the last 30 years (Riedo et al., 2021), only 5% of applied compounds reach their target with the remaining ending up in soil and increasing the possibility of negative effects on soil ecosystems (Mitchell et al., 2001). In soil, pesticides interact with microorganisms resulting in either growth-linked microbial degradation (Copley, 2009) or toxicity with associated inhibition of important microbial functions (Puglisi et al., 2012) or changes in the structure of the soil microbial community (Feld et al., 2015). Pesticide authorization within the EU is under the control of a strict regulatory framework that aims to minimize undesirable effects on non-target organisms. Regulation 1107/2009 describes the risk assessment procedures and lists all tests that should be undertaken to determine environmental exposure and the potential toxicity of pesticides on indicator organisms from different trophic levels (Schäfer et al., 2019). Such risk

assessment procedures are well established and standardized for aquatic and terrestrial macro-organisms, structured in a tiered scheme with increasing experimental complexity (Karpouzas et al., 2022). A similar risk assessment scheme is not available for soil microorganisms even though it is identified as a specific protection goal for environmental risk assessment of pesticides (European Food Safety Authority, 2010). To date the toxicity of pesticides on soil microorganisms is determined with the use of the OECD-216 N transformation test (European Food Safety Authority, 2010). This provides a bulk measurement of ammonification and nitrification, but it is considered limited as it fails to provide an accurate assessment of the potential toxicity of pesticides on soil microorganisms, particularly as standardized molecular methods are routinely used in soil microbiology (Karpouzas et al., 2022). In 2017, the EFSA identified arbuscular mycorrhizal fungi (AMF) as indicators of pesticide toxicity (European Food Safety Authority, 2017), while others (Karpouzas et al., 2016; Wessén & Hallin, 2011) proposed AOM as ideal microbial indicators of the toxicity of agrochemicals on soil microbiota. A three-tier risk assessment scheme was proposed for evaluating the toxicity of pesticides on soil microorganisms using both AOM and AMF as bioindicators (Karpouzas et al., 2016). In these proposed schemes, Tier I involves single species *in vitro* assays of pesticides on soil-derived and ecotoxicologically relevant AOM and AMF strains, aligned with the single species tests used at Tier I in aquatic and terrestrial ecotoxicology. If undesirable effects are observed in these assays, further testing at tiers II and III should be undertaken involving soil microcosm and field scale studies, respectively.

Aerobic chemolithoautotrophic AOM in soil comprise three groups: AOB of the genera *Nitrosomonas*, *Nitrosospira* and *Nitrosococcus*, AOA of the class *Nitrososphaerales*, and complete ammonia-oxidizing *Nitrospira* (comammox) (Prosser et al., 2020). AOB and AOA perform the first and rate-limiting step of nitrification, the oxidation of ammonia to nitrite (NO_2^-) via a series of intermediates (Beeckman et al., 2018; Prosser et al., 2020) with canonical nitrite-oxidizing bacteria (NOB), typically *Nitrospira* and *Nitrobacter* in soil, subsequently oxidizing NO_2^- to nitrate (NO_3^-). Comammox *Nitrospira* oxidize NH_3 through to NO_3^- and are ubiquitous in soil (Wang et al., 2022; Xu et al., 2020). However, there are currently no representative soil isolates.

Several traits of AOM support their use as potential bioindicators of pesticide toxicity on soil microbiota: (i) they have a key functional role in soil (Beeckman et al., 2018; Prosser & Nicol, 2008), (ii) they are sensitive to abiotic perturbations including exposure to xenobiotics (Wessén & Hallin, 2011) (iii) their diversity and ecophysiology have been extensively characterized (Prosser et al., 2020), and (iv) functional and molecular tools are well-established for measuring their activity and abundance *in vitro* (Lehtovirta-Morley et al., 2013) and in soil (Junier et al., 2010). Several studies have assessed the toxicity of pesticides on the activity and abundance of AOM in soil using molecular and biochemical tools (Karas et al., 2015; Papadopoulou et al., 2016;

Sim et al., 2022; Vasileiadis et al., 2018). In contrast, single species *in vitro* assays with AOM have been mostly used for activity screening of nitrification inhibitors (Kaur-Bhambra et al., 2022; Papadopoulou et al., 2020; Shen et al., 2013; Wright et al., 2020; Zhao et al., 2020), and only rarely in an ecotoxicological context (Vasileiadis et al., 2018).

We aimed to establish and standardize single species *in vitro* assays to determine the toxicity of pesticides on AOM for use as a first tier ecotoxicity test. The toxicity of 10 pesticides (including three selected transformation products) representing all major groups of plant protection products (insecticides, fungicides, and herbicides) were tested on isolated nitrifier strains that represent the dominant lineages of AOA and AOB found in soil (Alves et al., 2018; Kowalchuk & Stephen, 2001). As nitrite-oxidizing bacteria (NOB) are functionally associated with AOM via utilization of AOM-derived nitrite, tests were expanded to include *Nitrobacter* and *Nitrospira* strains that represent the two dominant NOB genera found typically in soil (Daims et al., 2016). We hypothesized that at least one microorganism from each group of nitrifiers would be systematically more sensitive to pesticides and exhibit a clear ecotoxicological response to exposure, and selected strains would be successfully utilized as surrogate microorganisms in single species *in vitro* assays.

2.2 Material and Methods

2.2.1 Microbial strains

Twelve strains were used in this study: six AOB representing the two dominant genera found in soil (*Nitrosospira multiformis* ATCC25196, *Nitrosospira briensis* C-128, *Nitrosomonas europaea* ATCC25978, *Nitrosomonas ureae* Nm 10, *Nitrosomonas oligotropha* Nm 45 and *Nitrosomonas communis* DSM 28436), three AOA strains with contrasting ecophysologies and representing widely distributed lineages in soil ("*Candidatus Nitrosocosmicus franklandianus*" C13, *Nitrosotalea sinensis* Nd2 and *Nitrososphaera viennensis* EN76), and three NOB strains representing the two dominant genera found in soil ("*Candidatus Nitrobacter laanbroekii* NHB1", *Nitrobacter winogradskyi* DSM 10237 and *Nitrospira defluvii*). The culture conditions of all tested strains are presented in the supplementary material (Annex I).

2.2.2 Pesticides

The range of pesticides used in this study are presented in Table 2.1. Their selection was based on (i) different targets or modes of action and those potentially interacting with AOM and NOB metabolic pathways and (ii) their widespread use in agricultural practice in Europe, although some were selected as historic model compounds (e.g., chlorpyrifos) for comparison purposes. All isolates were challenged with 7 pesticides: chlorpyrifos (insecticides); metsulfuron-methyl, clethodim,

glyphosate (herbicides); pyraclostrobin, etridiazole and hymexazol (fungicides). In addition, for chlorpyrifos, glyphosate and iprodione, the impact of their transformation products 3,5,6-trichloro-2-pyridinol (TCP), aminomethylphosphonic acid (AMPA) and 3,5-dichloroaniline (3,5-DCA), respectively, were investigated as hallmark pesticide transformation products.

The concentration levels used per compound (Supplementary Table S1, Annex I) were selected based on a prediction of the concentration found in soil solution upon application of the maximum recommended dose taking into consideration factors of the crop species receiving the application, the mode of application (foliar or soil drench) and frequency of application per season. A detailed description of the calculation is provided in Supplementary Material, Annex I.

Upon calculation of expected pesticide concentrations after application of the recommended dose, concentrations were tested at 0.1x, 1x, 10x and 100x the recommended dose (Supplementary Table S1, Annex I). It should be noted that for some pesticides an application of the maximum concentration (100x) was not possible due to solubility limitations and the maximum working concentration was determined after a solubility test.

Analytical standards of all pesticides ($\geq 92\%$) were purchased from HPC Standards (Germany) and were used for both *in vitro* assays and analytical methods.

2.2.3 Single species *in vitro* assays

The toxicity of pesticides was determined in liquid cultures of the selected AOM and NOB strains at four concentrations to establish inhibition threshold values (EC_{50}) per strain and pesticide. Cultures were established in triplicate for each strain x pesticide x concentration combination in 100-mL culture bottles containing 50 mL growth medium and inoculated with a 1 or 2% (v/v) transfer of exponentially growing cultures of AOB or AOA/NOB, respectively. All pesticides, except for glyphosate and its metabolite AMPA, were added to the cultures as filter-sterilized DMSO solutions (50 μ L). The final concentration of DMSO was 0.1% (v/v) with no inhibitory effect on the growth of any of the isolates tested. Glyphosate and AMPA were dissolved in sterile ddH₂O before addition of 50 μ L (0.1% v/v) to the tested cultures. All pesticides were added to cultures at the beginning of the exponential growth phase. For all assays, control cultures (no pesticide) were amended with 50 μ L DMSO or ddH₂O.

As rates of NO₂⁻ production or consumption are concomitant with growth rates of AOM or NOB, respectively (Lehtovirta-Morley et al., 2014; Evangelia S. Papadopoulou et al., 2020), the effect of pesticide concentration on growth and calculation of inhibition threshold values were determined from regular measurement of NO₂⁻ concentrations. The stability of the pesticides in the liquid cultures was also determined for a minimum of two concentration levels and two time points: immediately after the addition of the pesticides (early logarithmic phase) and at the

end of the experiment (early stationary phase) (see Supplementary Material, Annex I).

2.2.4 Nitrite measurements

The NO_2^- concentration in liquid cultures was determined colorimetrically in a 96-well plate assay using Griess diazotizing and coupling solutions (Shinn, 1941) as described by Hink et al. (Hink et al., 2018). For each measurement, duplicate standards ranged between 0 and 100 μM NaNO_2 were used. Absorbance was recorded at 540 nm using a Multiskan GO microplate spectrophotometer (Thermo Scientific).

2.2.5 Calculation of toxicity endpoint values

Determination of half maximal effective concentrations (EC_{50}) was performed according to Papadopoulou et al. (Papadopoulou et al., 2020). Specifically, EC_{50} values were calculated using the R package *drc: Analysis of Dose-Response Curves v3.0-1* package (Ritz & Streibig, 2015) based on a dose-response model with normalized data whereby nitrite concentration values were divided by the mean value of the control. A description of the tested models can be found in Ritz et al. (Ritz et al., 2015). For the selection of the best fitting model, an empirical modeling approach was used based on goodness of fit indices, followed by the choice of the four-parameter log logistic model as the best compromise among tested models for comparing toxicity endpoint values.

Table 2.1. Chemical classification, mode of action and potential inhibition characteristics of pesticides and selected transformation products used in this study.

| Pesticide type | Pesticide | Chemical classification | Mode of action | Reference | Potential effect on nitrifiers | References |
|----------------|---|-------------------------|--|-----------------------|--|---|
| Insecticides | Chlorpyrifos | Organophosphate | Blocks nerve system of insects - inhibiting the acetylcholinesterase enzyme | (Sparks et al., 2020) | No potential effect | - |
| | 3,5,6-trichloro-2-pyridinol (TCP) (Transformation product of chlorpyrifos) | Organophosphate | Antimicrobial metabolite which inhibits proliferation of chlorpyrifos-degradation by soil microorganisms | (Racke et al., 1990) | Antimicrobial activity | - |
| Herbicides | Glyphosate | Organophosphate | Inhibition of the plant enzyme 5-enolpyruvylshikimate-3-phosphate synthase, that catalyses biosynthesis of essential aromatic amino acids in plants, fungi, and bacteria | (Secor & Cséke, 1988) | Target enzyme produced by <i>Nitrosomonas</i> , <i>Nitrospira</i> (AOB) and <i>Nitrososphaerales</i> (AOA) | (Chain et al., 2003; Herbold et al., 2017; Kerou et al., 2016; Kozłowski et al., 2016a, 2016b; Nicol et al., 2019; Norton et al., 2008; Rice et al., 2016; Wang et al., 2014) |

Table 2.1. Continued

| Pesticide type | Pesticide | Chemical classification | Mode of action | Reference | Potential effect on nitrifiers | References |
|----------------|---|-------------------------|---|--------------------------|--|--|
| Herbicides | Aminomethylphosphonic acid (AMPA) (Transformation product of Glyphosate) | Organophosphate | A mimetic of both D- and L-alanine, inhibited D-Ala-D-Ala synthetase, alanine racemase, and UDP-N-acetylmuramyl-L-alanine synthetase | (Atherton et al., 1982) | Antimicrobial activity | (Atherton et al., 1982) |
| | Metsulfuron-methyl | Sulfonylurea | Blocks biosynthesis of branched chain amino acids by inhibition of acetolactate synthase (ALS) | (Lonhienne et al., 2018) | Target enzyme produced by <i>Nitrosomonas</i> , <i>Nitrospira</i> (AOB) and <i>Nitrososphaerales</i> (AOA) | (Chain et al., 2003; Herbold et al., 2017; Kerou et al., 2016; Kozlowski et al., 2016a, 2016b; Nicol et al., 2019; Norton et al., 2008; Rice et al., 2016; Wang et al., 2014) |
| | Clethodim | Cyclohexanedione | Inhibition of enzyme acetyl coenzyme A carboxylase (ACCase), that interrupts lipid synthesis and consequently harms development of the plant. | (Secor & Cséke, 1988) | Target enzyme produced by <i>Nitrosomonas</i> , <i>Nitrospira</i> (AOB) and <i>Nitrososphaerales</i> (AOA) | Kerou et al. 2016; Chain et al. 2003; L. Wang et al. 2014; Norton et al. 2008; Kozlowski, Kits, and Stein 2016a, 2016b; Rice et al. 2016; Herbold et al. 2017; Nicol et al. 2019 |

Table 2.1. Continued

| | | | | | | |
|-------------------|--|---------------|--|--------------------------|--|--|
| Fungicides | Pyraclostrobin | Strobilurin | Inhibition of ubiquinone, an electron-transfer associated coenzyme | (Karadimos et al., 2005) | Target enzyme involved in ammonia oxidizer energy metabolism | (Chain et al., 2003; Whittaker et al., 2000) |
| | Etridiazole | Triazole | Stimulation of hydrolysis of membrane-bound phospholipids to free fatty acids and lysophosphatides | (Radzuhn & Lyr, 1984) | Inhibition of nitrification | (Beeckman et al., 2023; Jog et al., 2022; McCarty & Bremner, 1989; Taggert et al., 2021) |
| | Hymexazol | Isoxazole | Inhibition of DNA and RNA synthesis | (Alonzo & Alfonso, n.d.) | Inhibition of nitrification | (Beeckman et al., 2023; Jog et al., 2022; McCarty & Bremner, 1989; Taggert et al., 2021) |
| | 3,5 DCA (Transformation product of iprodione) | Dicarboximide | Unknown | (Mercadier et al., 1996) | Unknown | (Vasileiadis et al., 2018) |

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2.2.6 Data analysis

Nitrite data, as well as the variance between the EC₅₀ values of pesticides were analysed by one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test ($p < 0.05$). To test the hypothesis that AOA, AOB and NOB show different sensitivities to the different pesticide groups, statistically significant differences between EC₅₀ values were analysed using the non-parametric Wilcoxon signed-rank test ($p < 0.05$).

2.3 Results

2.3.1 Impact of pesticides on the activity of AOA strains

2.3.1.1. Insecticides

Chlorpyrifos and its transformation product TCP significantly inhibited ammonia oxidation by *N. sinensis* at all concentrations (Supplementary Figure S1, Annex I). Comparatively, *Ca. N. franklandianus* and *N. viennensis* were only inhibited by chlorpyrifos at concentrations $> 2.9 \mu\text{M}$.

2.3.1.2. Herbicides

Metsulfuron-methyl, clethodim, glyphosate and AMPA inhibited the activity of *N. sinensis* at concentrations $\geq 0.26 \mu\text{M}$, $\geq 83 \mu\text{M}$, $\geq 590 \mu\text{M}$, and $\geq 4500 \mu\text{M}$, respectively (Supplementary Figure S1, Annex I). In contrast these compounds did not affect *Ca. N. franklandianus* and *N. viennensis* at any of the tested concentrations.

2.3.1.3. Fungicides

Pyraclostrobin was inhibitory to all AOA strains with *N. sinensis* and *Ca. N. franklandianus* being almost fully inhibited at concentrations $\geq 7.7 \mu\text{M}$, and *N. viennensis* at concentrations $\geq 77 \mu\text{M}$ (Supplementary Figure S1, Annex I). Etridiazole inhibited *Ca. N. franklandianus* and *N. viennensis* at all concentrations ($\geq 4 \mu\text{M}$), and *N. sinensis* at concentrations $\geq 40.4 \mu\text{M}$. Hymexazol had a significant inhibitory effect on all AOA strains only at the highest concentration level (1009 μM), although complete inhibition was evident only for *N. sinensis*. Finally, 3,5-DCA significantly inhibited *N. sinensis* at concentrations $\geq 31 \mu\text{M}$ while partial suppression was observed at the concentration 3.1 μM . *Ca. N. franklandianus* and *N. viennensis* were completely inhibited only at the highest concentration of 310 μM .

2.3.1.4. EC₅₀ values for AOA

The calculated EC₅₀ values for *Ca. N. sinensis* were significantly lower compared to *Ca. N. franklandianus* and *N. viennensis* for seven out of the 10 pesticides (Figure 2.1). *Ca. N. franklandianus* and *N. viennensis* showed near equivalent EC₅₀ values for the different

pesticides. The EC₅₀ values of chlorpyrifos, etridiazole and pyraclostrobin, the most toxic compounds to AOA, and were lower than their recommended dose rates for all AOA strains (Figure 2.1).

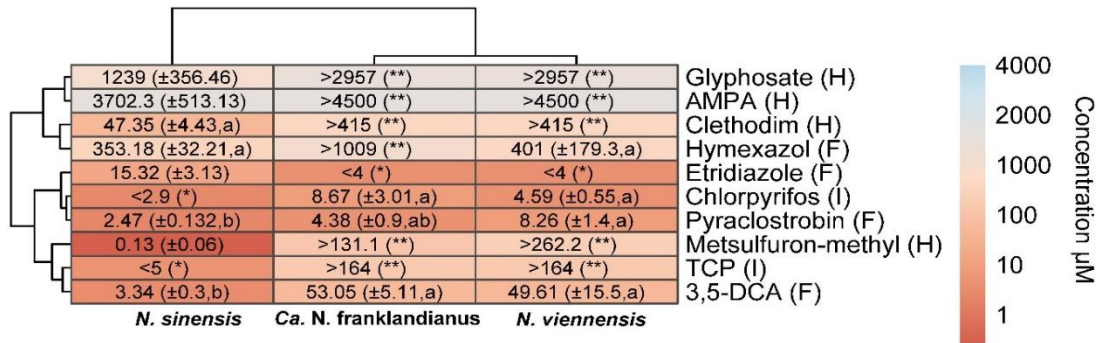


Figure 2.1. Mean EC₅₀ values (µM) of pesticide inhibitory concentrations on AOA strains, *N. sinensis*, *Ca. N. franklandianus* and *N. viennensis*. Standard error of mean values (denoted by ±) are given in parentheses. Lower case letters indicate significant differences ($p < 0.05$) in EC₅₀ values between tested strains for each pesticide. Asterisks denote the minimum (*) or the maximum tested concentration (**) in cases where no EC₅₀ could be determined from the statistical analysis. The capital letters I, F, and H in parentheses next to the tested pesticides denote the categories insecticides, fungicides, and herbicides, respectively. Dendrograms based on Euclidean distances and the complete linkage clustering method using log-transformed mean EC₅₀ values are presented for clustering strains and pesticides according to their sensitivity and toxicity, respectively. Values are color-coded by orders of the tested concentrations.

2.3.2. Impact of pesticides on the activity of AOB strains

2.3.2.1. Insecticides

Chlorpyrifos and TCP did not significantly affect the activity of any AOB strain (Supplementary Figure S2, Annex I).

2.3.2.2. Herbicides

Glyphosate completely inhibited *N. europaea* and *N. multiformis* at concentrations ≥ 2957 µM, and *N. communis*, *N. briensis*, *N. ureae* and *N. oligotropha* at concentrations ≥ 590 µM, with the effect on the latter being temporary at ≥ 590 µM (Supplementary Figure S2, Annex I). In contrast, the transformation product AMPA fully inhibited the activity of *N. briensis* and *N. ureae* only at a concentration ≥ 900 µM. Metsulfuron-methyl significantly and completely inhibited *N. europaea*, *N. oligotropha*

and *N. communis*, only at the highest concentration (262.2 μM and 131.1 μM , respectively), while *N. multiformis* was only temporarily suppressed by the highest concentration (262.2 μM). However, metsulfuron-methyl significantly inhibited *N. briensis* and *N. ureae* at concentrations $\geq 2.6 \mu\text{M}$. Clethodim completely inhibited *N. europaea* at concentrations $\geq 83 \mu\text{M}$, but it had no or only transient effects (*N. multiformis*) on the other AOB.

2.3.2.3. Fungicides

Pyraclostrobin completely inhibited *N. europaea* at all concentrations ($\geq 0.77 \mu\text{M}$), *N. briensis*, *N. ureae* at concentrations $\geq 7.7 \mu\text{M}$, and *N. oligotropha* at concentrations $\geq 77 \mu\text{M}$ (Supplementary Figure S2, Annex I). Etridiazole inhibited all AOB strains at all concentrations. Hymexazol did not affect *N. communis* and *N. multiformis*, temporarily inhibited *N. oligotropha*, and completely inhibited *N. europaea*, *N. briensis* and *N. ureae* at the highest concentration (1009 μM). The transformation product of iprodione, 3,5-DCA completely inhibited *N. europaea*, *N. multiformis*, *N. communis* and *N. oligotropha* at concentrations $\geq 31 \mu\text{M}$, and *N. briensis* and *N. ureae* at concentrations $\geq 3.1 \mu\text{M}$.

2.3.2.4. EC₅₀ values for AOB

The calculated EC₅₀ values showed that *N. briensis* and *N. ureae* were consistently the most sensitive AOB strains (Figure 2.2). The sensitivity to glyphosate increased as follows: *N. europaea*, *N. multiformis* > *N. oligotropha* > *N. communis*, *N. briensis*, *N. ureae*. AMPA affected only *N. briensis* and *N. ureae*. Sensitivity to metsulfuron-methyl increased as follows: *N. europaea*, *N. oligotropha*, *N. communis* > *N. multiformis*, *N. ureae* and *N. briensis*. Pyraclostrobin was more toxic to *N. europaea*, *N. briensis*, *N. ureae* and *N. communis* (EC₅₀ $\leq 7.98 \mu\text{M}$) compared to *N. multiformis* and *N. oligotropha* (EC₅₀ ≥ 15.94). *N. briensis* (EC₅₀ = $1.08 \pm 0.4 \mu\text{M}$) and *N. ureae* (EC₅₀ = $1.64 \pm 0.29 \mu\text{M}$) were the most sensitive AOB strains to 3,5-DCA. Metsulfuron-methyl, 3,5-DCA, pyraclostrobin and etridiazole were the most inhibitory compounds to AOB. Amongst them only etridiazole (all tested strains) and pyraclostrobin (four of six strains) showed EC₅₀ values which were lower than the recommended dose rate (Figure 2.2).

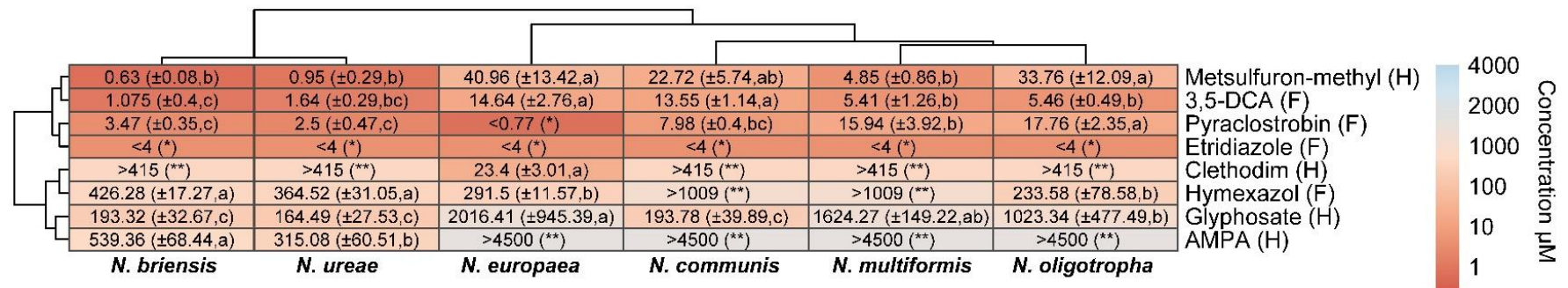


Figure 2.2. Mean EC₅₀ values (µM) of pesticide inhibitory concentrations on AOB strains *N. briensis*, *N. ureae*, *N. europaea*, *N. communis*, *N. multiformis* and *N. oligotropha*. Standard errors of mean values (denoted by ±) are given in parentheses. Pesticides with no effect on all tested strains were excluded from the analysis. Lower case letters indicate significant differences ($p < 0.05$) in the EC₅₀ values between the tested strains for each pesticide. Asterisks denote the minimum (*) or the maximum tested concentration (**) in cases where no EC₅₀ could be descent from the statistical analysis. The capital letters I, F, and H in parentheses next to the tested pesticides denote the categories insecticides, fungicides, and herbicides, respectively. Dendrograms based on the Euclidean distances and the complete linkage clustering method using log-transformed mean EC₅₀ values are presented for clustering strains and pesticides according to their sensitivity and toxicity, respectively. Values are color-coded by orders of the tested concentrations.

2.3.3 Impact of pesticides on the activity of NOB strains

2.3.3.1. Insecticides

Chlorpyrifos did not affect any of the NOB strains. However, its transformation product TCP completely inhibited *Ca. Nitrobacter laanbroekii* and NHB1, *N. defluvii* at concentrations $\geq 75\mu\text{M}$ and $164\mu\text{M}$, respectively (Supplementary Figure S3, Annex I).

2.3.3.2. Herbicides

Glyphosate significantly inhibited *N. winogradskyi* and *N. defluvii* only at the highest concentration ($2957\mu\text{M}$), unlike AMPA that temporarily inhibited all NOB strains at $4500\mu\text{M}$ (Supplementary Figure S3, Annex I). Metsulfuron-methyl temporarily reduced nitrite consumption by *Ca. Nitrobacter laanbroekii* NHB1 at concentrations $\geq 13.11\mu\text{M}$, while clethodim completely inhibited the same strain at the highest concentration ($415\mu\text{M}$).

2.3.3.3. Fungicides

Pyraclostrobin had a temporary inhibitory effect on the activity of *Ca. Nitrobacter laanbroekii* NHB1 at concentrations $\geq 7.7\mu\text{M}$ (Supplementary Figure S3, Annex I). Etridiazole inhibited *Ca. Nitrobacter laanbroekii* NHB1 and *N. defluvii* at concentrations $\geq 404\mu\text{M}$, and *N. winogradskyi* only at the highest concentration ($1010\mu\text{M}$). Hymexazol transiently inhibited only *Ca. Nitrobacter laanbroekii* NHB1 at the highest concentration ($1009\mu\text{M}$). Finally, 3,5-DCA, completely inhibited *Ca. Nitrobacter laanbroekii* NHB1 and *N. defluvii* only at the highest concentration of $310\mu\text{M}$.

2.3.3.3.4. EC₅₀ values for NOB

Based on the calculated EC₅₀ values, *Ca. Nitrobacter laanbroekii* NHB1 was the most sensitive NOB strain, compared to *N. winogradskyi* and *N. defluvii*, which showed near equivalent sensitivity (Figure 2.3). Pyraclostrobin was the most inhibitory compound for *Ca. Nitrobacter laanbroekii* NHB1 and 3,5-DCA for *N. winogradskyi* and *N. defluvii*. None of the pesticides had EC₅₀ values for NOB strains that were lower than their recommended dose rate.

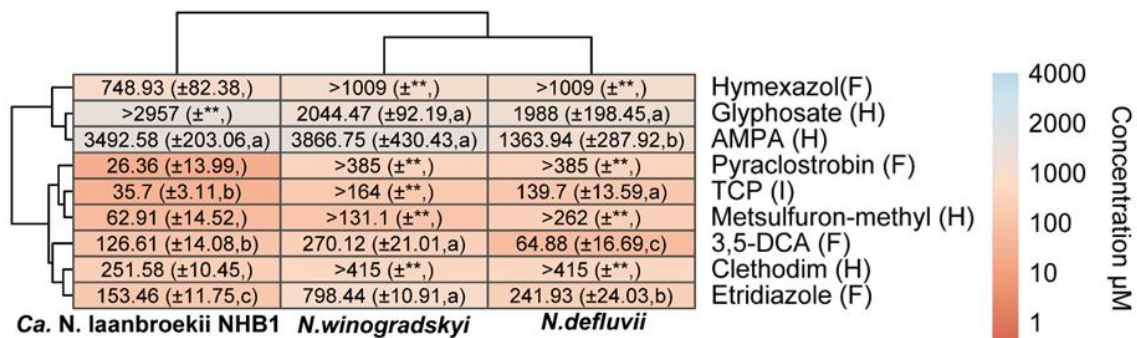


Figure 2.3. Mean EC_{50} values (μM) of pesticide inhibitory concentrations on NOB strains *Nitrobacter* sp. NHB1, *N. winogradskyi* and *N. defluvii*. Standard errors of the mean values (denoted by \pm) are given in parentheses. Pesticides with no effect on all tested strains were excluded from the analysis. Lower case letters indicate significant differences ($p < 0.05$) in the EC_{50} values between the tested strains for each pesticide. Asterisks denote the minimum (*) or the maximum (**) tested concentration in cases where no EC_{50} could be descent from the statistical analysis. The capital letters I, F, and H in parentheses next to the tested pesticides denote the categories insecticides, fungicides, and herbicides, respectively. Dendrograms based on the Euclidean distances and the complete linkage clustering method using log-transformed mean EC_{50} values are presented for clustering strains and pesticides according to their sensitivity and toxicity respectively. Values are color-coded by orders of the tested concentrations.

2.3.4 Sensitivity of AOA, AOB and NOB to each pesticide category

Based on the EC_{50} values, toxicity patterns for each pesticide category (insecticides, fungicides, herbicides) were identified (Figure 4a). Insecticides seem to be more toxic to AOA compared to AOB ($p = 0.006$) but more tests are needed due to the low number of the tested compounds. Herbicides showed no significant differences in their toxicity to the different groups. Fungicides were significantly more toxic to AOA ($p = 0.027$) and AOB ($p = 0.032$) compared to NOB. Also, variation in sensitivity patterns to each pesticide category were observed between each nitrifiers group (Figure 4b). AOA showed higher sensitivity to insecticides ($p = 0.012$) and fungicides ($p = 0.034$) than herbicides. AOB were more sensitive to fungicides ($p = 0.043$). NOB were more sensitive to insecticides compared to herbicides ($p = 0.012$).

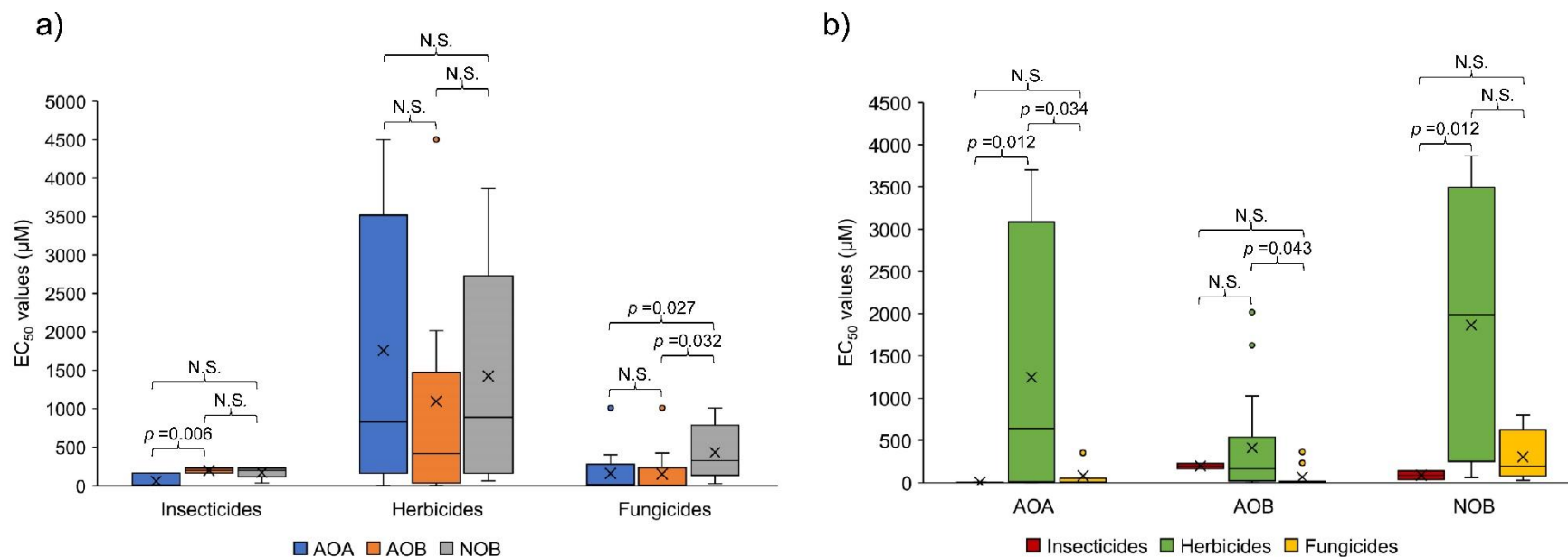


Figure 2.4. Comparison of (a) the toxicity of different pesticide categories (insecticides, fungicides, herbicides) to AOA, AOB, and NOB, and (b) sensitivity of AOA, AOB, and NOB to the different pesticide groups (insecticides, fungicides, herbicides) based on the mean EC₅₀ values calculated for each pesticide category. N.S. indicates no significant difference ($p > 0.05$). In cases where no EC₅₀ could be determined in statistical analyses the lowest or the highest tested concentrations were used in the calculation.

2.4 Discussion

The impact of pesticides on soil microbial communities has attracted considerable attention in recent years from scientists and regulatory bodies due to the essential role of soil microorganisms in ecosystem functioning. However, we still lack fundamental knowledge, relevant tools, testing procedures and bioindicators to assess pesticide impacts. We previously proposed the use of AOM as bioindicators for assessing the toxicity of pesticides on the soil microbiota in the frame of a tiered risk assessment scheme (Karpouzias et al., 2022). Here we present the development and validation of *in vitro* bioassays with soil AOM and functionally related NOB as a standard tier I type assay, equivalent to single species tests used in aquatic and terrestrial ecotoxicology.

We first characterized the sensitivity of 12 AOA, AOB and NOB strains from dominant lineages found in soil and identified representatives that consistently exhibited the highest sensitivity with a clear ecotoxicological response to pesticides from all major categories. Acidophilic *N. sinensis* was the most sensitive AOA strain with neutrophilic AOA *Ca. N. franklandianus* and *N. viennensis* being equally more tolerant. The different sensitivity of the acidophilic and neutrophilic strains to pesticides, has been reported previously for iprodione and its transformation product 3,5-DCA (Vasileiadis et al., 2018). Similar sensitivity has recently been identified in response to nitrification inhibitors (Kaur-Bhambra et al., 2022; Papadopoulou et al., 2020; Zhao et al., 2020). The variation in the responses of the tested AOA strains to pesticide exposure are likely associated with variation in their biochemical and structural characteristics. AOA membranes contain a variety of glycerol dialkyl glycerol tetraether (GDGT) lipids which vary in composition between different strains (Elling et al., 2016), and which may have consequences for differences in permeability and sensitivity to abiotic stressors (Lehtovirta-Morley et al., 2016). In addition, the higher tolerance of *Ca. N. franklandianus* and *N. viennensis* to pesticides and other abiotic stressors may be associated with its capability to form aggregates and biofilms and production of extracellular polymeric material, reducing access of xenobiotic compounds to the cell surface (Gao et al., 2008; Kerou et al., 2016; Lehtovirta-Morley et al., 2016).

The response of the six AOB strains, representing both *Nitrosospira* and *Nitrosomonas* genera that typically dominate soil AOB communities, was tested for 10 different pesticides. *N. briensis* and *N. ureae* were consistently the most sensitive strains followed by *N. europaea*, with *N. communis*, *N. multiformis* and *N. oligotropha* the most tolerant. Previous studies have tested the inhibitory activity of ethoxyquin, a preservative potent nitrification inhibitor, on only two AOB strains, *N. europaea* and *N. multiformis*, which showed equivalent sensitivity (Papadopoulou et al., 2020). In another recent study, a clear strain-specific sensitivity pattern among tested AOB strains (*N. briensis*, *N. europaea*, *N. multiformis*, *N. tenuis*) was not observed when

challenged with a range of biological nitrification inhibitors (Kaur-Bhambra et al., 2022). Amongst the two most sensitive AOB strains identified in our study, *N. briensis* seems the most appropriate candidate for use in ecotoxicology assessments as *Nitrosospira* is the dominant AOB genus in soil ecosystems (Kowalchuk & Stephen, 2001), while members of this species appear as dominant in metataxonomic analyses of AOB diversity in agricultural soils (Assémien et al., 2017; Papadopoulou et al., 2022). Overall, of all pesticides tested only etridiazole and pyraclostrobin showed toxicity to AOB strains at concentrations below the EC₅₀ values suggesting a high risk for soil AOB by their regular agronomic use.

We expanded our single species *in vitro* assays to NOB which are dependent on AOB for substrate provision and to complete the process of nitrification when ammonia is oxidized by canonical AOM. Reduction in the activity of NOB by pesticides might increase nitrogen loss from agricultural ecosystems and aggravate the problem of atmospheric pollution (Prosser et al., 2020). Amongst the NOB strains tested, the acidophilic *Ca. Nitrobacter laanbroekii* NHB1 strain showed the greatest sensitivity to most of the studied pesticides followed by *N. defluvii*, and *N. winogradskyi* the most tolerant. Few data are available regarding the *in vitro* inhibition of NOB by agrochemicals except by nitrification inhibitors (Matsuba et al., 2003; Papadopoulou et al., 2020). It has been demonstrated that *Ca. Nitrobacter laanbroekii* NHB1 was significantly more tolerant to nitrapyrin, DCD and DMPP compared to AOA and AOB strains (Papadopoulou et al., 2020). This is in accordance with our findings where NOB were less sensitive to fungicides and to insecticides compared to AOA and AOB. This might reflect their versatile metabolism that enable some *Nitrobacter* strains to degrade complex organic compounds (John & Okpokwasili, 2012). However, our measurements did not show an appreciable pesticide degradation by NOB, except for *N. winogradskyi* where degradation of the fungicides pyraclostrobin, etridiazole and hymexazol was evident (Supplemental Material, Annex I). Unlike AOA and AOB, the tested pesticides showed inhibitory activity to NOB at concentration levels that are much higher than their recommended dose rates suggesting low potential risk for this group of nitrifiers in agricultural soils.

Toxicity patterns among pesticide groups or specific pesticides warranted further investigation. With the notable exception of hymexazol, fungicides were the most toxic pesticide group to AOA and AOB. Amongst them etridiazole was toxic to all tested AOM strains, followed by pyraclostrobin. The high toxicity of etridiazole, a thiodiazole molecule, on AOM is not surprising considering that diazoles and triazoles are long known as highly potent and specific nitrification inhibitors in soil and in activated sludge (Beeckman et al., 2023; McCarty & Bremner, 1989; Taggert et al., 2021). However, their inhibitory activity varies depending on the number and the nature of substituents in the azole ring (Jog et al., 2022; Taggert et al., 2021). This might explain the limited toxicity of hymexazol, another azole fungicide even though it belongs to the class isoxazoles that are less potent inhibitors of nitrification

compared to diazoles and triazoles (Li et al., 2020). Pyraclostrobin, a quinone inhibitor (QoI)-type fungicide, acts on ubiquinone, an electron-transfer associated coenzyme (Karadimos et al., 2005) that is also involved in ammonia oxidation and nitrification (Chain et al., 2003; Whittaker et al., 2000).

Herbicides are characterized by a wide range of biochemical modes of action that are also often relevant for soil microorganisms (Thiour-Mauprivez et al., 2019). Glyphosate and clethodim, which act on enzymes also found in soil microorganisms like 5-enolpyruvylshikimate-3-phosphate synthase (Wang et al., 2014) and acetyl-CoA carboxylase (Secor & Cséke, 1988) respectively, showed no toxicity on AOM or inhibited only the most sensitive AOM strains at concentrations higher than the recommended agronomic dose rate. In contrast, metsulfuron-methyl was inhibitory on *N. ureae*, *N. briensis*, *Ca. N. sinensis* and *N. multiformis* at concentrations lower or at the range of the recommended dose rate (*N. multiformis*). Metsulfuron-methyl is a sulfonylurea herbicide disrupting the synthesis of branched chain amino acids in plants by specifically acting on acetolactate synthase (Lonhienne et al., 2018). The gene *ilv* encoding for acetolactate synthase is also present in soil bacteria, including the genomes of AOB strains belonging to *Nitrosomonas* and *Nitrosospira* (Kanissery et al., 2019; Nelemans et al., 2017), with two of its isozymes (Type II and III) being sensitive to sulfonylureas (Nelson & Duxbury, 2008). Previous *in vitro* assays with several sulfonylurea herbicides tested on a range of pathogenic (Kreisberg et al., 2013) and soil bacteria (Boldt & Jacobsen, 1998) identified metsulfuron-methyl as the most inhibitory compound. Variation in the sensitivity of the different AOB and AOA strains to metsulfuron-methyl might be associated with its affinity to the *ilv* gene of these strains, a hypothesis that will be further pursued in following studies.

The insecticide chlorpyrifos had a contrasting effect on AOA and AOB. It was highly inhibitory to all tested AOA strains at concentrations far below the recommended dose but was not inhibitory to AOB. The higher toxicity of chlorpyrifos on AOA over AOB could not be assigned to its site of action (acetylcholinesterase inhibitor), which is not relevant for microorganisms. A similar selective inhibition of AOA over AOB by chlorpyrifos was also reported in a previous soil study (Karas et al., 2018). A potential reason for this selective toxicity of the particularly lipophilic chlorpyrifos on AOA might be associated with the permeability of AOA membranes, which have been shown to be selectively permeable to lipophilic compounds like chlorpyrifos (Łapińska et al., 2023).

In addition to individual pesticides, we identified transformation products that were equally or more toxic to AOM compared to their parent compound, reinforcing our earlier proposal that the toxicity of transformation products should be assessed in addition to parent compounds (Karpouzias et al., 2022). For example, the transformation product 3,5-DCA was inhibitory to most AOB strains and *Ca. N. sinensis* at concentrations predicted to be present in soil after application of the recommended dose rate of its parent compound iprodione. Our data expand upon our earlier

observations that 3,5-DCA is the main driver of soil nitrification inhibition upon application of iprodione (Vasileiadis et al., 2018). In contrast, TCP and AMPA are transformation products of chlorpyrifos and glyphosate, respectively, and showed similar toxicity patterns to their parent compounds.

2.5 Conclusion

We describe the development and standardization of novel single species *in vitro* assays as a new tool for the assessment of the toxicity of pesticides on soil microorganisms, using nitrifying microorganisms as relevant microbial indicators. The testing of 10 pesticides against three AOA, six AOB and three NOB strains, representing all dominant lineages of soil nitrifiers, allowed us to identify the most sensitive strain per group that could be used as bioindicator species for toxicity assays: (i) the AOA *Ca. N. sinensis*; (ii) the AOB *N. ureae* and *N. briensis* (with the latter being a better representative as a dominant member of AOB communities in soil ecosystems), and (iii) *Ca. Nitrobacter laanbroekii* from NOB. We further identified (i) pesticide category-specific patterns with fungicides being the most toxic to AOM, and (ii) NOB being considerably more tolerant than AOM. These results suggest that certain fungicides, and particularly etridiazole and pyraclostrobin, could be highly inhibitory to AOM at agronomical relevant concentrations. In summary, single species *in vitro* assays could be used as a standard tier I type ecotoxicity assays for providing a conservative assessment of the potential toxicity of pesticides on soil microbiota. Current efforts are developing these assays into a fast-track high-throughput format that would facilitate their implementation into pesticide toxicity testing practice.

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Chapter 3

Assessment of pesticide toxicity on sensitive soil nitrifiers: validation of single-species *in vitro* bioassay

Abstract

Single-species tests are a fundamental component of both aquatic and terrestrial ecotoxicology, widely used to evaluate the environmental impact of pesticides. Despite the critical ecological functions of soil microorganisms, equivalent testing methods for assessing pesticides ecotoxicity are notably lacking in current frameworks. This study expands upon our previous research by evaluating the toxicity of 14 pesticides - encompassing insecticides, herbicides, and fungicides - on three most sensitive nitrifying strains: *Nitrosotalea sinensis* (AOA), *Nitrosospira briensis* (AOB), and *Ca. Nitrobacter laanbroekii* NHB1 (NOB). Using the proposed single-species *in vitro* assay, we determined the impact of pesticides on the activity of the nitrifying strains and calculated the EC₅₀ values of each pesticide for the strains, aiming to validate their effectiveness as bioindicators in a Tier I ecotoxicity testing framework. Fungicides were most toxic to *N. sinensis*, causing significant inhibition near field doses. Tembotrione affected both *N. sinensis* and *Ca. N. laanbroekii* NHB1 at higher concentrations, while spiromesifen was the only insecticide to inhibit *N. sinensis*. Overall, *N. sinensis* was the most sensitive strain, followed by *N. briensis* and *Ca. N. laanbroekii* NHB1, indicating these strains are promising bioindicators for pesticide toxicity in soil. The differential sensitivities of these strains to various pesticide classes highlight the need for a comprehensive approach that captures a broad spectrum of microbial responses.

Keywords: ammonia-oxidizing bacteria, ammonia-oxidizing archaea, nitrite-oxidizing bacteria, *Nitrosotalea sinensis*, *Nitrosospira briensis*, *Ca. Nitrobacter laanbroekii* NHB1, single species *in vitro* tests, ecotoxicity, pesticide

3.1 Introduction

Nitrification is a key process in the global nitrogen cycle and contributes significantly to soil nitrogen with estimates of around 330 Tg (330×10^{12} g) of nitrogen per year (Kuypers et al., 2018). This process involves the conversion of ammonia (NH₃) to nitrate (NO₃⁻), increasing nitrogen availability for plants and improving soil fertility (Schleper & Nicol, 2010). The first and rate-limiting step of nitrification is the oxidation of ammonia to hydroxylamine (NH₂OH), which is subsequently converted to nitrite (NO₂⁻) via nitric oxide (NO) by ammonia oxidizing microorganisms (AOM) and finally to nitrate by nitrite-oxidizing bacteria (NOB) such as *Nitrospira* and *Nitrobacter* (Beeckman et al., 2018; Prosser et al., 2020). Ammonia oxidation is carried out by several microbial groups that have been identified in the past two decades: (i) ammonia-oxidizing bacteria (AOB), primarily from the β -proteobacteria, including *Nitrosomonas* and *Nitrosospira* (Kowalchuk & Stephen, 2001; Prosser et al., 2020), (ii) ammonia-oxidizing archaea (AOA) from the Thaumarchaeota phylum, with *Nitrososphaerales* and *Nitrosotaleales* dominating neutral-alkaline and acidic soils,

respectively (Könneke et al., 2005; Prosser et al., 2020), and (iii) comammox bacteria, which are capable of performing the entire nitrification process within a single cell (Daims et al., 2015; van Kessel et al., 2015). The organisms and genes involved in nitrification have been extensively studied, underscoring the economic and environmental importance of this process (You et al., 2009).

Disruptions in the nitrification process, such as by the application of pesticides, can alter the nitrogen balance in soil. While pesticides are crucial in modern agriculture for minimizing crop losses and boosting yields to meet the demands of a growing global population (Tudi et al., 2021), only about 5% of applied pesticides reach their intended targets, with the remainder accumulating in the soil and potentially harming soil microorganisms (Karpouzas et al., 2022). Numerous studies have reported the effects of pesticides on soil microbial communities. Commonly applied herbicides have been found to inhibit microbial growth and activity and alter microbial diversity (Cycoń & Piotrowska-Seget, 2015; Lu et al., 2018; Tang et al., 2023). Several pesticides also impact key nitrogen-cycling microbes, including AOA, AOB, and NOB (Karas et al., 2015; Papadopoulou et al., 2016; Sim et al., 2022; Vasileiadis et al., 2018).

Despite being recognized as protection goals by the European Food Safety Authority (EFSA Panel on Plant Protection Products and their Residues (PPR), 2010), there is an absence of a comprehensive environmental risk assessment framework specifically addressing the impact of pesticides on soil microorganisms, equivalent to the models used in aquatic ecotoxicology. Karpouzas et al. (2022) proposed a tiered system to assess pesticide impacts on soil microbial communities, focusing on key groups like ammonia-oxidizing microorganisms (AOM) and arbuscular mycorrhizal fungi (AMF). The system progresses from *in vitro* screening (Tier I) to soil microcosm or pot studies (Tier II), and finally, to field-scale assessments (Tier III), with advanced molecular and biochemical tools for monitoring bioindicator activity, abundance, and diversity. This approach ensures that potential pesticide toxicity is evaluated at increasing levels of complexity, from lab settings to real agricultural conditions, while tracking pesticide dissipation and transformation (Karpouzas et al., 2022).

Several characteristics make AOM ideal candidates for bioindicators of pesticide toxicity on soil microbiota: (i) they play a pivotal ecological role in soil environments (Prosser & Nicol, 2008), (ii) they are highly sensitive to abiotic disturbances, including exposure to xenobiotics (Wessén & Hallin, 2011), and (iii) extensive knowledge exists about their ecology, physiology, and life cycle, along with well-established tools for measuring their activity and abundance both *in vitro* (Lehtovirta-Morley et al., 2013) and in soil environments (Junier et al., 2010).

In our previous work (see Chapter 2), we aimed to develop single-species *in vitro* ecotoxicological assays as a Tier I ecotoxicity testing method to conservatively assess the potential toxicity of pesticides on soil microbiota, using nitrifying microorganisms as key indicators. We evaluated the toxicity of 10 pesticides on a range of soil nitrifiers, including three AOA, six AOB, and three NOB strains grown in liquid

cultures. The results revealed that at least one non-target ammonia-oxidizing microorganism was affected by each pesticide, with varying sensitivities among AOA and AOB strains, likely due to their structural and biochemical differences (Schleper & Nicol, 2010; Shen et al., 2013). This approach helped identify the most sensitive strains in each group: *Nitrosotalea sinensis* (AOA), *Nitrosomonas ureae* and *Nitrospira briensis* (AOB), with the latter being a more representative AOB strain in soil ecosystems, and *Ca. Nitrobacter laanbroekii* NHB1 (NOB). Additionally, we found that fungicides were the most toxic to AOM, while NOB demonstrated significantly greater tolerance.

In this study, we aimed to investigate the effectiveness of our proposed single-species test by assessing the toxicity of 14 additional pesticides on the most sensitive nitrifying strains, which have been identified as reliable bioindicators of pesticide toxicity. We hypothesized that single-species *in vitro* ecotoxicological tests using key nitrifying microorganisms can serve as an effective Tier I ecotoxicity test method to conservatively assess the potential impact of pesticides on soil microbiota. We expect that AOM, due to their essential role in nitrification and previously demonstrated sensitivity, will show greater sensitivity to pesticide exposure compared to nitrite oxidizing bacteria (NOB), with fungicides being the most toxic. The pesticide sensitivity observed in the nitrifying strains is expected to reflect the broader ecological impact of different pesticide categories, confirming their suitability as bioindicators.

3.2 Material and Methods

3.2.1 Microbial strains

The three most sensitive strains based on our previous work were used in this study: *Nitrosotalea sinensis* Nd2 (AOA), *Nitrospira briensis* C-128 (AOB), and *Candidatus Nitrobacter laanbroekii* NHB1 (NOB). All strains were grown aerobically in the dark without shaking. AOA, *N. sinensis* Nd2, was incubated at 35°C in a medium supplemented with 0.5 mM NH₄⁺ (NH₄Cl) and MES-buffer (10mM) and pH 5.2 (Lehtovirta-Morley et al., 2011). AOB, *N. briensis* C-128, was grown at 28°C in Skinner and Walker's medium (Skinner & Walker, 1961) containing 1 mM NH₄⁺ [(NH₄)₂SO₄] and phenol red (0.5 mg L⁻¹) as a pH indicator. NOB, *Ca. Nitrobacter laanbroekii* NHB1 was grown at 28°C in freshwater medium at pH 6 and supplemented with 0.5 mM NO₂⁻ (Hink et al., 2024).

3.2.2 Pesticides

The selection of pesticides, presented in Table 3.1, was guided by two key criteria: (i) representing commonly used pesticides in European agriculture and (ii) targeting different mechanisms of action, particularly those that could interact with

AOM and NOB metabolic pathways. A total of fourteen pesticides were tested against the three isolates: acetamiprid, spiromesifen, acrinathrin, pirimicarb (insecticides), tembotrion, clopyralid, metribuzin, terbuthylazine (herbicides), as well as fludioxonil, flutolanil, iprodione, chlorothalonil, tebuconazole and epoxiconazole (fungicides).

The concentration levels for each compound were determined by predicting the concentration that would be present in soil solution after applying the maximum recommended dose. This prediction considered factors such as the crop species receiving the treatment, the application method (whether foliar or soil drench), and the frequency of application throughout the season. A detailed explanation of the calculation process is available in the Supplementary Material for Chapter 2 (Annex I). After determining the expected pesticide concentrations post-application, the compounds were tested at four levels: 0.1x, 1x, 10x, and 100x the recommended dose (Table 3.2). It is important to note that, for certain pesticides, the maximum concentration (100x) could not be tested due to solubility limitations. In such cases, the maximum working concentration was established following a solubility test.

Analytical standards of all pesticides ($\geq 92\%$) were purchased from HPC Standards (Germany) and were used for both *in vitro* assays and analytical methods.

3.2.3 Single species *in vitro* assays

The toxicity of the pesticides was assessed in liquid cultures of the selected strains at four different concentration levels for determination of the inhibition threshold values (EC_{50}) for each strain and pesticide. For this purpose, cultures were established in triplicate for each strain, pesticide, and concentration combination. These cultures were grown in 100-mL culture bottles containing 50 mL of growth medium and inoculated with a 1% or 2% (v/v) of exponentially growing cultures of AOB or AOA/NOB respectively.

All pesticides, except for pirimicarb, were added to the cultures as filter-sterilized DMSO solutions (50 μ L), resulting in a final DMSO concentration of 0.1% (v/v), which was confirmed to have no inhibitory effect on the growth of any of the isolates tested. Metribuzine and pirimicarb were dissolved in sterile double-distilled water (ddH₂O) and 50 μ L (0.1% v/v) was added to the test cultures. Pesticides were added to the cultures at the start of the exponential growth phase. Control cultures, without pesticide, were supplemented with 50 μ L of either DMSO or ddH₂O, depending on the solvent used for the pesticide.

The impact of pesticide concentration on growth and the calculation of inhibition threshold values were determined through regular measurements of NO₂⁻ concentrations in the liquid cultures.

3.2.4 Nitrite measurements

The NO_2^- concentration in liquid cultures was determined colorimetrically in a 96-well plate assay using Griess diazotizing and coupling solutions as described by Hink et al. (2018). For each measurement, duplicate standards ranging between 0 and 100 μM NaNO_2 were used. Absorbance was recorded at 540 nm using a Multiskan GO microplate spectrophotometer (Thermo Scientific).

3.2.5 Calculation of toxicity endpoint values

Determination of half maximal effective concentration (EC_{50}) values were calculated using the R package *drc: Analysis of Dose-Response Curves v3.0-1* package (Ritz & Streibig, 2015), based on a dose-response model with normalized data whereby nitrite concentration values were divided by the mean value of the control. A description of the tested models can be found in Ritz et al. (Ritz et al., 2015). For the selection of the best fitting model, an empirical modeling approach was used based on goodness of fit indices, followed by the choice of the four-parameter log logistic model as the best compromise among tested models for comparing toxicity endpoint values.

3.2.6 Data analysis

Nitrite data were analysed by one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test ($p < 0.05$). To test the hypothesis that the strains had different sensitivities to the different pesticide categories, statistical differences of the EC_{50} values were analysed using non-parametric Wilcoxon signed rank test ($p < 0.05$).

Table 3.1. Chemical classification, mode of action and potential inhibition characteristics of pesticides and selected transformation products.

| Pesticide type | Pesticide | Chemical classification | Mode of action | Reference | Potential effect on nitrifiers | References |
|----------------|--------------|--|---|-----------------------------|--|---|
| Insecticides | Acetamiprid | Neonicotinoid | Blocking the nerve system of insects- nicotinic agonist that reacts with nicotinic acetylcholine receptors (nACh-R) | (Kimura-Kuroda et al. 2012) | No potential effect | - |
| | Acrinathrin | Pyrethroid | Blocking of nerve system of insects- Na/K channel | (Dekeyser 2005) | No potential effect | - |
| | Spiromesifen | Spirocyclic tetronic/ tetramic acid derivative | Inhibition of the enzyme acetyl coenzyme A carboxylase (ACCase), that interrupts lipid synthesis | (Abdel-Fatah et al. 2019) | Potential effect- Enzyme present on <i>Nitrosospira</i> (AOB) and <i>Nitrosotaleales</i> (AOA) | (Herbold et al. 2017; Rice et al. 2016) |
| | Pirimicarb | Aminopyrimidine | Blocking the nerve system of insects - inhibiting the acetylcholinesterase enzyme | (Zhou et al. 2010) | No potential effect | -- |

Table 3.1. Continued.

| | | | | | | |
|-------------------|---------------|----------------|--|-------------------------------------|--|---|
| Herbicides | Tembotrione | Triketone | Inhibition of the enzyme 4-hydroxyphenylpyruvate dioxygenase that participates in catabolism of Tyr and biosynthesis of carotene | (Pinke et al. 2014) | Potential effect- Enzyme present on <i>Nitrosospira</i> (AOB) and <i>Nitrosotaleales</i> (AOA) | (Herbold et al. 2017; Rice et al. 2016) |
| | Metribuzin | Triazinone | Inhibiting of photosynthesis by disrupting photosystem II | (Shah et al. 2009) | No potential effect | - |
| | Clopyralid | Picolinic acid | Blocking of plant hormone system | (Valenzuela-Valenzuela et al. 2001) | No potential effect | - |
| | Terbuthylazin | Chlorotriazine | Inhibiting of photosynthesis by disrupting photosystem II | (Mendes et al. 2017) | No potential effect | - |
| Fungicides | Iprodione | Dicarboximide | Hyperactivation of the high osmolarity glycerol (HOG) signaling pathway through group III hybrid histidine kinases, affecting cellular homeostasis | (Yoshimi et al. 2005) | Potential effect- Soil microorganisms posses histidine kinases | (Stewart 2010) |

Table 3.1. Continued.

| | | | | | | |
|-------------------|----------------|----------------|--|-----------------------|---|---|
| Fungicides | Flutolanil | Benzamide | Inhibition of succinate dehydrogenase (Complex II) which participates on the citric cycle and aerobic respiration | (Mol et al. 2019) | Potential effect- Enzyme present on <i>Nitrosospira</i> (AOB) and <i>Nitrosotaleales</i> (AOA) | (Herbold et al. 2017; Rice et al. 2016) |
| | Fludioxonil | Phenylpyrrole | Hyperactivation of the high osmolarity glycerol (HOG) signaling pathway through group III hybrid histidine kinases, affecting cellular homeostasis | (Yoshimi et al. 2005) | Potential effect- Soil microorganisms posses histidine kinases | (Stewart 2010) |
| | Chlorothalonil | Organochloride | Inhibition of enzymes responsible for cellular respiration, block of fungal spores germination | (Baćmaga et al. 2018) | Potential effect- Inhibition of lyceraldehyde-3-phosphate dehydrogenase (GAPDH) enzyme | (Herbold et al. 2017; Rice et al. 2016; Baćmaga, et al. 2018) |
| | Tebuconazole | Triazoles | Inhibition of ergosterol synthesis, block of spore germination and fungus growth | (Dong 2024) | Potential effect- Inhibition of nitrification | (McCarty et al. 1991; Taggert et al. 2021; Jog et al. 2022) |

Table 3.1. Continued.

| | | | | | | |
|-------------------|---------------|-----------|---|-------------------------|--|---|
| Fungicides | Epoxiconazole | Triazoles | Inhibition of mycelial growth, block of spore germination | (Bertelsen et al. 2001) | Potential effect- Inhibition of nitrification | (McCarty et al. 1991; Taggart et al. 2021; Jog et al. 2022) |
|-------------------|---------------|-----------|---|-------------------------|--|---|

Table 3.2. Concentration levels used in the *in vitro* bioassays for each pesticide tested. The concentration of pesticides expected to be found in the soil solution upon application of the recommended agronomic dose are indicated in bold.

| Group of pesticides | Pesticide | Concentration level (μM) |
|-------------------------|----------------|---------------------------------------|
| Insecticides (I) | Acetamiprid | 0.45, 4.5 , 45, 450 |
| | Spiromesifen | 2.1, 21 , 210, 675 |
| | Acrinathrin | 0.073, 0.73 , 7.3, 73 |
| | Pirimicarb | 0.88, 8.8 , 88, 880 |
| Herbicides (H) | Tembotrion | 0.34, 3.4 , 34, 340 |
| | Clopyralid | 0.29, 2.9 , 29, 290 |
| | Metribuzin | 1.26, 12.6 , 126, 1166 |
| | Terbuthylazin | 3.52, 35.2 , 352, 1308 |
| Fungicides (F) | Fludioxonil | 1.55, 15.5 , 155, 1128 |
| | Flutolanil | 0.52, 5.2 , 52, 433 |
| | Iprodione | 0.3, 1.5 , 15, 150 |
| | Chlorothalonil | 3.76, 37.6 , 376, 1880 |
| | Tebuconazole | 0.87, 8.7 , 87, 870 |
| | Epoxiconazole | 0.58, 5.8 , 58, 580 |

3.3 Results

3.3.1 Impact of pesticides on the activity of *Nitrosotalea sinensis*

Among the tested insecticides, acetamiprid partially inhibited the growth of *N. sinensis* at the highest concentration tested (450 μM), while acrinathrin and pirimicarb had no significant effect. However, spiromesifen inhibited the growth at concentrations $\geq 2.1 \mu\text{M}$. Regarding herbicides, tembotrione and clopyralid inhibited the strain's activity at concentrations of 340 μM and 290 μM , respectively. Additionally, metribuzin and terbuthylazine suppressed activity at concentrations $\geq 126 \mu\text{M}$ and $\geq 352 \mu\text{M}$, respectively. For fungicides, strong inhibition was observed with fludioxonil ($\geq 15.55 \mu\text{M}$), flutolanil ($\geq 52 \mu\text{M}$), and iprodione ($\geq 15 \mu\text{M}$), while chlorothalonil significantly inhibited growth at concentrations $\geq 37.6 \mu\text{M}$. In contrast, tebuconazole and epoxiconazole affected growth only at higher concentrations ($\geq 87 \mu\text{M}$ and $\geq 58 \mu\text{M}$, respectively) (Figure 3.1).

After calculation of EC_{50} values, the fungicides were more toxic for the growth of *N. sinensis* as the values were close to the recommended dose. Fludioxonil and chlorothalonil were the most toxic compounds with their EC_{50} (3.67 ± 0.3 and 23.27 ± 9.35 , respectively) being significantly lower than the recommended tested dose. The EC_{50} of herbicides were higher from the recommended doses (197.56 ± 14.23 , 108.85 ± 16.04 , 149.58 ± 2.19 and 80.88 ± 3.73 for tembotrione, clopyralid, metribuzin and terbuthylazine, respectively), while the EC_{50} of the only insecticide that affected nitrite production, spiromesifen, was lower than the recommended dose (11.99 ± 1.63) (Figure 3.2).

3.3.2. Impact of pesticides on the activity of *Nitrospira briensis*

The insecticides did not influence the activity of the strain *N. briensis*. The same pattern was also observed for the herbicides with the exception of metribuzin, which partially affected the activity of the strain at concentration 1166 μM , and terbuthylazine, which inhibited the activity of the strain at concentrations $\geq 352 \mu\text{M}$. For the fungicides, only chlorothalonil, tebuconazole and epoxiconazole affected the growth of *N. briensis* at concentrations ≥ 376 , ≥ 870 and $\geq 580 \text{ M}\mu$, respectively (Figure 3.3).

The calculated EC_{50} illustrated that chlorothalonil is the most toxic compound (51.01 ± 8.13), however this value was higher than the recommended dose. The EC_{50} for terbuthylazine (253.85 ± 18.83), tebuconazole (136.5 ± 38.74) and epoxiconazole (256.92 ± 10.26) were also higher than the recommended concentrations (Figure 3.4).

Insecticides

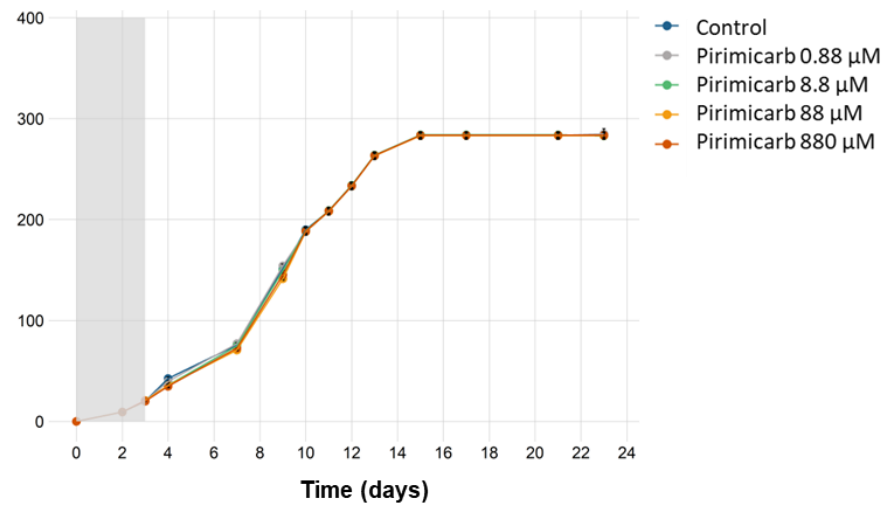
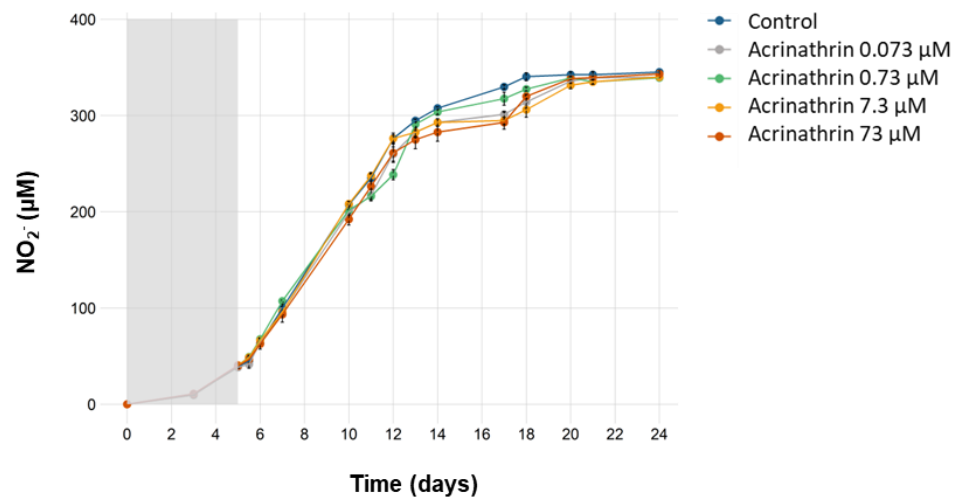
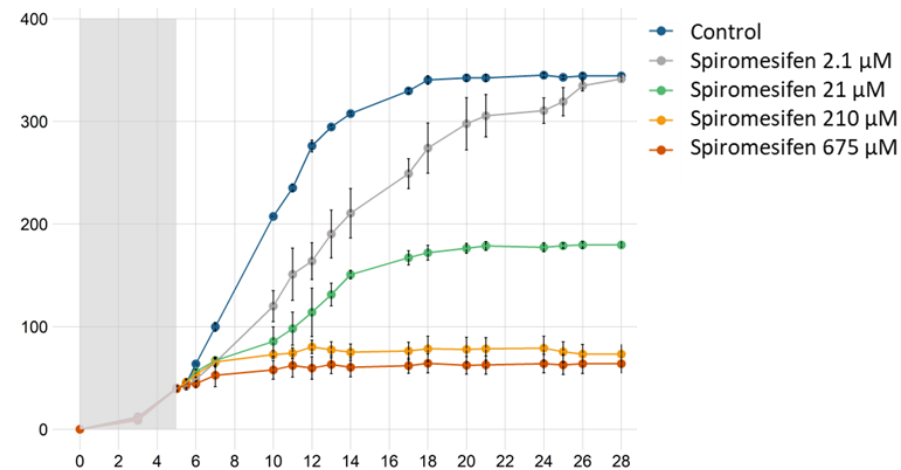
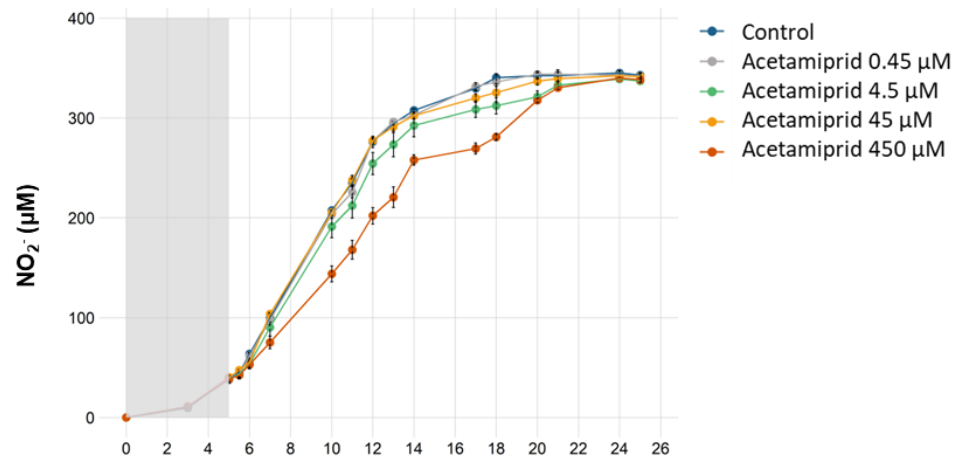


Figure 3.1. The effect of different concentrations of pesticides on nitrite production by the AOA strain *Nitrosotalea sinensis*. Error bars represent the standard error of the mean from triplicate cultures. Grey color indicates the period before pesticide application

Herbicides

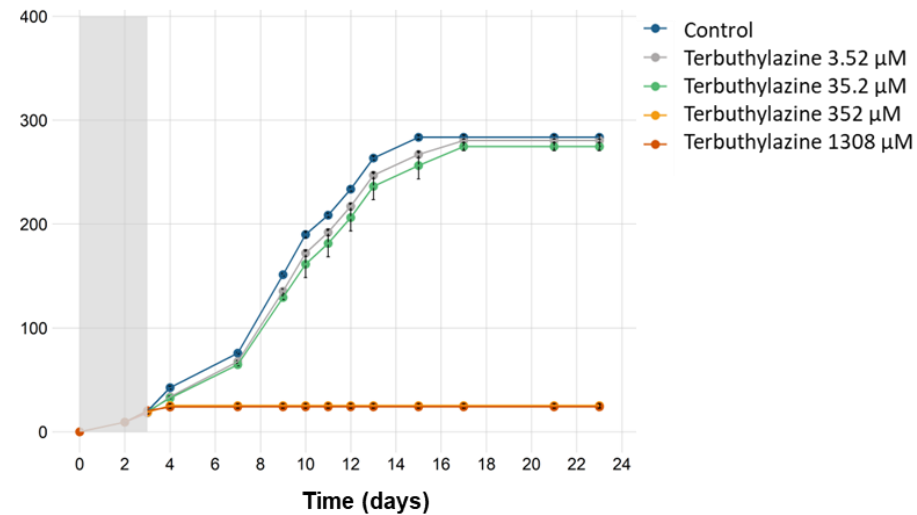
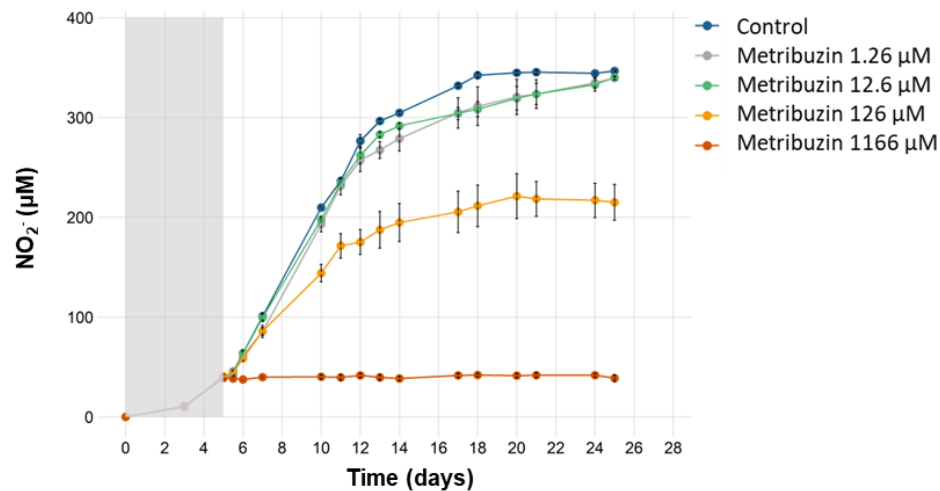
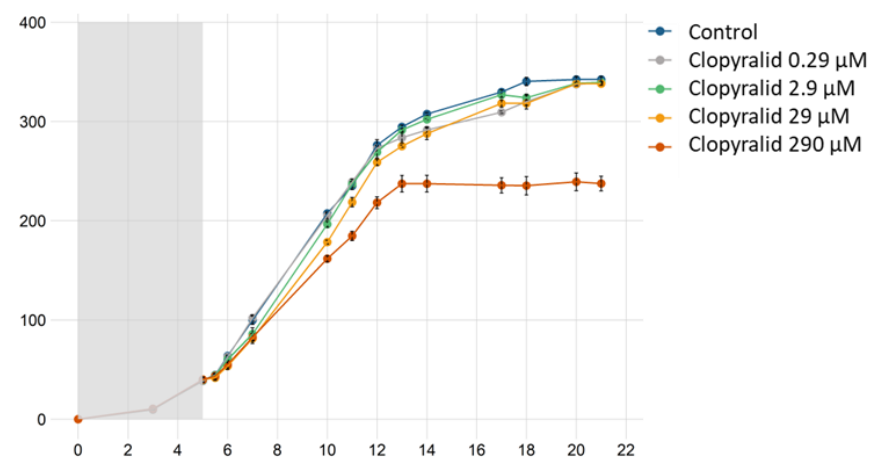
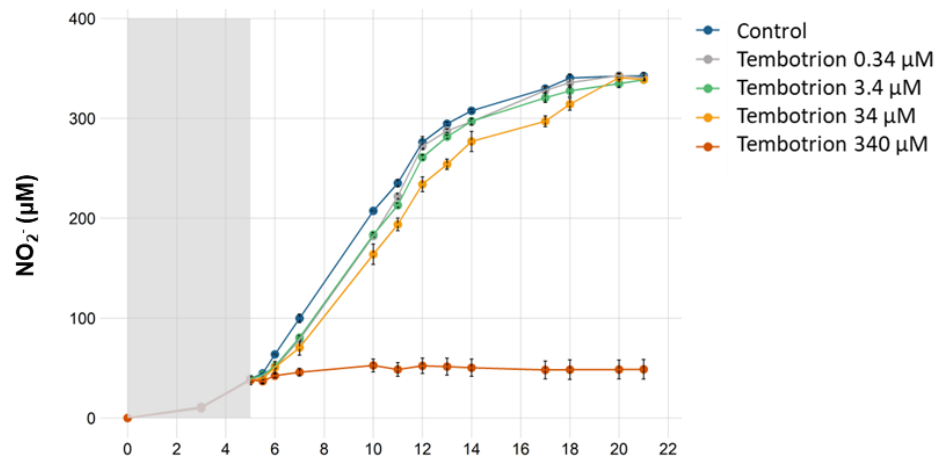


Figure 3.1. The effect of different concentrations of pesticides on nitrite production by the AOA strain *Nitrosotalea sinensis*. Error bars represent the standard error of the mean from triplicate cultures. Grey color indicates the period before pesticide application

Fungicides

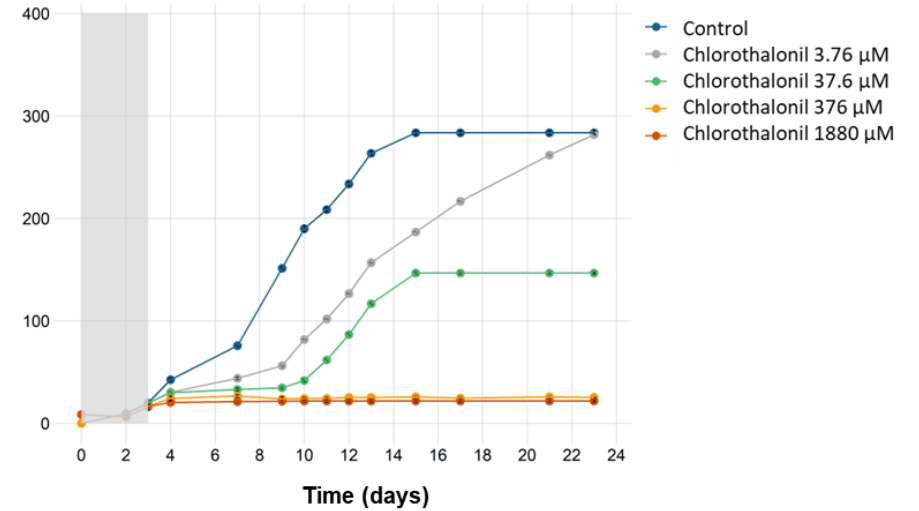
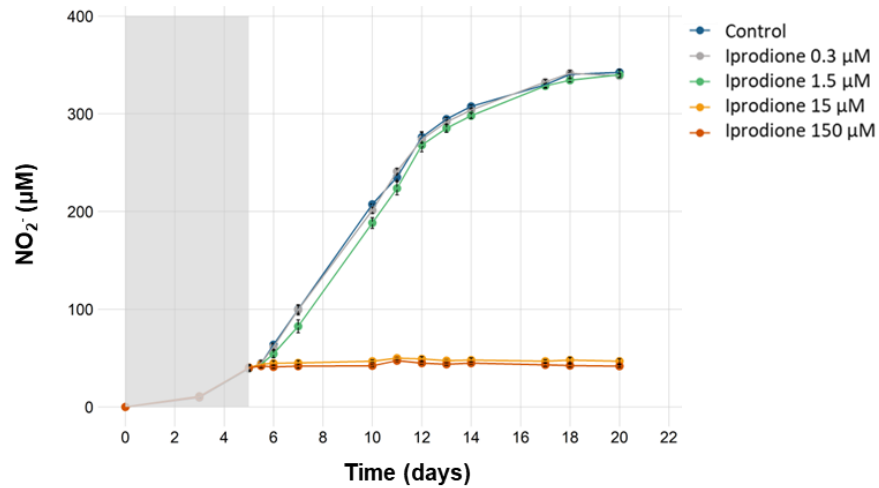
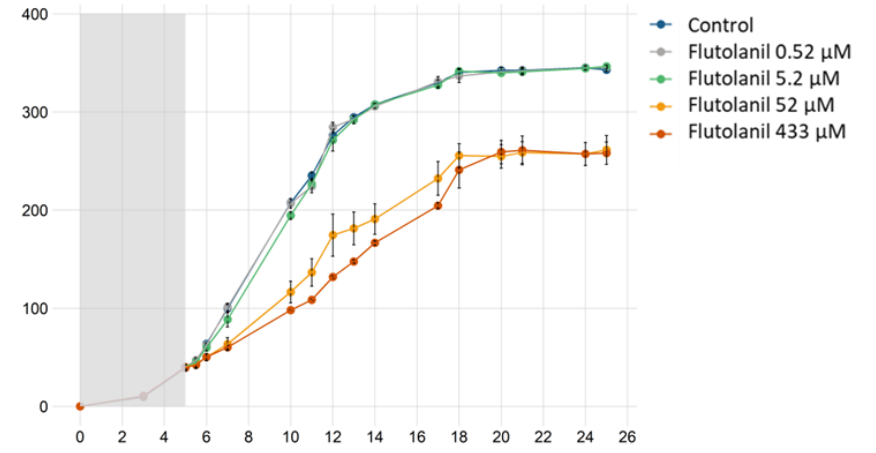
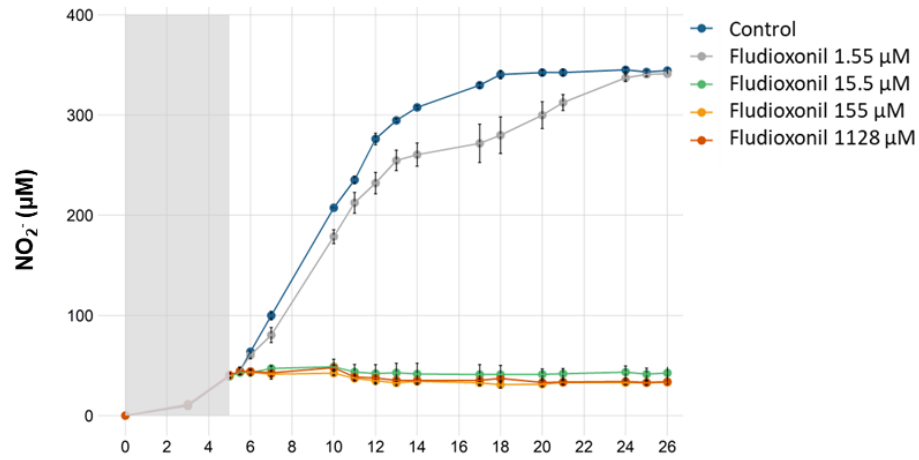


Figure 3.1. The effect of different concentrations of pesticides on nitrite production by the AOA strain *Nitrosotalea sinensis*. Error bars represent the standard error of the mean from triplicate cultures. Grey color indicates the period before pesticide application

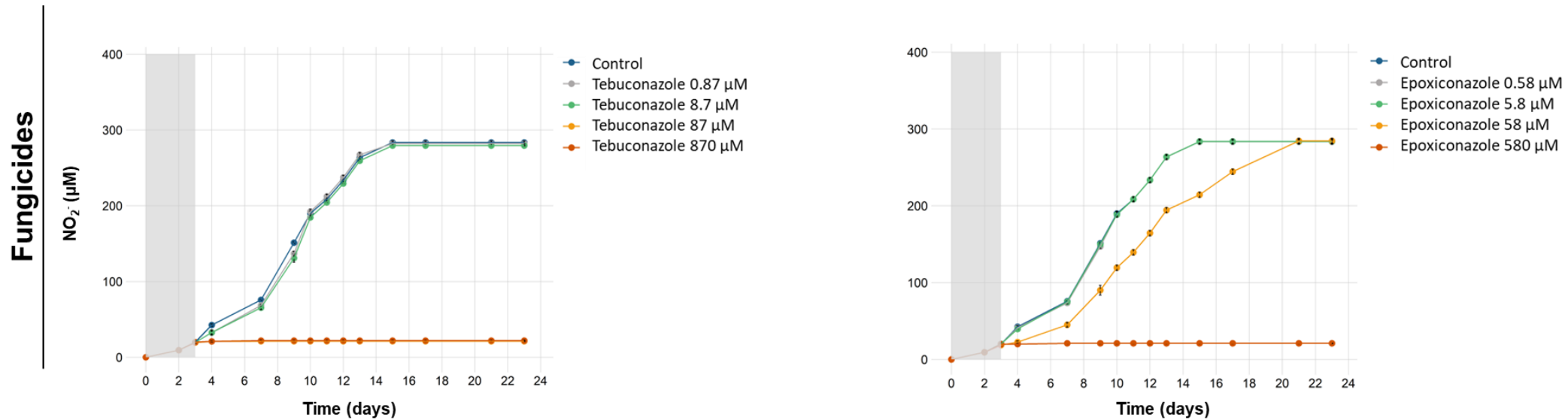


Figure 3.1. The effect of different concentrations of pesticides on nitrite production by the AOA strain *Nitrosotalea sinensis*. Error bars represent the standard error of the mean from triplicate cultures. Grey color indicates the period before pesticide application

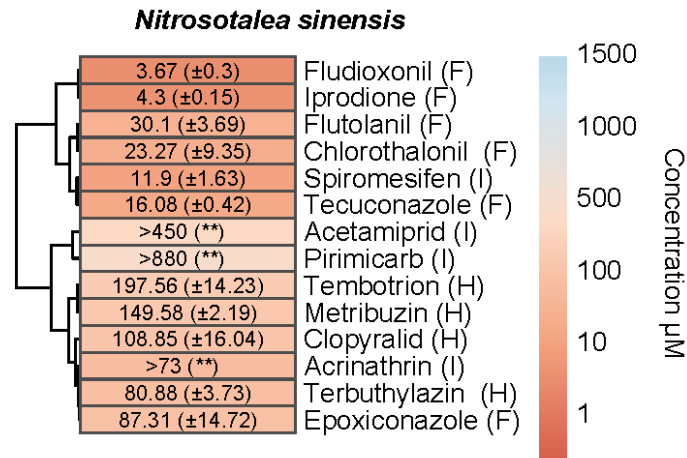


Figure 3.2. Mean EC₅₀ values (μM) of pesticide inhibitory concentrations on the AOA strain *Nitrosotalea sinensis*. Standard error of mean values (denoted by ±) is given in parentheses. Asterisks denote the maximum tested concentration (**). The capital letters I, F, and H in parentheses next to the tested pesticides denote the categories insecticides, fungicides, and herbicides, respectively. Dendrograms based on Euclidean distances and the complete linkage clustering method using log-transformed mean EC₅₀ values are presented for clustering pesticides according to their toxicity, respectively. Values are color-coded by orders of the tested concentrations.

Insecticides

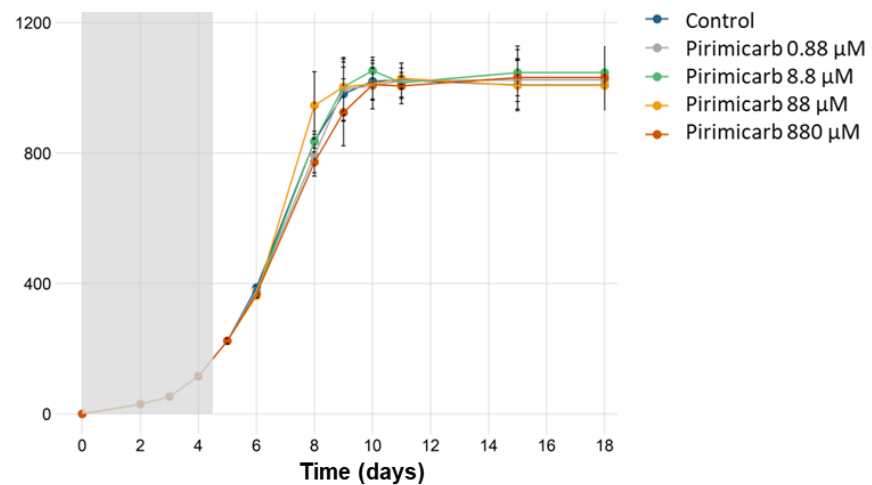
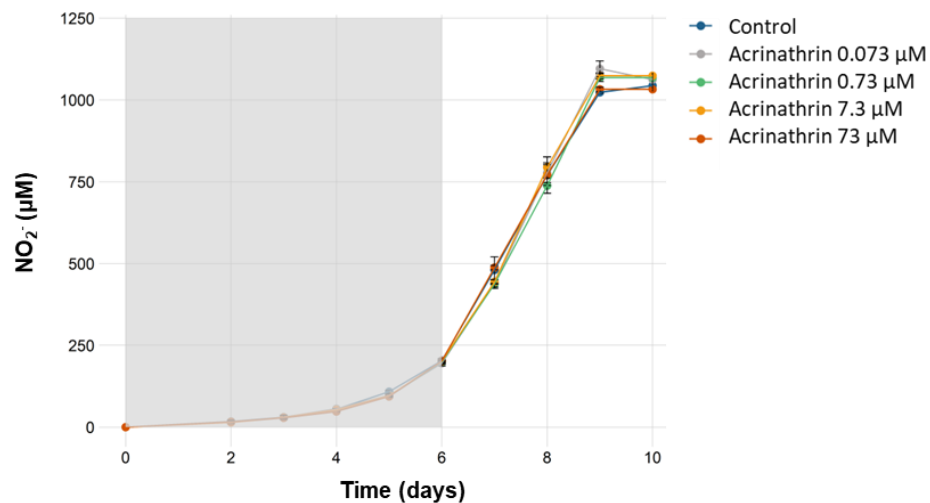
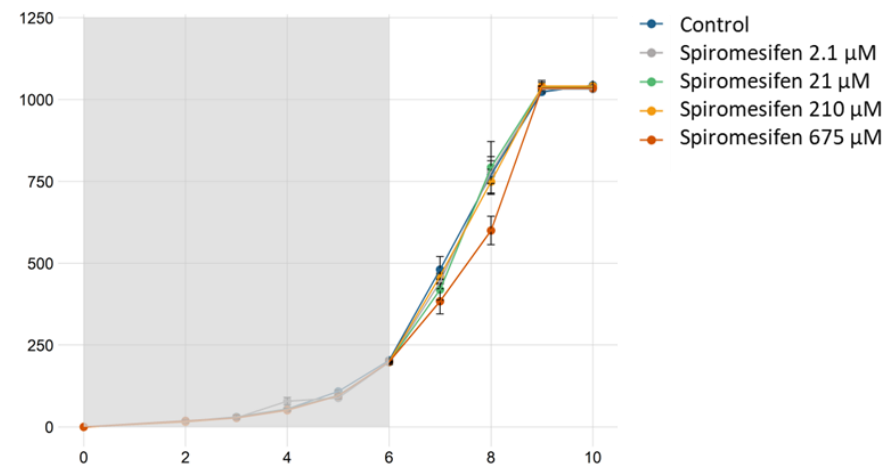
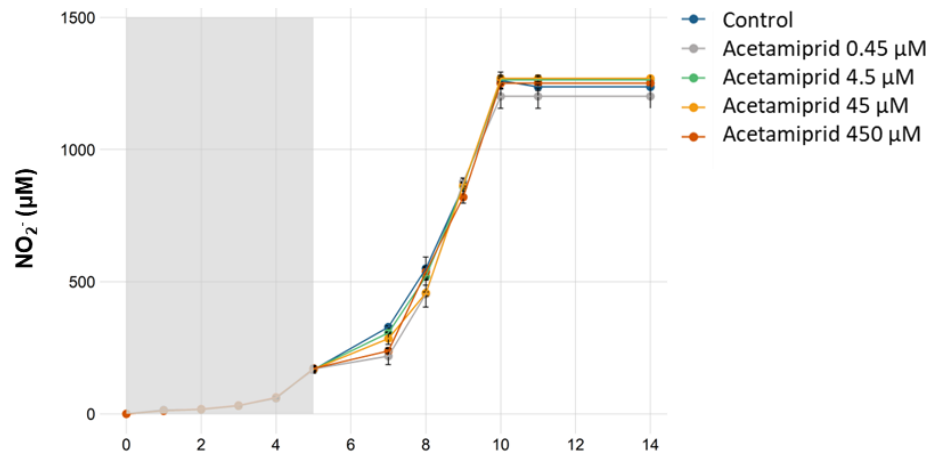


Figure 3.3. The effect of different concentrations of pesticides on nitrite production by the AOB strain *Nitrosospira briensis*. Error bars represent the standard error of the mean from triplicate cultures. Grey color indicates the period before pesticide application.

Herbicides

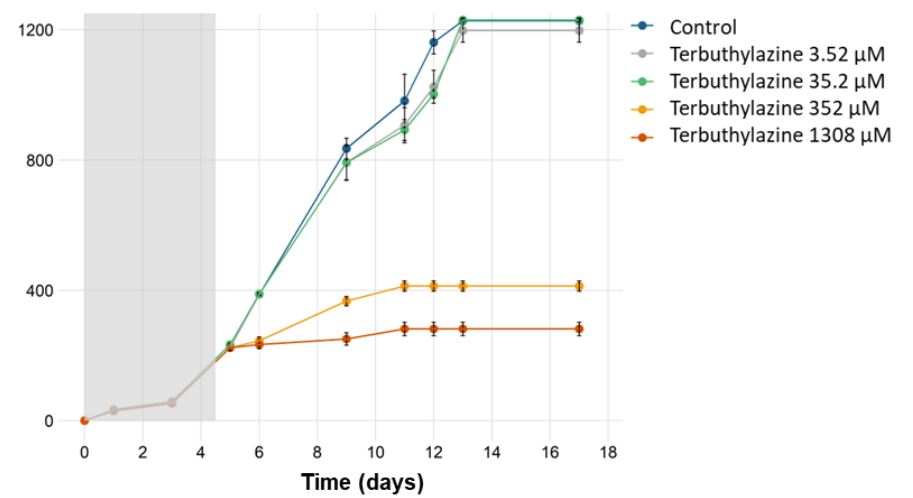
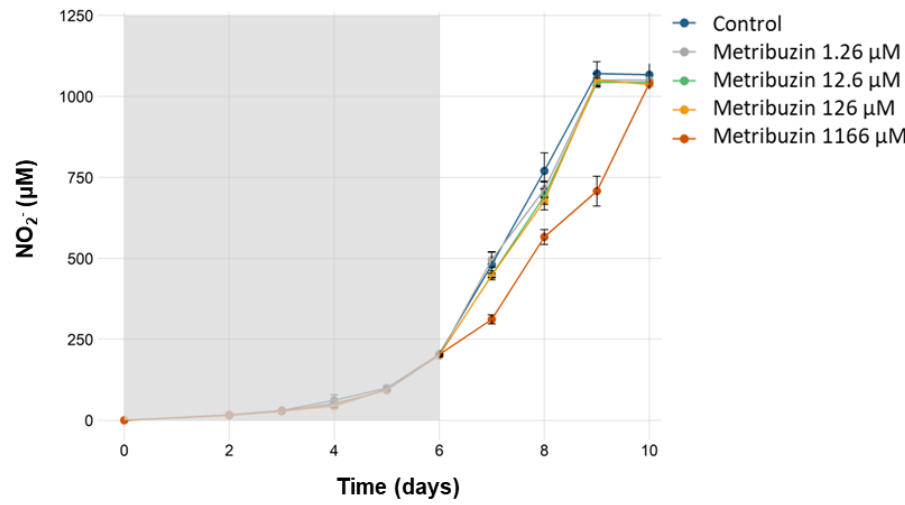
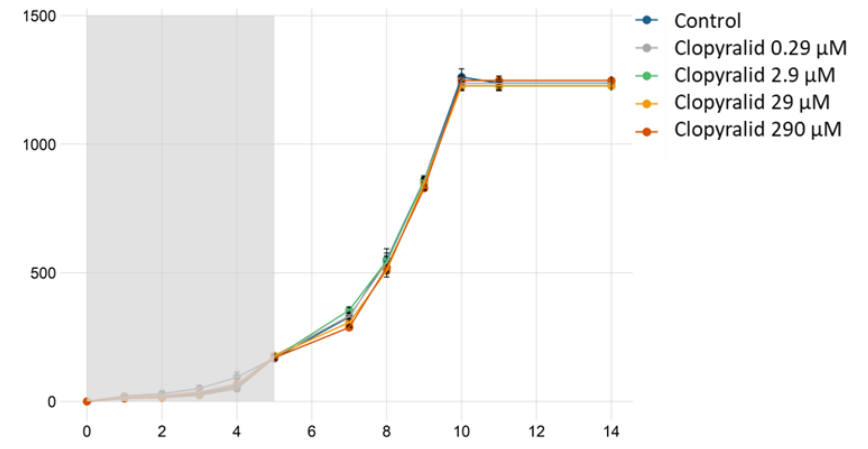
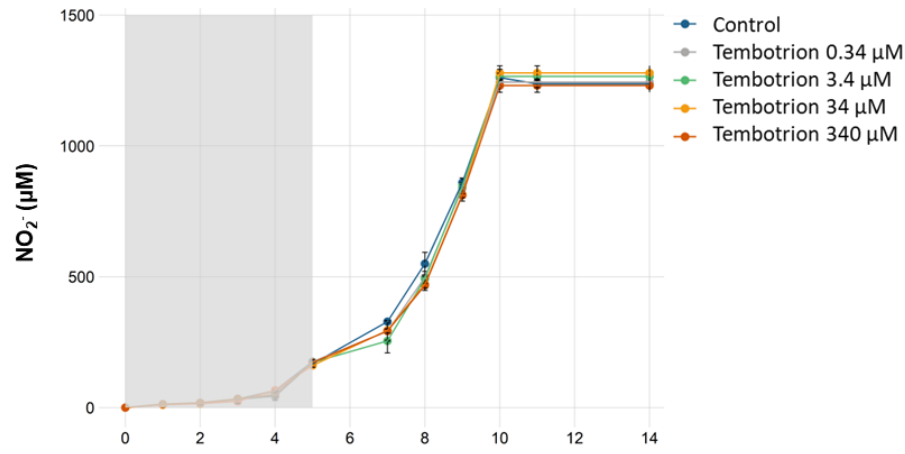


Figure 3.3. The effect of different concentrations of pesticides on nitrite production by the AOB strain *Nitrosospira briensis*. Error bars represent the standard error of the mean from triplicate cultures. Grey color indicates the period before pesticide application.

Fungicides

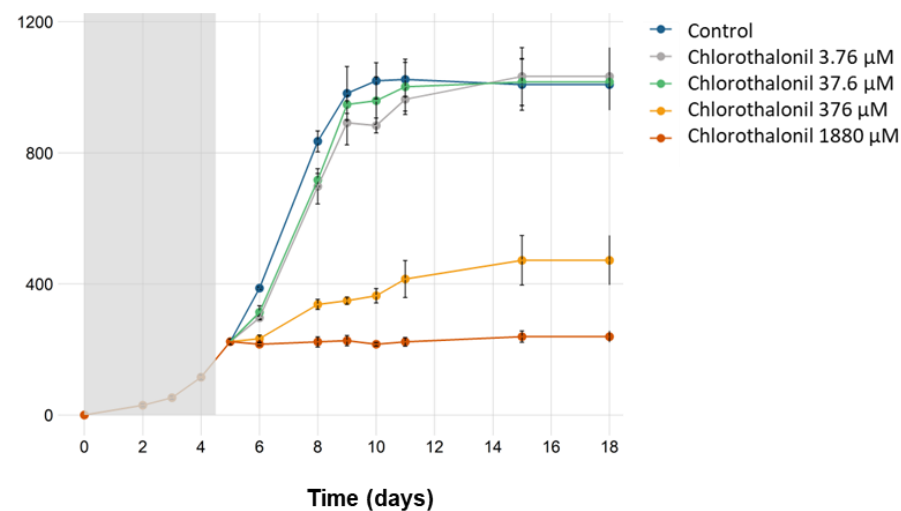
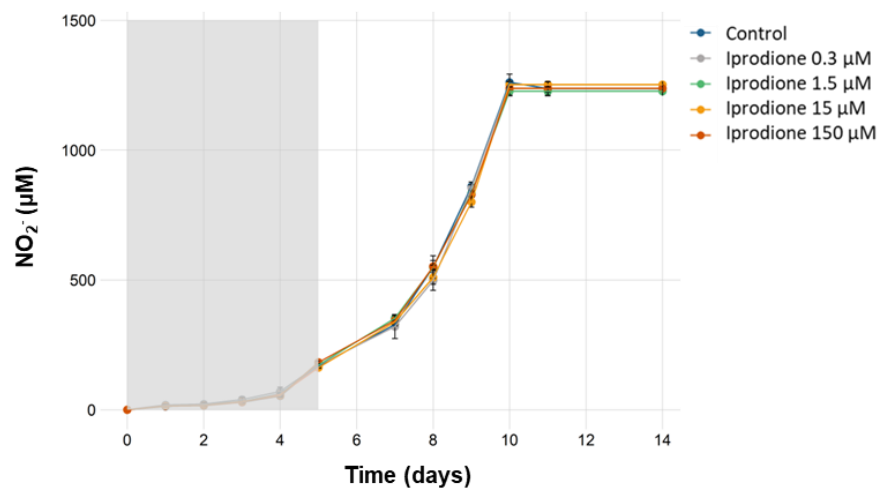
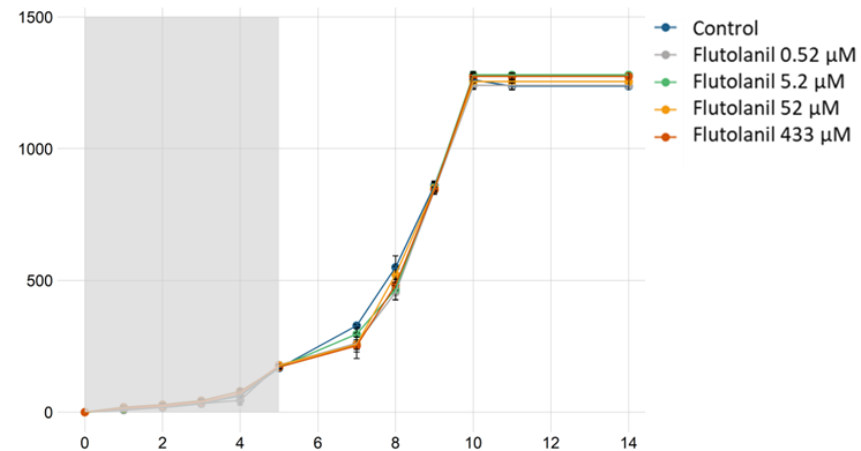
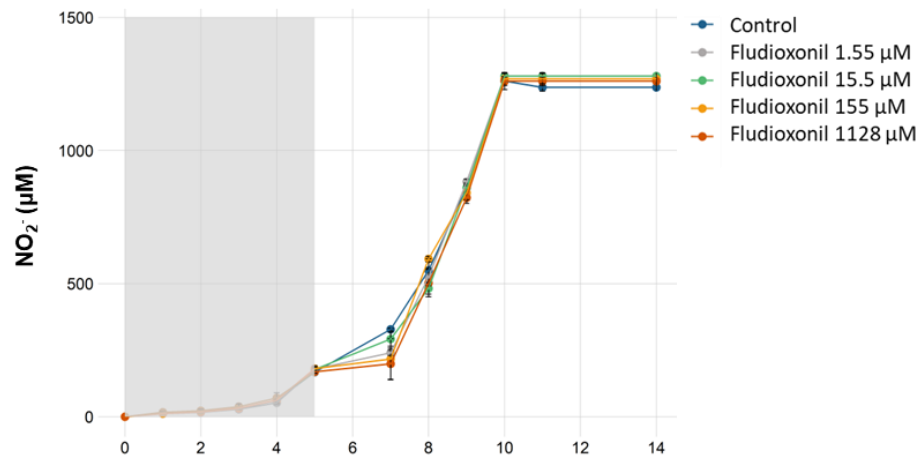


Figure 3.3. The effect of different concentrations of pesticides on nitrite production by the AOB strain *Nitrosospira briensis*. Error bars represent the standard error of the mean from triplicate cultures. Grey color indicates the period before pesticide application.

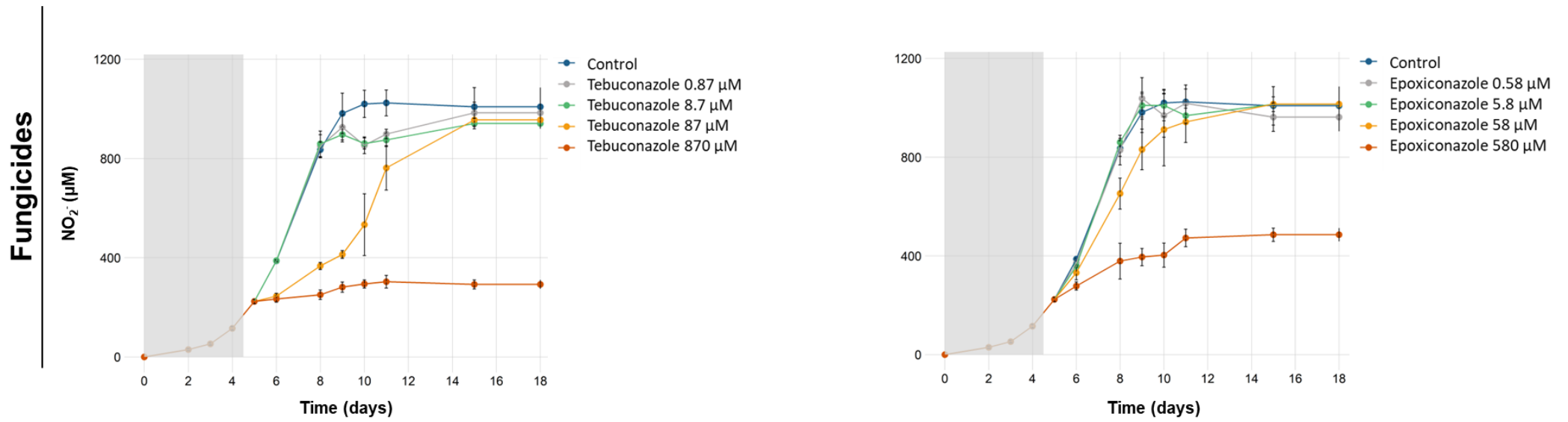


Figure 3.3. The effect of different concentrations of pesticides on nitrite production by the AOB strain *Nitrosospira briensis*. Error bars represent the standard error of the mean from triplicate cultures. Grey color indicates the period before pesticide application.

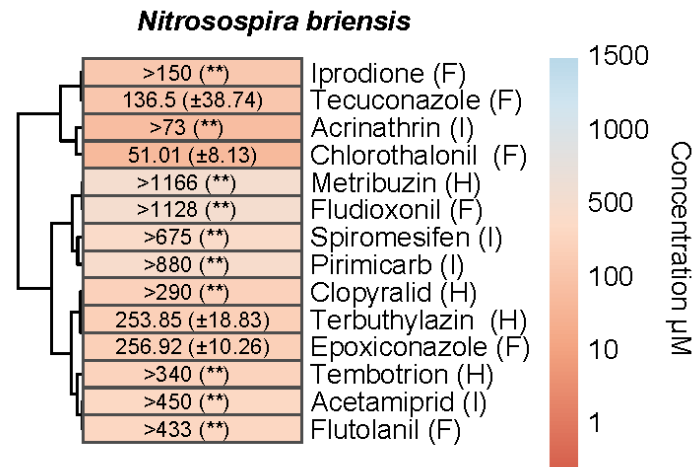


Figure 3.4. Mean EC_{50} values (μM) of pesticide inhibitory concentrations on the AOB strain *Nitrosospira briensis*. Standard error of mean values (denoted by \pm) are given in parentheses. Asterisks denote the maximum tested concentration (**). The capital letters I, F, and H in parentheses next to the tested pesticides denote the categories insecticides, fungicides, and herbicides, respectively. Dendrograms based on Euclidean distances and the complete linkage clustering method using log-transformed mean EC_{50} values are presented for clustering pesticides according to their toxicity, respectively. Values are color-coded by orders of the tested concentrations.

3.3.3. Impact of pesticides on the activity of *Ca. Nitrobacter laanbroekii* NHB1

The activity of the NOB strain *Ca. Nitrobacter laanbroekii* NHB1 was not affected after the application of insecticides, except for spiromesifen, which inhibited the strain at the highest tested concentration (675 μ M). Of the herbicides, only tembotrion suppressed the activity of the strain and only at the concentration of 340 μ M. The fungicide chlorothalonil caused full inhibition at concentration 1880 μ M and partial inhibition at concentrations \geq 37.6 μ M. Partial inhibition was also observed after application of tebuconazole at concentration 870 μ M (Figure 3.5).

The calculated EC₅₀ values of spiromesifen, tembotrion, chlorothalonil, tebuconazole and epoxiconazole for *Ca. N. laanbroekii* NHB1 (510.21 \pm 13.64, 115.75 \pm 27.64, 137.45 \pm 29.32, 548.84 \pm 213.3 and 415.12 \pm 3.89, respectively) were significantly higher than the recommended dose (Figure 3.6).

3.3.4 Sensitivity of the different tested strains to each pesticide category

According to the calculated EC₅₀ values, toxicity patterns for each pesticide category (insecticides, fungicides, herbicides) were observed (Figure 3.7). The toxicity of the insecticides was not significantly different between the strains. Herbicides and fungicides were more toxic to the strain *N. sinensis* compared to *N. briensis* ($p=0.035$ and $p=0.039$, respectively) and *Ca. N. laanbroekii* NHB1 ($p=0.047$ and $p=0.028$, respectively), while no significant differences in toxicity were observed between *N. briensis* and *Ca. N. laanbroekii* NHB1.

Insecticides

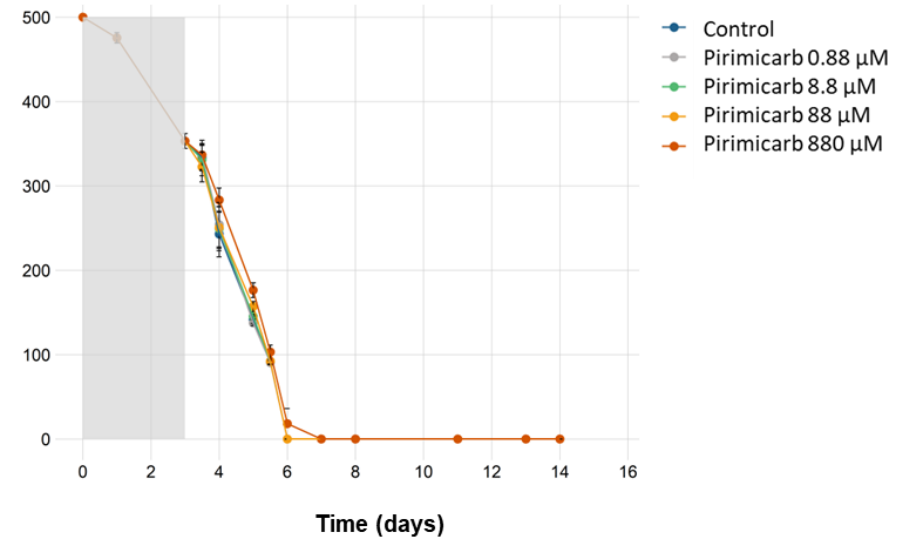
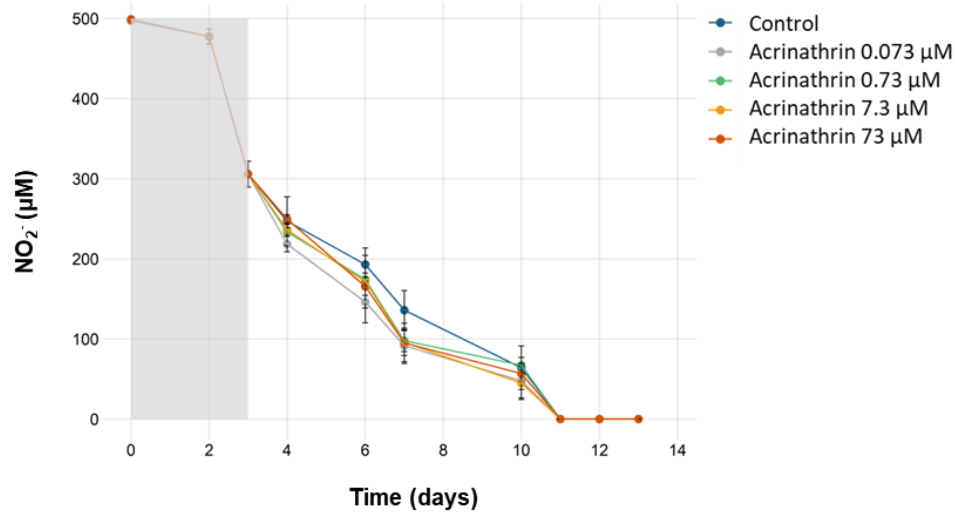
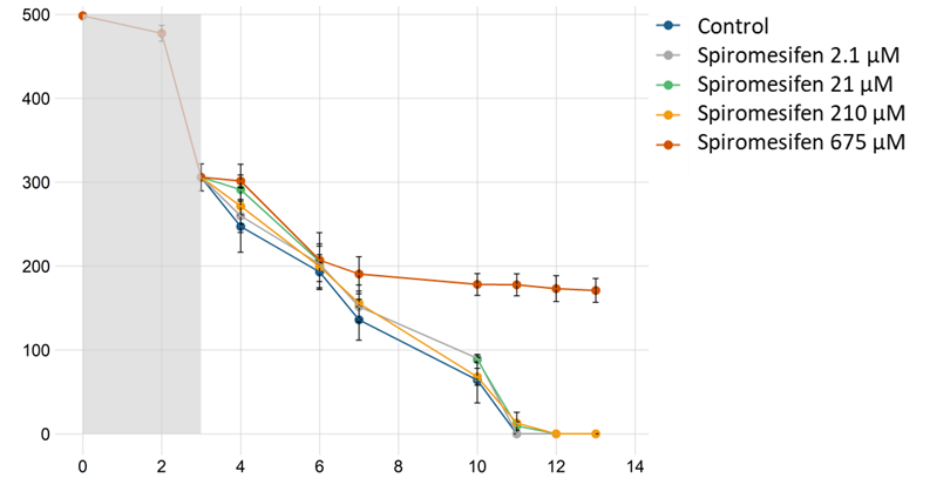
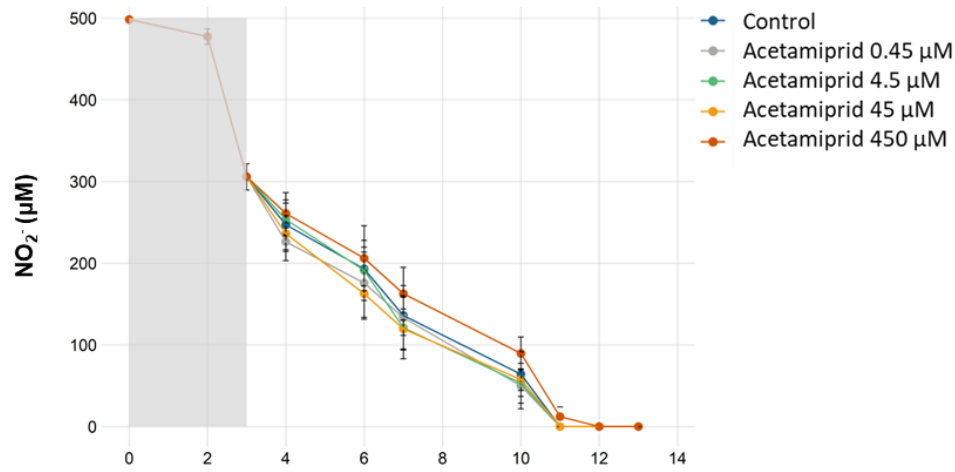


Figure 3.5. The effect of different concentrations of pesticides on nitrite consumption by the NOB, *Ca. Nitro bacter laanbroekii* NHB1. Error bars represent the standard error of the mean from triplicate cultures. Grey color indicates the period before pesticide application.

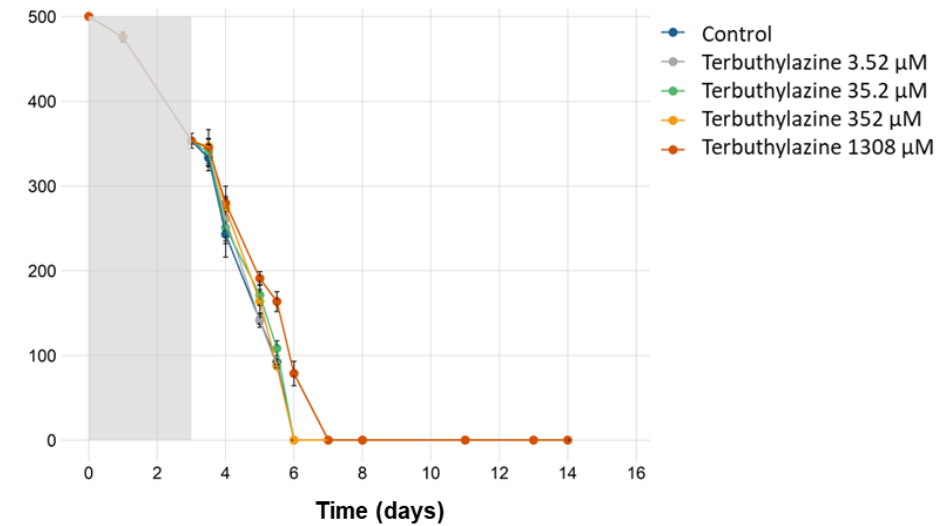
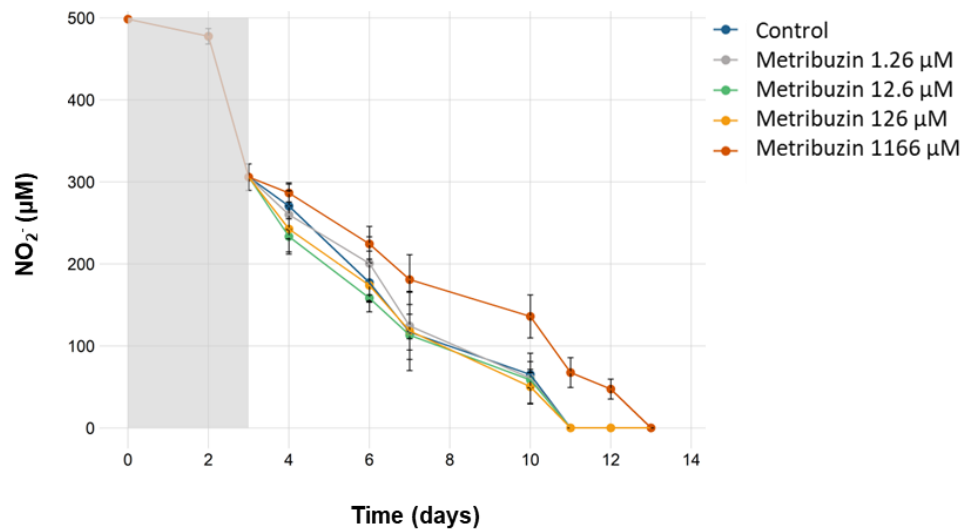
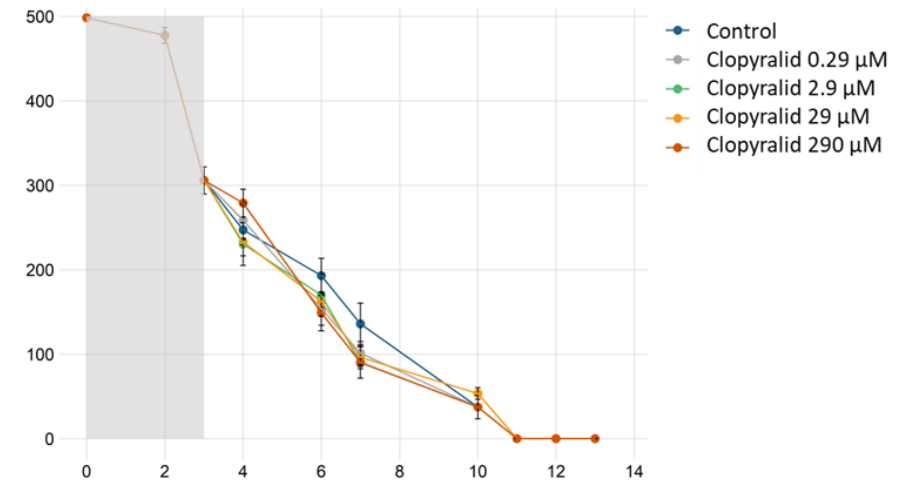
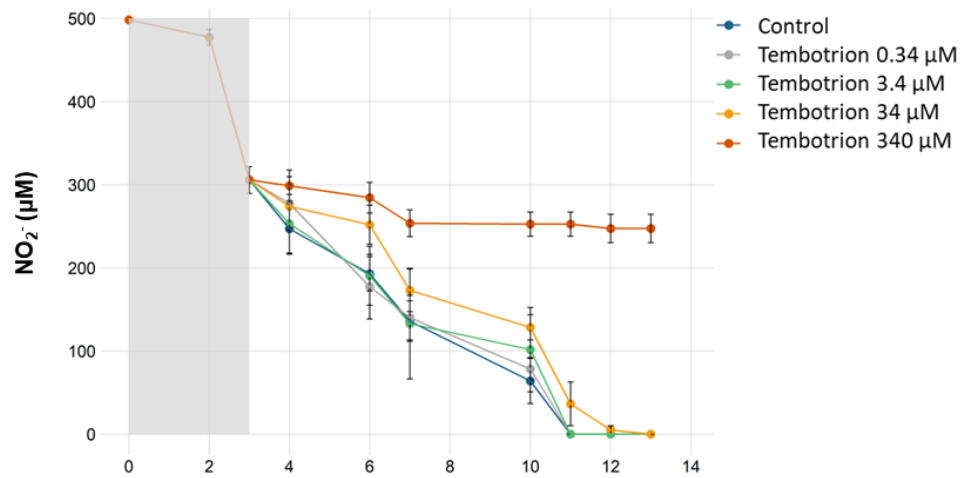


Figure 3.5. The effect of different concentrations of pesticides on nitrite consumption by the NOB, *Ca. Nitrobacter laanbroekii* NHB1. Error bars represent the standard error of the mean from triplicate cultures. Grey color indicates the period before pesticide application.

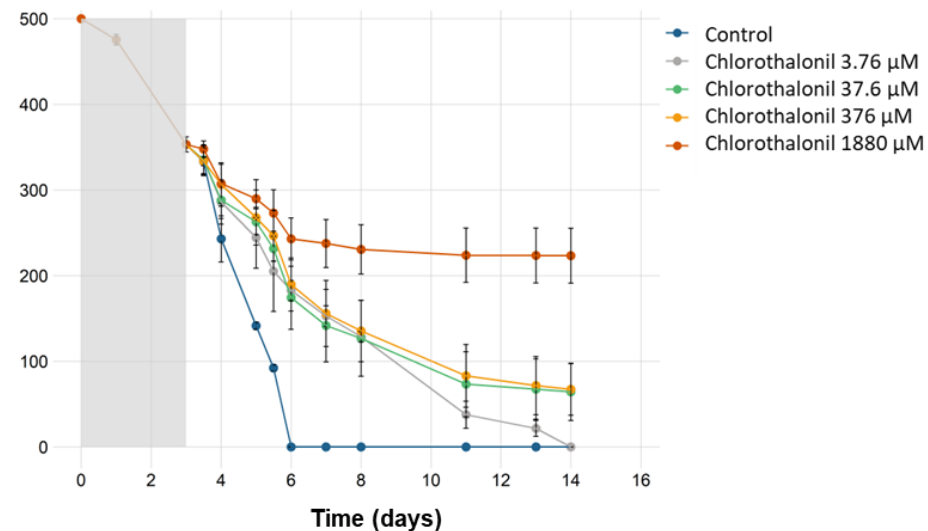
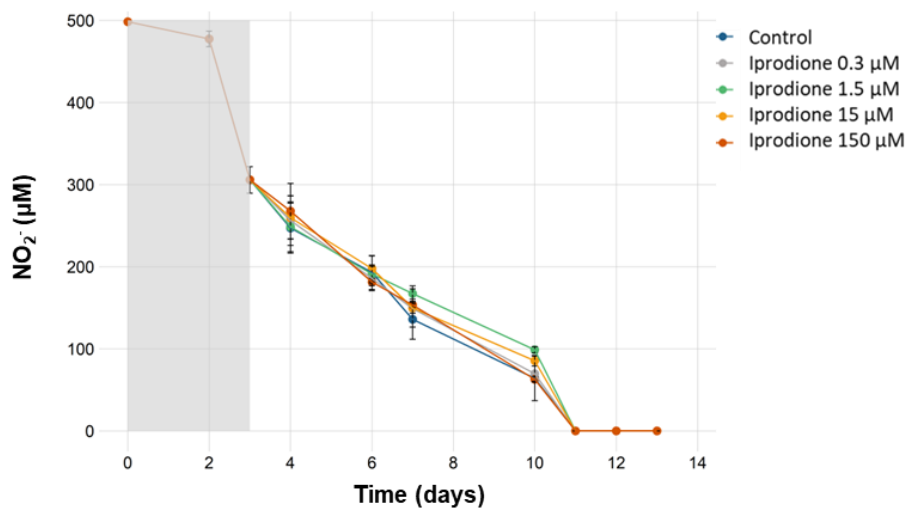
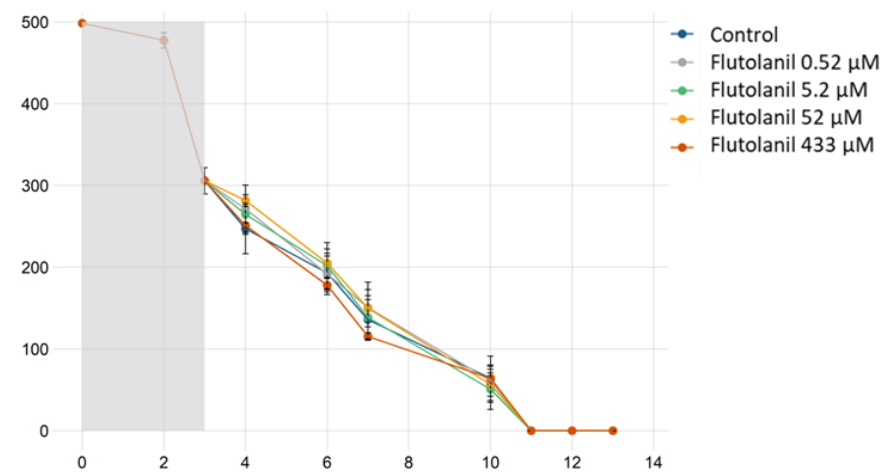
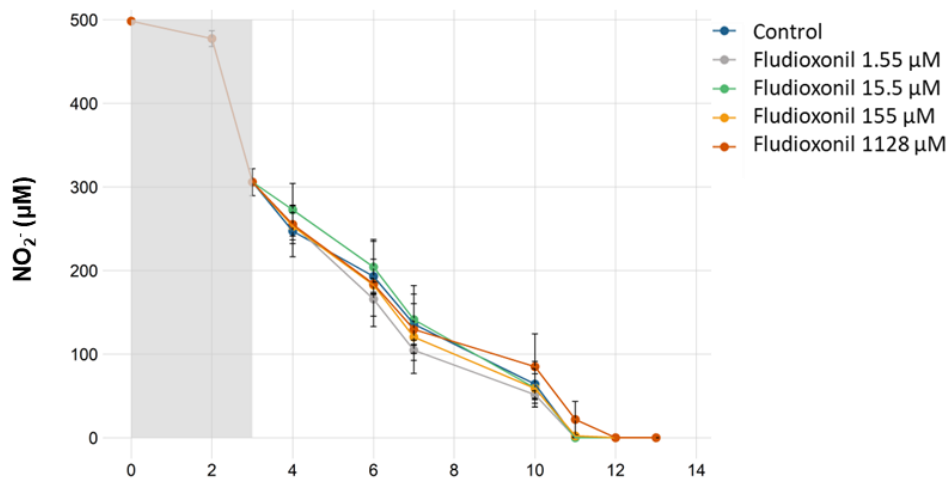


Figure 3.5. The effect of different concentrations of pesticides on nitrite consumption by the NOB, *Ca. Nitro bacter laanbroekii* NHB1. Error bars represent the standard error of the mean from triplicate cultures. Grey color indicates the period before pesticide application.

Fungicides

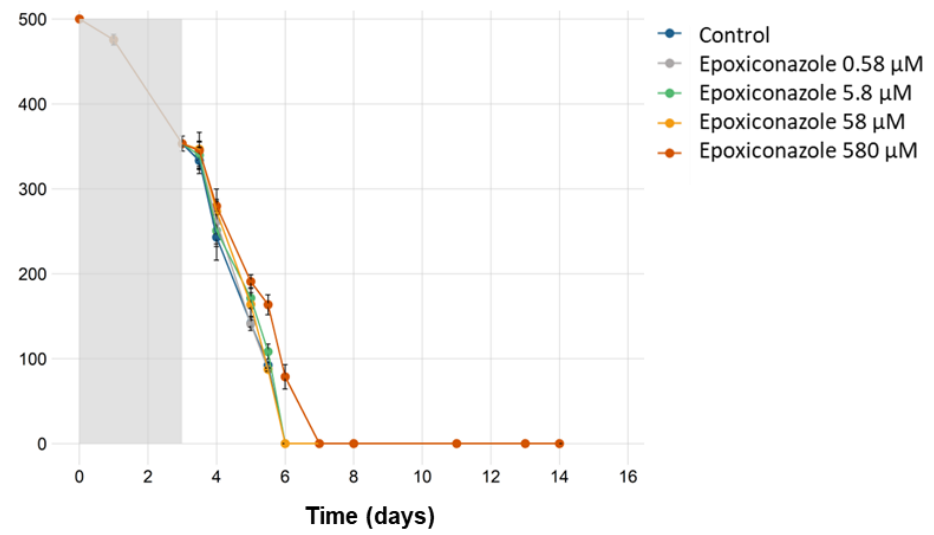
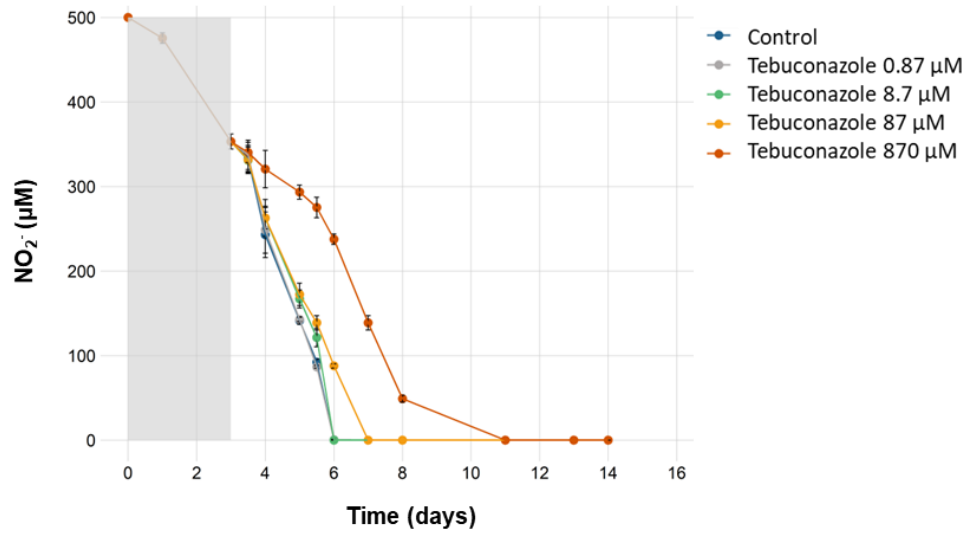


Figure 3.5. The effect of different concentrations of pesticides on nitrite consumption by the NOB, *Ca. Nitrobacter laanbroekii* NHB1. Error bars represent the standard error of the mean from triplicate cultures. Grey color indicates the period before pesticide application.

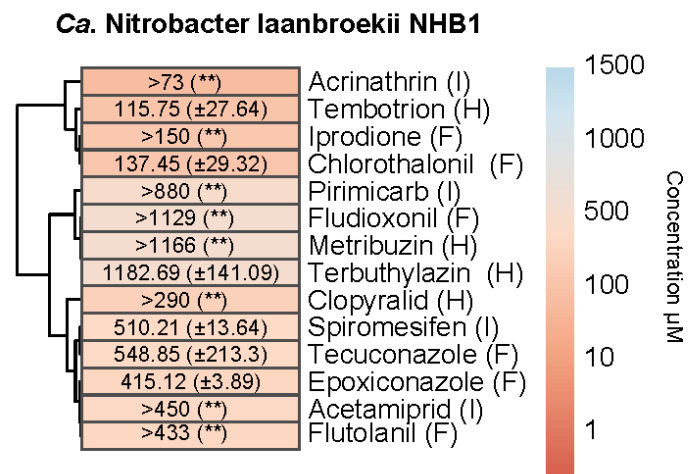


Figure 3.6. Mean EC₅₀ values (μM) of pesticide inhibitory concentrations on NOB strain *Ca. Nitrobacter laanbroekii* NHB1. Standard error of mean values (denoted by ±) are given in parentheses. Asterisks denote the maximum tested concentration (**). The capital letters I, F, and H in parentheses next to the tested pesticides denote the categories insecticides, fungicides, and herbicides, respectively. Dendrograms based on Euclidean distances and the complete linkage clustering method using log-transformed mean EC₅₀ values are presented for clustering pesticides according to their toxicity, respectively. Values are color-coded by orders of the tested concentrations.

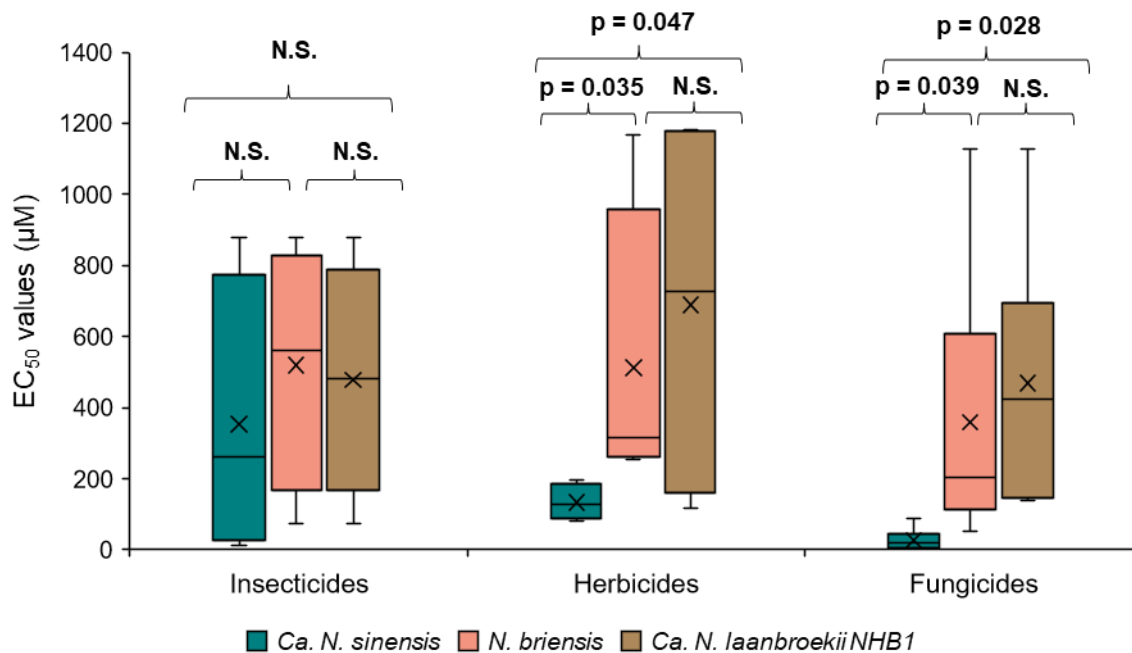


Figure 3.7. Comparison of the toxicity of different pesticide categories (insecticides, fungicides, herbicides) to *Nitrosotalea sinensis*, *Nitrospira briensis* and *Ca. Nitrobacter laanbroekii* NHB1 based on the mean EC₅₀ values calculated for each pesticide category. N.S. indicates no significant difference ($p > 0.05$). In cases where no EC₅₀ could be determined in statistical analyses the highest tested concentrations were used in the calculation.

3.4 Discussion

The growing concern over the effects of pesticides on soil microbial communities has drawn significant attention from both scientists and regulatory agencies, noting the crucial role of these microorganisms in maintaining ecosystem functions. However, a significant gap remains in our understanding of pesticides effects on microbial communities, as well as a lack of tools, testing methods, and bioindicators to effectively assess pesticides impact. In our previous work, we proposed a potential single species test using the most sensitive AOM and NOB strains as bioindicators to evaluate pesticide toxicity on soil microbiota within a tiered risk assessment framework comparable to the single-species tests employed in aquatic and terrestrial ecotoxicology (Chapter 2). In this study, we applied the proposed *in vitro* bioassay to assess the toxicity of 14 additional pesticides commonly used in agricultural practices aiming to validate and confirm the effectiveness of our previous findings.

The acidophilic *N. sinensis*, identified in our previous work as the most sensitive AOA strain, consistently displayed high sensitivity to most of the tested pesticides across all the major categories. The strain was affected by all pesticides except for the insecticides acetamiprid, acrinathrin, and pirimicarb. This high sensitivity aligns with our previous findings (Chapter 2), where the strain's activity was significantly inhibited by all pesticides tested.

Other previous studies have also reported similar results for *N. sinensis* with iprodione, which inhibited strain's activity at concentrations similar to those in our study (Vasileiadis et al., 2018). Additionally, *N. sinensis* has demonstrated high sensitivity to other agrochemicals, such as nitrification inhibitors, compared to neutrophilic AOA strains (Kaur-Bhambra et al., 2022; Papadopoulou et al., 2020; Zhao et al., 2020). This sensitivity is likely due to the strain's membrane composition, particularly its glycerol dialkyl glycerol tetraether (GDGT) lipids, which influence membrane permeability and its response to stress factors (Elling et al., 2016; Lehtovirta-Morley et al., 2016). This suggests that *N. sinensis* could serve as an early warning bioindicator for pesticide-induced disruption in sensitive soil microbial communities.

Among the two most sensitive AOB strains identified in our previous study, *N. briensis* was chosen as the most suitable candidate for ecotoxicology assessments. This decision was based on its prevalence as a *Nitrosospira* species in soil ecosystems (Kowalchuk & Stephen, 2001) and its dominant presence in metataxonomic analyses of AOB diversity in agricultural soils (Assémien et al., 2017; Papadopoulou et al., 2022). The selection was influenced by the strain's response to the four most toxic compounds tested. In this study, only tebuconazole, chlorothalonil, epoxiconazole, and terbuthylazine affected *N. briensis*, while insecticides did not impact the strain, consistent with our previous findings (Chapter 2). Additionally, a recent study confirmed that *N. briensis* does not exhibit a distinct sensitivity pattern to various biological nitrification inhibitors (Kaur-Bhambra et al., 2022). This reduced sensitivity to the tested pesticides is likely due to the mode of action of these chemicals. Most of the tested pesticides do not appear to affect AOB strains significantly (Table 3.1), and those targeting enzymes present in the AOB genome may have target sites that are rather different between AOB and the organisms typically affected by these compounds.

To fully capture the ecological implications of pesticides use on nitrification, we expanded our in vitro assays to include NOB, which rely on AOB for substrates and complete nitrification, as pesticide-induced reductions in NOB activity could increase nitrogen loss and exacerbate atmospheric pollution (Prosser et al., 2020). The NOB strain that we proposed as a potential bioindicator is Ca. *Nitrobacter laanbroekii* NHB1. This strain was affected by only a few tested pesticides and only at the highest concentrations, consistent with our previous findings (Chapter 2), indicating a low risk to this nitrifier group in agricultural soils. Only a few studies on the effects of agrochemicals on NOB strains have been conducted in vitro, and highlighted Ca. *Nitrobacter laanbroekii* NHB1's high tolerance to nitrification inhibitors compared to AOA and AOB (Kolovou et al., 2023; Matsuba et al., 2003; Papadopoulou et al., 2020) is comparable to our findings with pesticides. The tolerance of this strain is likely due to its versatile metabolism, which enables some *Nitrobacter* strains to degrade complex organic compounds (John & Okpokwasili, 2012).

Toxicity patterns across different pesticide groups were also analysed between the studied strains. Among the pesticide groups analysed, fungicides showed greater toxicity towards *N. sinensis* compared to the other strains. This trend aligns with our earlier work (Chapter 2), although previous findings indicated significant effects on AOB strains as well. In contrast, the current study found higher EC₅₀ values for *N. briensis* compared to *N. sinensis*,

which could be attributed to their physiological structural differences. The cell membrane of AOA consists of glycerol-ether lipids, with isoprenoid side chains attached via ether bonds to a glycerol backbone, unlike the fatty acid-based membranes of AOB. These structural differences influence membrane permeability and consequently susceptibility to xenobiotics like pesticides (Schleper & Nicol, 2010; Shen et al., 2013). The observed toxicity of tebuconazole and epoxiconazole was anticipated given that triazoles are well-known nitrification inhibitors in soil and wastewater ecosystems (Beeckman et al., 2023; McCarty et al., 1991; Taggert et al., 2021). However, the extent of their inhibitory effects can vary depending on the substituents present in the azole ring (Jog et al., 2022; Taggert et al., 2021). Additionally, chlorothalonil's toxicity across all tested strains is likely due to its inhibition of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a key enzyme in glycolysis found in many soil microorganisms, including AOM (Yang et al., 2023).

A similar toxicity pattern to that observed with fungicides was also found for herbicides, with *N. sinensis* being the most sensitive strain. This contrasts with our previous findings where no significant herbicide toxicity differences were observed between soil nitrifying groups (Chapter 2). It is important to note that these results are based on EC₅₀ values that are higher than the recommended field application rates that we tested. The observed low toxicity may be linked to the mode of action of the tested herbicides, which target fundamental plant processes such as photosynthesis and plant hormonal systems (Mendes et al., 2017; Shah et al., 2009; Valenzuela-Valenzuela et al., 2001). The exception was tembotrione, which inhibits 4-hydroxyphenylpyruvate dioxygenase, an enzyme involved in tyrosine catabolism and carotenoid biosynthesis, and is also found in AOM (Table 1) (Pinke et al., 2014). Tembotrione only affected *N. sinensis* and *Ca. Nitrobacter laanbroekii* NHB1 at the highest concentrations tested.

The tested insecticides did not affect the activity of the strains, with the exception of spiromesifen, which significantly inhibited the activity of *N. sinensis*. Spiromesifen was the only pesticide tested that showed potential effects on nitrifying strains, likely due to its mode of action - targeting acetyl coenzyme A carboxylase (ACCase), which disrupts lipid synthesis, a process also present in microbial genomes (Abdel-Fatah et al., 2019). In our previous study, insecticides primarily affected AOA strains, though the number of pesticides tested was limited, making the observation less robust (Chapter 2). Combined with the current findings, it appears that insecticides generally exhibit low toxicity to soil nitrifiers, likely due to their primary mode of action, which targets the nervous system of insects.

3.4 Conclusion

This study evaluated the effectiveness of single-species in vitro assays in assessing the toxicity of 14 commonly used pesticides on key nitrifying microorganisms. We focused on three bioindicator strains: *Nitrosotalea sinensis* (AOA), *Nitrosospira briensis* (AOB), and *Ca. Nitrobacter laanbroekii* (NOB) to monitor their sensitivity to various pesticide categories. Our findings revealed that *N. sinensis* exhibited the highest sensitivity to most pesticides, particularly fungicides, underscoring its effectiveness as a bioindicator for pesticide toxicity in soil microbial communities. In contrast, *N. briensis* and *Ca. Nitrobacter laanbroekii* demonstrated greater tolerance, with only a few pesticides causing notable inhibition. Despite their lower sensitivity, these AOB and NOB strains are crucial for a comprehensive risk assessment, as they provide valuable insights into a broader range of microbial responses. The results confirm that single-species in vitro assays are a promising method for assessing pesticide toxicity. By including both sensitive and less sensitive strains, our approach provides a well-rounded assessment of pesticide effects, capturing a wider range of microbial responses. This method can play an important role in early risk assessment of pesticides and contributes to the protection of essential ecological functions performed by soil microorganisms.

3.6 Acknowledgments

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Chapter 4

Assessment of pesticide toxicity using the Microbial Assay for Risk Assessment (MARA): A comparative study with soil nitrifying microorganisms

Abstract

The widespread use of pesticides in agriculture has led to significant soil contamination, posing risks to soil ecosystems and affecting the sustainability of agriculture. Soil microorganisms, particularly those involved in key biochemical processes such as nitrification, are critical for maintaining soil health. This study evaluated the effectiveness of a commercial test kit that utilizes 11 heterotrophic microbial strains (Microbial Assay for Risk Assessment (MARA)) in detecting pesticide toxicity and compares its sensitivity to that of soil nitrifiers, specifically ammonia-oxidizing microorganisms (AOM), including ammonia-oxidizing archaea (AOA) and bacteria (AOB) and nitrite-oxidizing bacteria (NOB). We observed that the MARA strains exhibited relatively low sensitivity, with most pesticides affecting strains only at high concentrations. In contrast, based on our previous results, AOM exhibited a significantly higher sensitivity to pesticide exposure, suggesting that AOM could serve as a more effective bioindicator of pesticide toxicity in soil environments. The results support the concept of developing a dedicated assay kit based on soil nitrifiers, which could provide a more accurate and sensitive tool for high throughput pesticide testing.

Keywords: ammonia-oxidizing bacteria, ammonia-oxidizing archaea, nitrite-oxidizing bacteria, ecotoxicity, pesticides, MARA

4.1 Introduction

The application of pesticides is one of the most widespread agricultural practices globally, primarily aimed at increasing crop yields to meet rising food demands (Tudi et al. 2021). In recent years, pesticide use has increased by approximately 50% (Riedo et al. 2021). However, only about 5% of the applied pesticides reach their targets, with the remainder accumulating in soil which represents the largest proportion in agricultural ecosystems. This accumulation increases the risk of negative effects on soil ecosystems (Karpouzas et al. 2022). Consequently, soil contamination by pesticides can have negative impacts on food quality and agricultural sustainability (Tudi et al. 2021). Soil microorganisms are vital to maintaining soil health, as they play essential roles in key biochemical processes such as the nitrogen and carbon cycles (Li et al. 2021). In soil environments, pesticides interact with microorganisms, which can result in either toxic effects on microbial functions and communities (Puglisi et al. 2012; Feld et al. 2015) or on increased growth linked to microbial degradation (Copley 2009). These outcomes depend on a variety of biotic and abiotic factors (Karpouzas et al. 2016; Karpouzas et al. 2022).

Recent studies have proposed ammonia oxidizing microorganism (AOM) as promising toxicity bioindicators of pesticides toxicity on soil microorganisms (Karpouzas et al. 2016; Karpouzas et al. 2022). AOM include three key groups: AOA, AOB, and complete ammonia-oxidizing bacteria (comammox). In canonical nitrification, AOA and AOB are responsible for the first and rate-limiting step of nitrification, where they oxidize ammonia to hydroxylamine

(NH_2OH), which is then converted to nitric oxide (NO) and finally to nitrite (NO_2^-) (Beeckman, Motte, and Beeckman 2018). In the second step, nitrite-oxidizing bacteria (NOB) convert nitrite to nitrate (NO_3^-) (Coskun et al. 2017; Beeckman, Motte, and Beeckman 2018). Recent studies have identified bacteria from the genus *Nitrospira*, which also includes canonical NOB, that can perform the complete oxidation of ammonia to nitrate within a single cell, a process known as complete ammonia oxidation (comammox) (Daims et al., 2015; van Kessel et al., 2015).

Several characteristics make AOM strong candidates as bioindicators for pesticide toxicity of soil microbiota: i. they play a crucial ecological role in soil environments (Prosser and Nicol 2008; Beeckman, Motte, and Beeckman 2018), ii. they are highly sensitive to abiotic disturbances, including exposure to xenobiotics (Wessén and Hallin 2011), and iii. there is extensive knowledge about their ecology, physiology, and diversity, with well-established tools available to measure their activity and abundance both *in vitro* (Lehtovirta-Morley et al., 2013) and in soil (Junier et al. 2010). Numerous studies have examined the toxicity of pesticides on AOM activity and abundance in soil using advanced molecular and biochemical techniques (Karas et al. 2015; Papadopoulou et al. 2016; Vasileiadis et al. 2018; Sim et al. 2022). However, *in vitro* assays with single AOM species have been rarely used in ecotoxicological studies.

In the previous work presented in Chapter 2, the toxicity of 10 pesticides was tested against a range of soil nitrifiers, including three AOA, six AOB, and three NOB strains grown in liquid culture and together represent the dominant lineages of soil nitrifiers. The tested pesticides affected at least one non-target ammonia-oxidizing microorganism (AOM), with varying levels of sensitivity observed among the AOA and AOB strains, likely due to their structural and biochemical differences (Shen et al., 2013). This study also revealed specific pesticide group patterns, with fungicides emerging as the most toxic group to AOM. Additionally, NOB were found to be considerably more tolerant than AOM. Notably, these results suggest that certain fungicides, particularly etridiazole and pyraclostrobin, could be highly inhibitory to AOM at agronomically relevant concentrations.

The development of soil microbiology analyses has led to the establishment of various International Organization for Standardization (ISO) standards. These standards encompass techniques such as soil DNA extraction, phospholipid fatty acid (PLFA) analysis, measurements of soil enzymatic activity, and the determination of microbial abundances through quantitative PCR (qPCR) and analysis of soil microbial community structures (Karpouzas et al. 2016; Karpouzas et al. 2022). These methods are not commonly used in pesticide toxicity analysis as they require specialized knowledge and are time intensive.

Another approach for assessing the toxicity of pesticides and other chemicals is through the use of commercially available toxicity kits that rely on bacterial growth bioassays (Gabrielson et al. 2003). These include Microtox (Modern Water, United Kingdom) and Polytox (Bethlehem, Pennsylvania) (Regno, Arulgnanendran, and Nirmalakhandan 1998; Farré et al. 2002). These systems, particularly Microtox, have been widely used but have certain limitations. Microtox relies on a single bacterial strain (*Allivibrio fischeri*), while Polytox

produces only a single result based on the respiration of a mixture of bacteria (Farré et al. 2002; Regno et al. 1998). This limits the depth and scope of the data that can be gathered from each assay. To address these limitations, multi-species assays have been developed. The Microbial Assay for Risk Assessment (MARA) and LumiMARA (both produced by NCIMB, Aberdeen, UK) provide more comprehensive ecotoxicity assays by utilizing a higher number of microorganisms isolated from different environments and which are analyzed individually (Gabrielson et al. 2003). This multi-species approach allows for a more detailed assessment of toxicity, overcoming the constraints posed by kits that use a single strain.

MARA is a multi-species assay designed to evaluate the ecotoxicity of chemicals and environmental samples (Gabrielson et al. 2003). Unlike single-species assays, MARA provides a comprehensive toxicity profile using a 24-hour test of 11 different microbial species, including both prokaryotic organisms and one eukaryote (yeast), which are lyophilized in a microplate. These species were carefully selected to represent a broad taxonomic diversity with the aim of ensuring a more accurate and reliable assessment of potential toxic effects (Wadhia and Thompson 2007). The toxic impact is measured by observing the growth of these organisms when exposed to various concentrations of the test substance or environmental sample. This growth is quantified by the reduction of tetrazolium red (TTC), a process that can be visualized using a flatbed scanner, microplate spectrophotometer, or digital camera. The captured images are then analysed using specialized software to create a unique "fingerprint" that represents the toxic profile of the sample (Wadhia and Thompson 2007). MARA offers several key advantages. It enables the simultaneous testing of multiple microbial species, enhancing and producing a distinctive toxic fingerprint for each sample, providing an overview of its potential ecological impact. Additionally, MARA has been developed to be user-friendly and utilized by non-specialists. Due to its low cost it can be adapted for high-throughput screening, and suitable for large-scale studies (Gabrielson et al. 2003; Wadhia and Thompson 2007).

This study aimed to evaluate the effectiveness of MARA as a high-throughput tool for assessing the ecotoxicity of commonly used agricultural pesticides. Specifically, we compared the sensitivity of MARA strains to that of soil nitrifiers when exposed to the same compounds. By contrasting the microbial toxic concentration (MTC) values obtained from MARA with the EC₅₀ values (half maximal effective concentration) of soil nitrifiers, this study aimed to determine the relative sensitivity of these organisms to pesticide exposure. Furthermore, the study intended to assess whether MARA could provide a comprehensive and accurate toxicity profile of pesticides for soil microbes compared to more specialized assays focused on nitrifying microorganisms, which were proposed as potential bioindicators of pesticide toxicity (see Chapter 2).

4.2 Material and Methods

4.2.1 Pesticides

The pesticides evaluated in this study were identical to those used in Chapter 2 to evaluate the toxicity of pesticides on nitrifying microorganisms. Table 4.1 lists the pesticides used in this study and the concentrations tested. As detailed previously, the selection criteria for these pesticides included i. different target organisms or employing distinct modes of action, and ii. their extensive use in European agriculture, although some, such as chlorpyrifos, were chosen as historical model compounds.

The pesticide concentrations tested are shown in Table 4.1. In most cases, the maximum concentration tested was 100 times the recommended dose, consistent with our previous work (Chapter 2). However, for certain pesticides, testing the highest concentration (100x) was not feasible due to solubility limitations. In these instances, the maximum concentration was determined based on solubility testing. The remaining concentrations were selected using a dilution factor appropriate for the MARA assay, ensuring they were comparable to or close to the concentrations used in nitrifier testing (Chapter 2). Analytical standards for all pesticides, with a purity of $\geq 92\%$, were obtained from HPC Standards (Germany) and were used for both *in vitro* assays and standard curve preparations.

Table 4.1. Pesticides and concentrations assessed with the MARA kit.

| Group | Pesticide | Concentration level (μM) |
|--------------|--|---|
| Insecticides | Chlorpyrifos | 7.1, 14.52, 28.5, 57, 114, 228 |
| | 3,5,6-trichloro-2-pyridinol (TCP) ^a | 5.12, 10.25, 20.5, 41, 82, 164 |
| Herbicides | Glyphosate | 5.9, 29.5, 59, 295, 590, 2957 |
| | Aminomethylphosphonic acid (AMPA) ^a | 9, 45, 90, 450, 900, 4500 |
| | Metsulfuron-methyl | 0.26, 1.31, 2.62, 13.11, 26.22, 262.2 |
| | Clethodim | 1.7, 5.1, 15, 46, 138, 415 |
| Fungicides | Pyraclostrobin | 1.58, 4.75, 14.25, 42.71, 128.3, 385 |
| | Etridiazole | 4, 20.2, 40.4, 202, 404, 1010 |
| | Hymexazol | 4.15, 12.45, 37.37, 112.11, 336.3, 1009 |
| | 3,5-Dichloroaniline (3,5-DCA) ^a | 1.3, 3.82, 11.18, 34.44, 103.3, 310 |

^a Transformation products

Table 4.2. Microorganisms that are used in the MARA assay.

| Row no. in the MARA plate | Microbial strain |
|---------------------------|---|
| 1 | <i>Microbacterium sp.</i> (bacterium) |
| 2 | <i>Brevundimonas diminuta</i> (bacterium) |
| 3 | <i>Citobacter freundii</i> (bacterium) |
| 4 | <i>Comamonas testosterone</i> (bacterium) |
| 5 | <i>Enterococcus casseliflavus</i> (bacterium) |
| 6 | <i>Delfia acidovorans</i> (bacterium) |
| 7 | <i>Kurthia gibsonii</i> (bacterium) |
| 8 | <i>Staphylococcus warneri</i> (bacterium) |
| 9 | <i>Pseudomonas aurantiaca</i> (bacterium) |
| 10 | <i>Serratia rubidaea</i> (bacterium) |
| 11 | <i>Pichia anomalia</i> (yeast) |

4.2.2 Preparation of pesticides working solution

The MARA kit is more effective for assessing the toxicity of aqueous samples. However, it can also be used with toxicants based in organic solvents, provided that the solvent is confirmed to be non-toxic to the MARA strains. All pesticides, except for glyphosate and its metabolite AMPA, were dissolved in sterilized dimethyl sulfoxide (DMSO) solutions. The toxicity of DMSO was tested at concentrations of 0.01%, 0.05%, 0.1%, 0.2%, 0.5%, and 1%. The final concentration of DMSO used for dissolving the pesticides was 0.1% (v/v), and did not exert a significant inhibitory effect on any of the strains. Glyphosate and AMPA were dissolved in sterile deionized water (ddH₂O).

4.2.3 MARA assay

The MARA assay (NCIMB, Aberdeen, UK) was conducted in 96-well plates following the manufacturer's instructions. Table 2 lists the microorganisms included on the MARA plate. For each pesticide, three independent determinations were performed. Each plate included both positive controls (without pesticides but with DMSO) and negative controls (without microorganisms). Typically, tetrazolium red dye reduction is used to detect growth differences among microorganisms exposed to varying concentrations of toxicants. However, in this study, absorbance at 600 nm was measured using a Thermo Scientific Multiskan GO Microplate Spectrophotometer after an 18-hour exposure to the tested samples at 30°C in the dark. The absorbance results were analysed using the MARA software provided by the assay manufacturer, with the results expressed as microbial toxic concentration (MTC) values for each microorganism. For the calculation of the MTC values the following formula is used:

$$MTC=c^{\min} d^{(P_{\text{total}}/P_0)-0.5}$$

c^{\min} : lowest tested concentration

d : dilution factor

P total : sum of the pellet sizes in all wells exposed to the tested concentrations

Po: pellet size in the control well

In this work the absorbance was used in the formulation instead of the pellet size.

4.2.4 Calculation of toxicity endpoint values

For the comparison of pesticides toxicity on MARA microbial strains and nitrifying strains the EC_{50} values (half maximal effective concentrations) were determined using the data from the MARA assay. Specifically, EC_{50} values were calculated using the R package drc: Analysis of Dose-Response Curves v3.0-1 package (Ritz and Streibig 2005) based on a dose-response model with normalized data whereby nitrite concentration values were divided by the mean value of the control. A description of the tested models can be found in Ritz et al. (Ritz et al. 2015). For the selection of the best fitting model, an empirical modeling approach was used based on goodness of fit indices, followed by the choice of the four-parameter log logistic model as the best compromise among tested models for comparing toxicity endpoint values.

4.3 Results

4.3.1 Effects of DMSO on MARA microbial strains

Relative to control cultures, DMSO concentrations $\geq 0.2\%$ had inhibitory effects on the MARA strains (Figure 1). A concentration of 1% (v/v) DMSO caused 10-40% inhibition and 0.2% and 0.5% (v/v) also resulted in reduced growth, with approximately 5-30% inhibition. The lowest tested concentrations exhibited a minimal inhibitory effect, with the growth of all strains remaining at or above 96% relative to the control (no DMSO) (Figure 4.1).

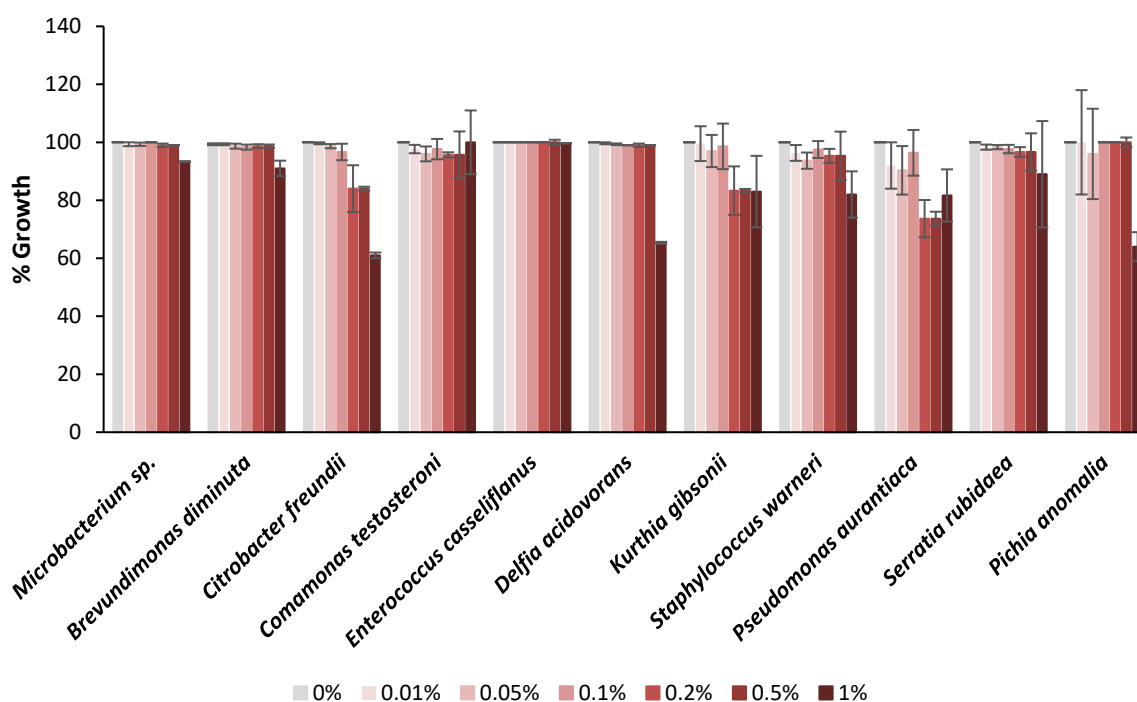


Figure 4.1. Impact of different concentration of dimethyl sulfoxide (DMSO) on the growth of the MARA strains.

4.3.2 Toxicity of pesticides on MARA assay

The results of the MARA kit were estimated for each pesticide based on the average MTC calculated values. The insecticide chlorpyrifos and its transformation product TCP affected the growth of the strains only at high tested concentration with their MTC values being higher than the recommended dose (Table 4.3). The lowest MTC values for chlorpyrifos were observed for *Microbacterium sp.* (41.3 μM), *Kurthia gibsonii* (41.7 μM), *Citrobacter freundii* (45.3 μM) and *Pichia anomalia* (40.3 μM), while for TCP, *Delfia acidovorans* showed the highest sensitivity. The impact of the tested herbicides was also low with all MTC values being higher than the recommended doses of the pesticides. Glyphosate and AMPA were more toxic against *Kurthia gibsonii* with MTC values of 417.5 and 563.6 μM respectively, whereas metsulfuron-methyl and clethodim showed higher toxicity against *Citrobacter freundii* (118 μM) and *Comamonas testosteroni* (138 μM), respectively. Fungicides had the same low toxicity patterns as herbicides and insecticides with the exception of pyraclostrobin, which exhibited high toxicity against *Microbacterium sp.*, with a calculated MTC value of 8.1 μM which was equivalent with the recommended dose. Etridiazole and hymexazol were more toxic to *Brevundimonas diminuta* with MTC values of 565.7 and 122.5 μM respectively, while the MTC for 3,5 DCA was 87.7 μM for *Kurthia gibsonii* (Table 4.3).

Table 4.3. Mean MTC values (μM) of the tested pesticides for each strain of the MARA kit. Standard errors of the mean values are given in brackets. The double asterisk denotes the maximum tested concentration.

| Pesticides | Chlorpyrifos | TCP | Glyphosate | AMPA | Metsulfuron-methyl | Clethodim | Pyraclostrobin | Etridiazole | Hymexazol | 3,5-DCA |
|-----------------------------------|-------------------|--------------------|----------------------|----------------------|--------------------|--------------------|--------------------|--------------------|--------------------|--------------------|
| Recommended dose | 29 | 25 | 59 | 90 | 2.62 | 8.3 | 7.7 | 40.4 | 10 | 3.1 |
| <i>Microbacterium sp.</i> | 41.3(\pm 8.3) | 68.7(\pm 12.4) | 1523.6(\pm 123.4) | 2957.6(\pm 45.4) | 262.2* | 245(\pm 81.5) | 8.1(\pm 0.9) | 648.7(\pm 10.8) | 516(\pm 19.9) | 118.7(\pm 11.5) |
| <i>Brevundimonas diminuta</i> | 54(\pm 16.6) | 115.7(\pm 16.3) | 796.3(\pm 16.6) | 963.3(\pm 34.3) | 250.5(\pm 7.8) | 240.3(\pm 58.7) | 180(\pm 9.3) | 565.7(\pm 24.9) | 122.5(\pm 19.1) | 114.7(\pm 16.5) |
| <i>Citrobacter freundii</i> | 45.3(\pm 15.4) | 68.7(\pm 14.4) | 1477.1(\pm 15.4) | 2563.6(\pm 45.3) | 118(\pm 36.7) | 253.3(\pm 21.2) | 136(\pm 14.8) | 670.3(\pm 23.7) | 372(\pm 10.5) | 185.7(\pm 29.7) |
| <i>Comamonas testosteroni</i> | 71.5(\pm 14.8) | 87.3(\pm 18.4) | 739.6(\pm 45.42) | 2254.1(\pm 18.4) | 239.3(\pm 36.4) | 138(\pm 37.2) | 292.7(\pm 11.4) | 1010* | 683(\pm 32) | 161(\pm 15.4) |
| <i>Enterococcus casseliflanus</i> | 72.3(\pm 6.5) | 156.5(\pm 4.9) | 1771.4(\pm 76.54) | 2963.2(\pm 76.5) | 262.2* | 372.5(\pm 23.3) | 385* | 1010* | 862.3(\pm 16.8) | 280(\pm 24) |
| <i>Delfia acidovorans</i> | 52(\pm 20.5) | 64.7(\pm 5.6) | 2500.7(\pm 23.4) | 3526.3(\pm 54.3) | 193.7(\pm 25.2) | 415* | 273(\pm 12.4) | 955(\pm 2.3) | 814(\pm 16.6) | 149.3(\pm 19.6) |
| <i>Kurthia gibsonii</i> | 41.7(\pm 14.5) | 136.7(\pm 6.5) | 417.5(\pm 23.32) | 563.6(\pm 24.6) | 212(\pm 25.6) | 413.5(\pm 29) | 68(\pm 17.3) | 859.3(\pm 19.4) | 1009* | 87.7(\pm 14.2) |
| <i>Staphylococcus warneri</i> | 54(\pm 11.3) | 85(\pm 16.1) | 534.3(\pm 11.3) | 1200.6(\pm 67.4) | 191.5(\pm 61.5) | 352.7(\pm 27) | 179(\pm 10.7) | 583(\pm 6.3) | 894(\pm 5.3) | 202.7(\pm 17.8) |
| <i>Pseudomonas aurantiaca</i> | 77(\pm 4.4) | 103.5(\pm 14.8) | 2655(\pm 34.5) | 3652.3(\pm 63.3) | 132.7(\pm 17) | 175.5(\pm 12.1) | 163.3(\pm 17.9) | 851.3(\pm 19.8) | 614(\pm 19.4) | 210(\pm 12.6) |
| <i>Serratia rubidaea</i> | 79(\pm 4.7) | 95.3(\pm 14.8) | 2401.3(\pm 85.6) | 3542.8(\pm 8.5) | 250.7(\pm 48.8) | 304.7(\pm 16.1) | 275.7(\pm 17.2) | 706(\pm 25.4) | 684.3(\pm 17.8) | 275(\pm 3) |
| <i>Pichia anomalia</i> | 40.3(\pm 13.1) | 164* | 683.3(\pm 356.4) | 1212.5(\pm 513.1) | 149(\pm 20.3) | 192.3(\pm 14.2) | 64.5(\pm 19.1) | 987(\pm 5.3) | 753.7(\pm 19.9) | 101.3(\pm 19.9) |

4.3.3 Comparison of pesticides toxicity among MARA microbial strains and nitrifying strains

The calculated EC₅₀ values for the MARA strains are presented in Tables 4.4, 4.5, and 4.6. In general, the EC₅₀ values for chlorpyrifos and TCP were higher than their corresponding MTC values across all MARA strains, with the exception of TCP for *Citrobacter freundii*, *Comamonas testosterone*, *Delftia acidovorans*, and *Pseudomonas aurantiaca*. The lowest EC₅₀ values for chlorpyrifos were observed in *Microbacterium sp.* (69.49 µM), *Kurthia gibsonii* (57.88 µM), and *Pichia anomala* (56.74 µM). For TCP, the lowest EC₅₀ was recorded in *Delftia acidovorans* (48.37 µM) (Table 4.4). A similar trend of higher EC₅₀ values was noted for both herbicides and fungicides. For glyphosate and AMPA, lower EC₅₀ values compared to MTC were only observed in *Pseudomonas aurantiaca* and *Serratia rubidaea*, respectively. The lowest EC₅₀ values were recorded for *Kurthia gibsonii* (455.69 µM for glyphosate and 658.95 µM for AMPA). EC₅₀ values for metsulfuron-methyl and clethodim were lower than MTC values only for *Staphylococcus warneri* and *Comamonas testosterone*, respectively. The lowest values were observed for metsulfuron-methyl in *Citrobacter freundii* (145.63 µM) and clethodim in *Comamonas testosterone* (115.63 µM), consistent with the MTC values (Table 4.5). For the fungicide pyraclostrobin, lower EC₅₀ values compared to MTC were seen in *Pichia anomala*, *Pseudomonas aurantiaca*, and *Microbacterium sp.*, the latter showing the lowest EC₅₀. For etridiazole and hymexazol, the lowest EC₅₀ values were observed in *Brevundimonas diminuta* (587.12 µM for etridiazole and 169.65 µM for hymexazol), consistent with the MTC values. For hymexazol, a lower EC₅₀ than the MTC was also observed in *Serratia rubidaea*. EC₅₀ values for 3,5-DCA were higher than the MTC values across all strains, except for *Citrobacter freundii*, *Kurthia gibsonii*, and *Pichia anomala*. The lowest EC₅₀ for this compound was found in *Kurthia gibsonii* (74.93 µM), consistent with the MTC values (Table 4.6).

Table 4.4. Mean EC₅₀ values (µM) of the tested insecticides for each strain of the MARA kit. Standard errors of the mean values are given in brackets. The double asterisk denotes the maximum tested concentration.

| Pesticides | Chlorpyrifos | | TCP | |
|-----------------------------------|--------------|------------------|--------------|------------------|
| | MTC | EC ₅₀ | MTC | EC ₅₀ |
| <i>Microbacterium</i> sp. | 41.3(±8.3) | 69.49(±21.54) | 68.7(±12.4) | 81.44(±9.54) |
| <i>Brevundimonas diminuta</i> | 54(±16.6) | 71.54(±32.65) | 115.7(±16.3) | 164* |
| <i>Citrobacter freundii</i> | 45.3(±15.4) | 71.12(±19.3) | 68.7(±14.4) | 59.77(±11.9) |
| <i>Comamonas testosteroni</i> | 71.5(±14.8) | 99.4(±24.52) | 87.3(±18.4) | 63.14(±14.65) |
| <i>Enterococcus casseliflanus</i> | 72.3(±6.5) | 102.63(±5.69) | 156.5(±4.9) | 164* |
| <i>Delfia acidovorans</i> | 52(±20.5) | 89.01(±8.3) | 64.7(±5.6) | 48.37(±17.96) |
| <i>Kurthia gibsonii</i> | 41.7(±14.5) | 57.88(±9.65) | 136.7(±6.5) | 164* |
| <i>Staphylococcus warneri</i> | 54(±11.3) | 78(±11.95) | 85(±16.1) | 101.91(±12.4) |
| <i>Pseudomonas aurantiaca</i> | 77(±4.4) | 98(±16.47) | 103.5(±14.8) | 94.79(±10.14) |
| <i>Serratia rubidaea</i> | 79(±4.7) | 105.64(±18.62) | 95.3(±14.8) | 107.06(±9.87) |
| <i>Pichia anomalia</i> | 40.3(±13.1) | 56.74(±20.69) | 164* | 164* |

Table 4.5. Mean EC₅₀ values (µM) of the tested herbicides for each strain of the MARA kit. Standard errors of the mean values are given in brackets. The double asterisk denotes the maximum tested concentration.

| Pesticides | Glyphosate | | AMPA | | Metsulfuron-methyl | | Clethodim | |
|-----------------------------------|----------------|------------------|---------------|------------------|--------------------|-----------------|--------------|-----------------|
| | MTC | EC50 | MTC | EC50 | MTC | EC50 | MTC | EC50 |
| <i>Microbacterium sp.</i> | 1523.6(±123.4) | 1654.98(±154.94) | 2957.6(±45.4) | 3214.65(±154.95) | 266.3(±11.6) | 262.2* | 245(±81.5) | 312.58(±43.95) |
| <i>Brevundimonas diminuta</i> | 796.3(±16.6) | 825.21(±35.96) | 963.3(±34.3) | 1054.44(±84.23) | 250.5(±7.8) | 262.2* | 240.3(±58.7) | 265.96(±9.41) |
| <i>Citrobacter freundii</i> | 1477.1(±15.4) | 1547.65(±98.02) | 2563.6(±45.3) | 2854(±98.66) | 118(±36.7) | 145.63(±10.85) | 253.3(±21.2) | 279.549(±10.52) |
| <i>Comamonas testosteroni</i> | 739.6(±45.42) | 754.96(±9.84) | 2254.1(±18.4) | 2463.54(±87.77) | 239.3(±36.4) | 262.2* | 138(±37.2) | 115.63(±29.62) |
| <i>Enterococcus casseliflanus</i> | 1771.4(±76.54) | 1954.01(±154.66) | 2963.2(±76.5) | 3254.14(±104.27) | 262.2* | 262.2* | 372.5(±23.3) | 415* |
| <i>Delfia acidovorans</i> | 2500.7(±23.4) | 2746(±102.69) | 3526.3(±54.3) | 3596.54(±11.09) | 193.7(±25.2) | 215.65 (±11.65) | 415* | 415* |
| <i>Kurthia gibsonii</i> | 417.5(±23.32) | 455.69(±8.02) | 563.6(±24.6) | 658.95(±47.21) | 212(±25.6) | 239.67(±18.54) | 413.5(±29) | 415* |
| <i>Staphylococcus warneri</i> | 534.3(±11.3) | 596.54(±44.06) | 1200.6(±67.4) | 1355.55(±50.95) | 191.5(±61.5) | 184.63 (±8.76) | 352.7(±27) | 375.69(±36.85) |
| <i>Pseudomonas aurantiaca</i> | 2655(±34.5) | 2602.33(±78.96) | 3652.3(±63.3) | 3654.51(±15.66) | 132.7(±17) | 156.52(±24.96) | 175.5(±12.1) | 205.63(±15.24) |
| <i>Serratia rubidaea</i> | 2401.3(±85.6) | 2451(±62.09) | 3542.8(±8.5) | 3254(±24.63) | 250.7(±48.8) | 262.2* | 304.7(±16.1) | 348.96(±22.25) |

Table 4.5. Continued

| Pesticides | Glyphosate | | AMPA | | Metsulfuron-methyl | | Clethodim | |
|------------------------|---------------|----------------|----------------|-----------------|--------------------|----------------|--------------|----------------|
| | MTC | EC50 | MTC | EC50 | MTC | EC50 | MTC | EC50 |
| <i>Pichia anomalia</i> | 683.3(±356.4) | 712.68(±50.77) | 1212.5(±513.1) | 1398.24(±69.58) | 149(±20.3) | 172.21(±29.87) | 192.3(±14.2) | 212.39(±30.05) |

Table 4.6. Mean EC₅₀ values (µM) of the tested fungicides for each strain of the MARA kit. Standard errors of the mean values are given in brackets. The double asterisk denotes the maximum tested concentration.

| Pesticides | Pyraclostrobin | | Etridiazole | | Hymexazol | | 3,5-DCA | |
|-----------------------------------|----------------|------------------|--------------|------------------|--------------|------------------|--------------|------------------|
| | MTC | EC ₅₀ | MTC | EC ₅₀ | MTC | EC ₅₀ | MTC | EC ₅₀ |
| <i>Microbacterium sp.</i> | 8.1(±0.9) | 5.68 (±3.54) | 648.7(±10.8) | 695.22(±56.95) | 516(±19.9) | 752.63(±156.36) | 118.7(±11.5) | 120.24 (±7.63) |
| <i>Brevundimonas diminuta</i> | 180(±9.3) | 206(±21.52) | 565.7(±24.9) | 587.12(±31.58) | 122.5(±19.1) | 169.65(±26.98) | 114.7(±16.5) | 136.21(±10.54) |
| <i>Citrobacter freundii</i> | 136(±14.8) | 151.56 (±15.64) | 670.3(±23.7) | 712.54(±33.54) | 372(±10.5) | 401.54(±35.65) | 185.7(±29.7) | 178.57(±13.47) |
| <i>Comamonas testosteroni</i> | 292.7(±11.4) | 315 (±46.96) | 1010* | 1010* | 683(±32) | 725.14(±21.32) | 161(±15.4) | 201.21(±40.65) |
| <i>Enterococcus casseliflanus</i> | 385* | 385* | 1010* | 1010* | 862.3(±16.8) | 835.24(±27.85) | 280(±24) | >310 |
| <i>Delfia acidovorans</i> | 273(±12.4) | 325(±56.87) | 955(±2.3) | 1010* | 814(±16.6) | 865.92(±47.32) | 149.3(±19.6) | 180.25(±11.5) |
| <i>Kurthia gibsonii</i> | 68(±17.3) | 99.18 (±35.68) | 859.3(±19.4) | 924.65(±20.01) | 1009* | 1009* | 87.7(±14.2) | 74.93(±8.65) |
| <i>Staphylococcus warneri</i> | 179(±10.7) | 215(±29.56) | 583(±6.3) | 612.47(±65.85) | 894(±5.3) | 952.63(±77.58) | 202.7(±17.8) | 227.59(±39.54) |

Table 4.6. Continued.

| Pesticides | Pyraclostrobin | | Etridiazole | | Hymexazol | | 3,5-DCA | |
|-------------------------------|----------------|------------------|--------------|------------------|--------------|------------------|--------------|------------------|
| | MTC | EC ₅₀ | MTC | EC ₅₀ | MTC | EC ₅₀ | MTC | EC ₅₀ |
| <i>Pseudomonas aurantiaca</i> | 163.3(±17.9) | 142.74 (±19.45) | 851.3(±19.8) | 935.85(±29.68) | 614(±19.4) | 658.96(±74.06) | 210(±12.6) | 281.35(±54.05) |
| <i>Serratia rubidaea</i> | 275.7(±17.2) | 335(±69.54) | 706(±25.4) | 774.52(±311.25) | 684.3(±17.8) | 665.91(±85.99) | 275(±3) | >310 |
| <i>Pichia anomalia</i> | 64.5(±19.1) | 54.95 (±14.82) | 987(±5.3) | 1010* | 753.7(±19.9) | 809.54(±35.65) | 101.3(±19.9) | 97.51(±25.84) |

4.3.4 Comparison of pesticides toxicity among MARA microbial strains and nitrifying strains.

Comparison between the calculated EC₅₀ values for MARA strains and nitrifying microorganisms demonstrated that the latter are more sensitive to the majority of the pesticides (Figure 4.2).

Chlorpyrifos exhibited the highest toxicity against AOA strains, with EC₅₀ values being lower than the recommended dose. In contrast, the activity of AOB and NOB was not affected by chlorpyrifos. MARA strains showed higher sensitivity than AOB and NOB, though still lower than AOA strains. TCP had a significantly weaker effect on all strains. Among the strains, only *Nitrosotalea sinensis* was affected by the lowest tested concentrations, resulting in low EC₅₀ values (Figure 4.2a).

The EC₅₀ values for glyphosate and its transformation product AMPA demonstrated a range of effects across different strains. *Brevundimonas diminuta*, *Comamonas testosterone*, *Kurthia gibsonii*, *Staphylococcus warneri*, *Pichia anomala*, *Nitrospira briensis*, *Nitrosomonas ureae*, and *Nitrosomonas communis* showed lower EC₅₀ values, although these were still higher than the recommended dose. The AOB strains were more sensitive to these compounds compared to the MARA strains. Metsulfuron-methyl exhibited higher toxicity toward all AOB strains and *Nitrosotalea sinensis*, with EC₅₀ values equivalent to or lower than the recommended dose. In contrast, its impact on the MARA strains was significantly lower, with EC₅₀ values exceeding the recommended dose. Clethodim displayed low toxicity across all tested strains, with the exception of *Nitrosotalea sinensis* and *Nitrosomonas europaea* (Figure 4.2b).

The tested fungicides impacted all AOA and AOB strains, with their EC₅₀ values markedly lower than those observed for the MARA strains. Pyraclostrobin exhibited high toxicity across all AOA and AOB strains, as well as the *Ca. Nitrobacter laanbroekii* NHB1 strain, with EC₅₀ values equal to or lower than the recommended dose. The MARA strains were only affected by the highest tested concentrations, except for *Microbacterium* sp., whose EC₅₀ value was equal to the recommended dose. Etridiazole inhibited all AOA and AOB strains across all tested concentrations, producing very low EC₅₀ values. In contrast, the MARA strains were only affected at the highest concentrations, with EC₅₀ values significantly higher than the recommended dose. Hymexazol demonstrated a consistently low level of toxicity across all studied strains. 3,5-DCA had the highest toxicity on all AOB strains and *Nitrosotalea sinensis*, with EC₅₀ values lower than the recommended dose, while the EC₅₀ values for these strains were higher than the recommended dose (Figure 4.2c).

Insecticides

a)

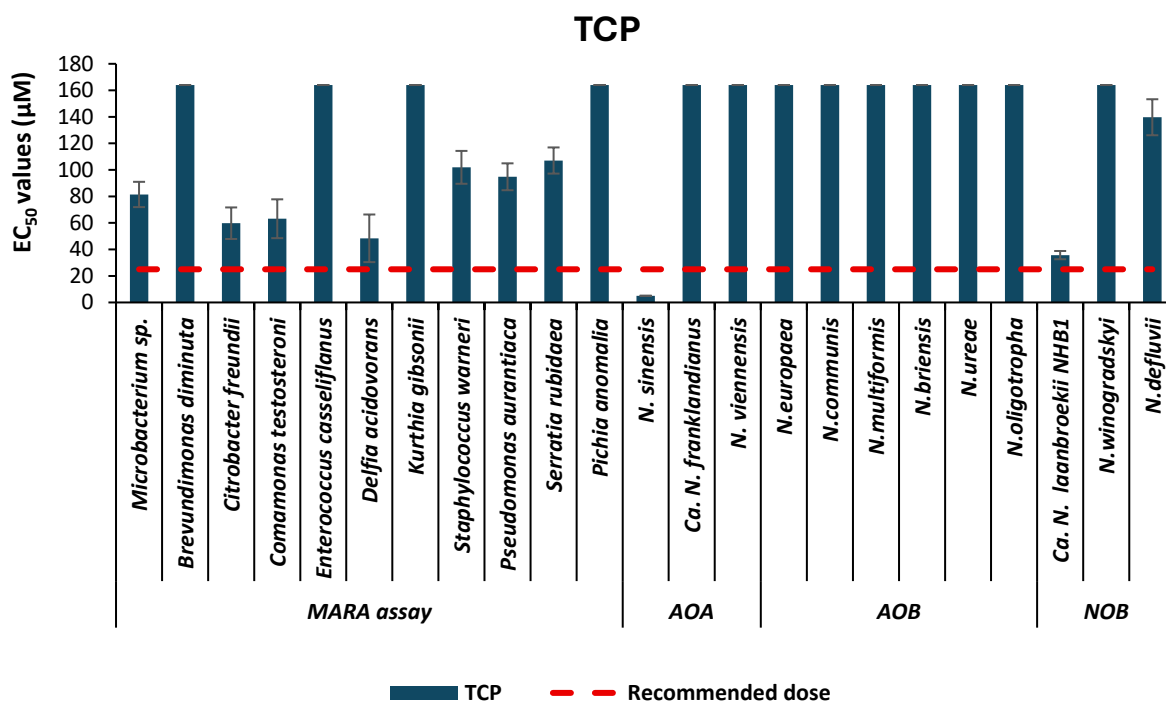
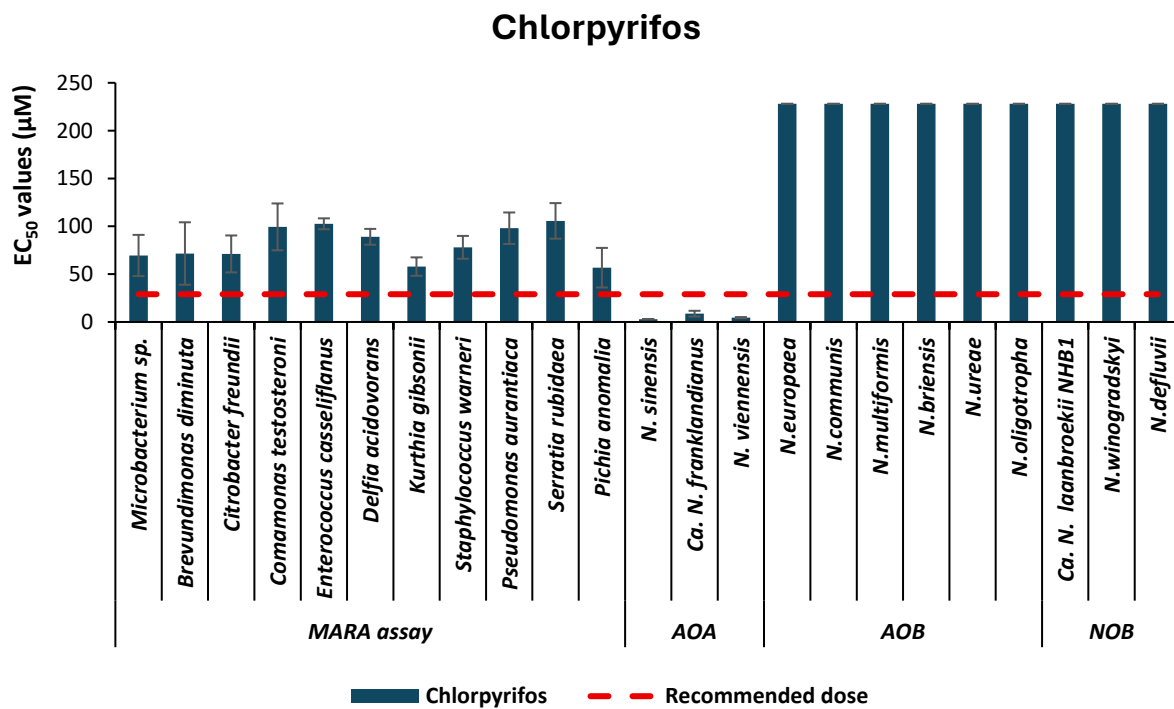


Figure 4.2. Comparison of the sensitivity of MARA strains, ammonia oxidizing archaea (AOA) and bacteria (AOB) and nitrite oxidizing bacteria (NOB), using calculated MTC and EC₅₀ values, respectively for (a) insecticides, (b) herbicides, and (c) fungicides.

Herbicides

b)

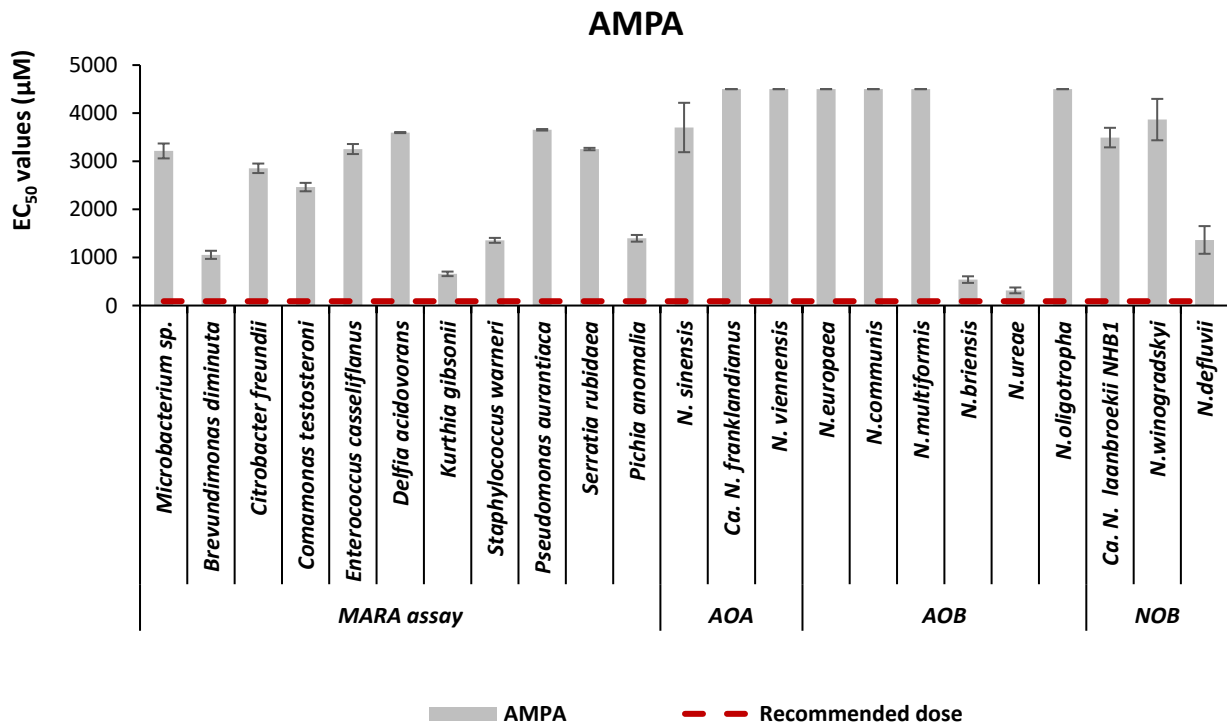
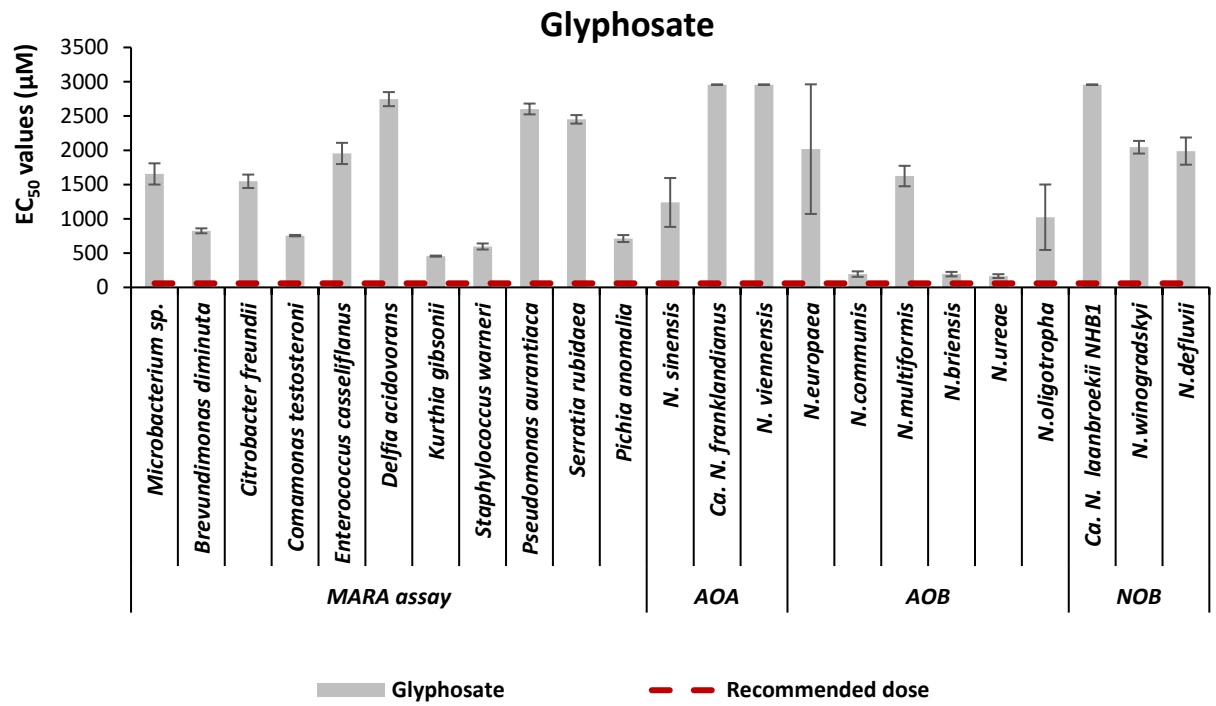


Figure 4.2. Comparison of the sensitivity of MARA strains, ammonia oxidizing archaea (AOA) and bacteria (AOB) and nitrite oxidizing bacteria (NOB), using calculated MTC and EC₅₀ values, respectively for (a) insecticides, (b) herbicides, and (c) fungicides. 141

Herbicides

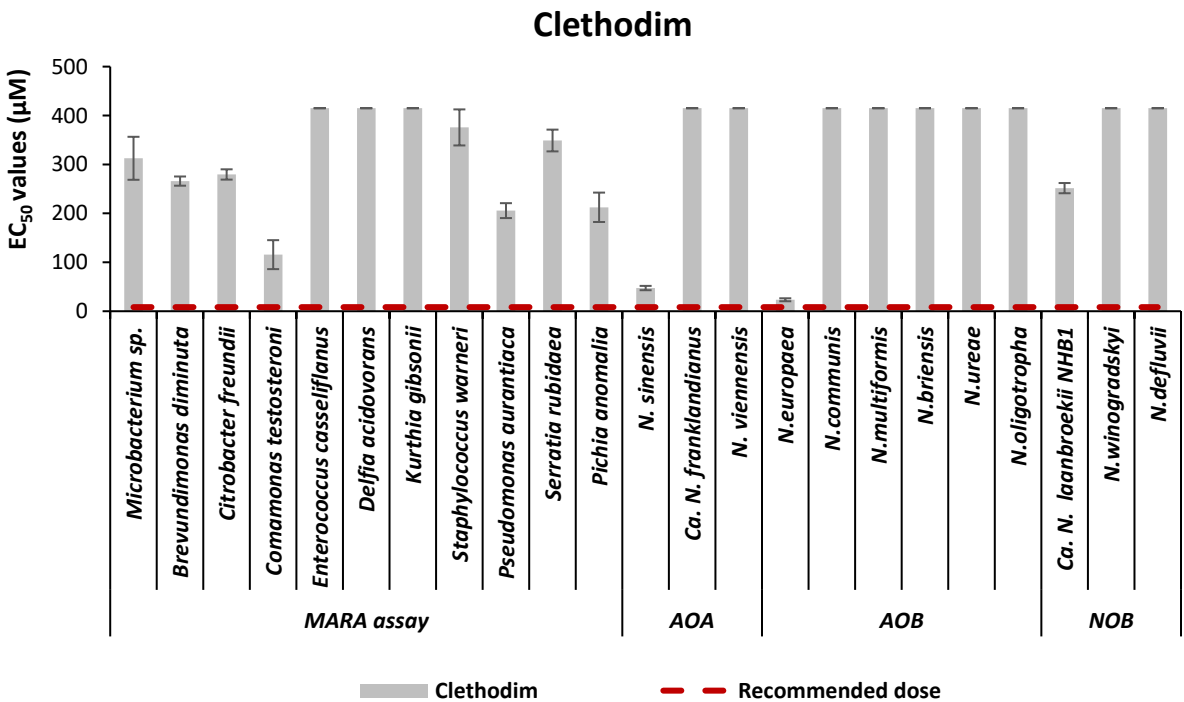
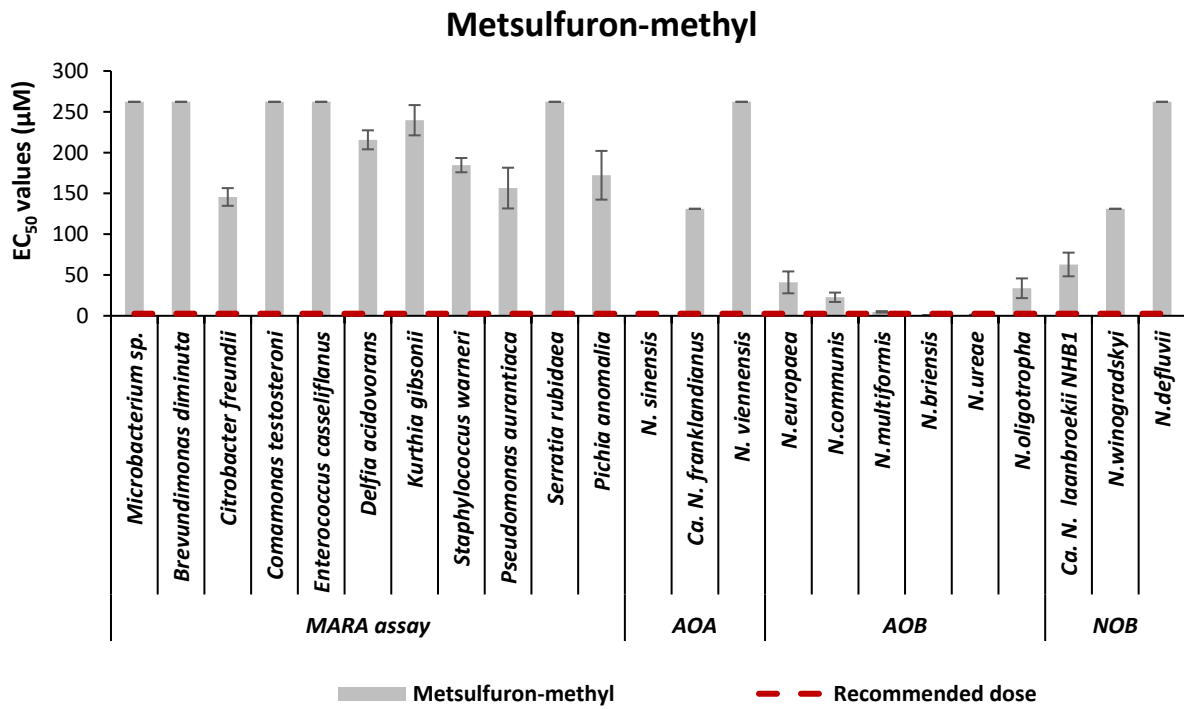


Figure 4.2. Comparison of the sensitivity of MARA strains, ammonia oxidizing archaea (AOA) and bacteria (AOB) and nitrite oxidizing bacteria (NOB), using calculated MTC and EC₅₀ values, respectively for (a) insecticides, (b) herbicides, and (c) fungicides.

Fungicides

c)

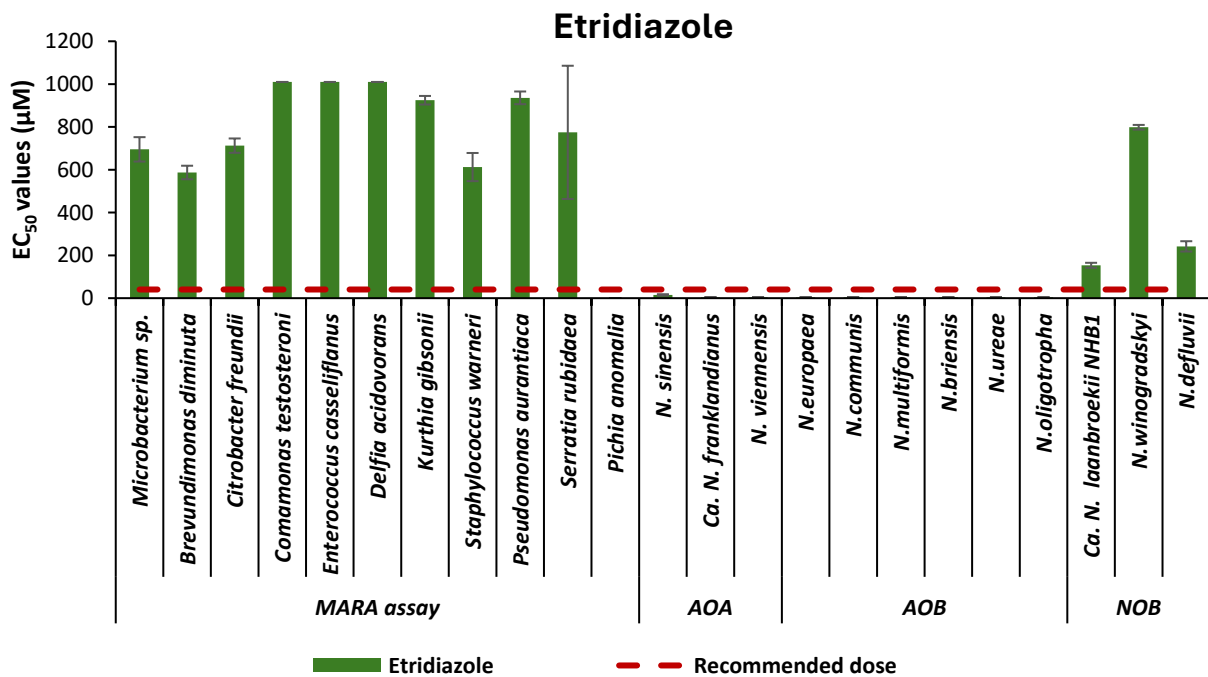
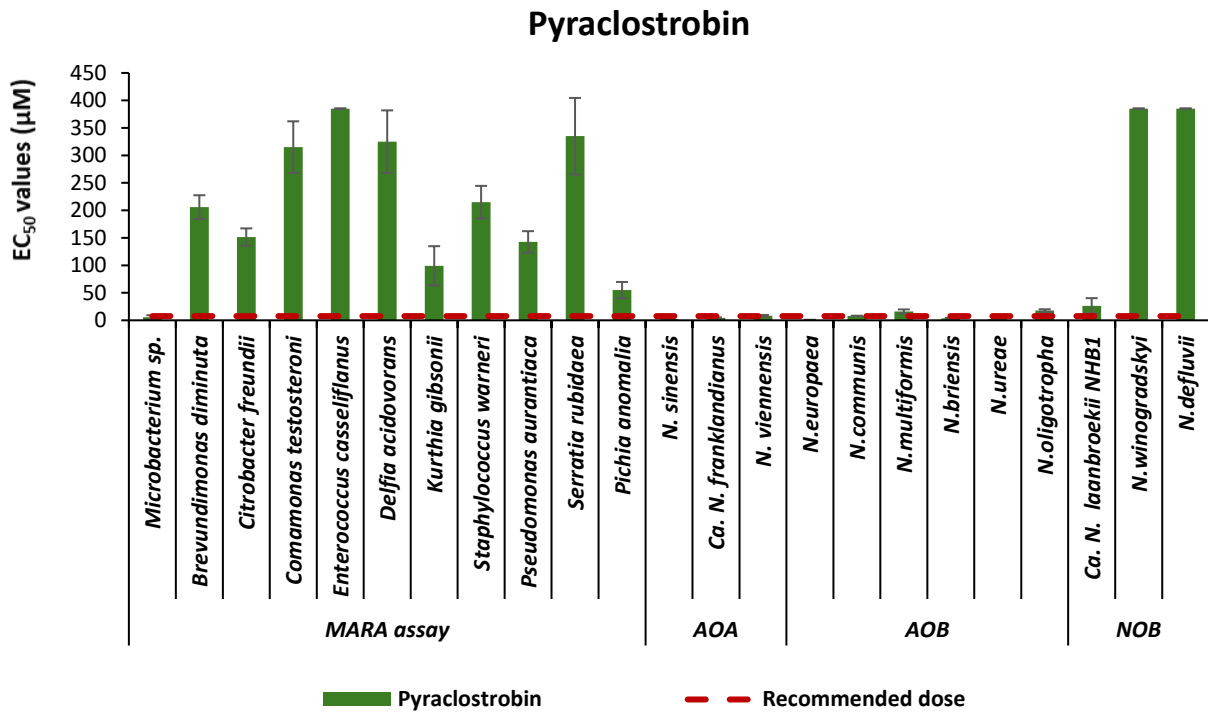


Figure 4.2. Comparison of the sensitivity of MARA strains, ammonia oxidizing archaea (AOA) and bacteria (AOB) and nitrite oxidizing bacteria (NOB), using calculated MTC and EC₅₀ values, respectively for (a) insecticides, (b) herbicides, and (c) fungicides.

Fungicides

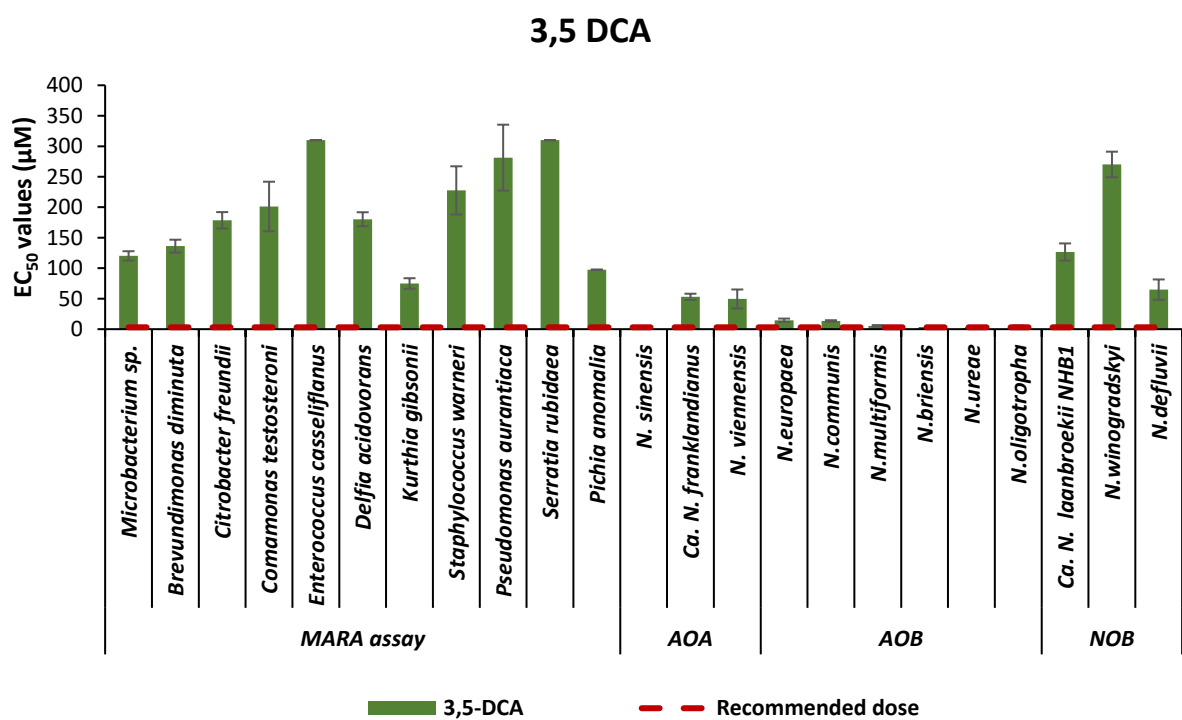
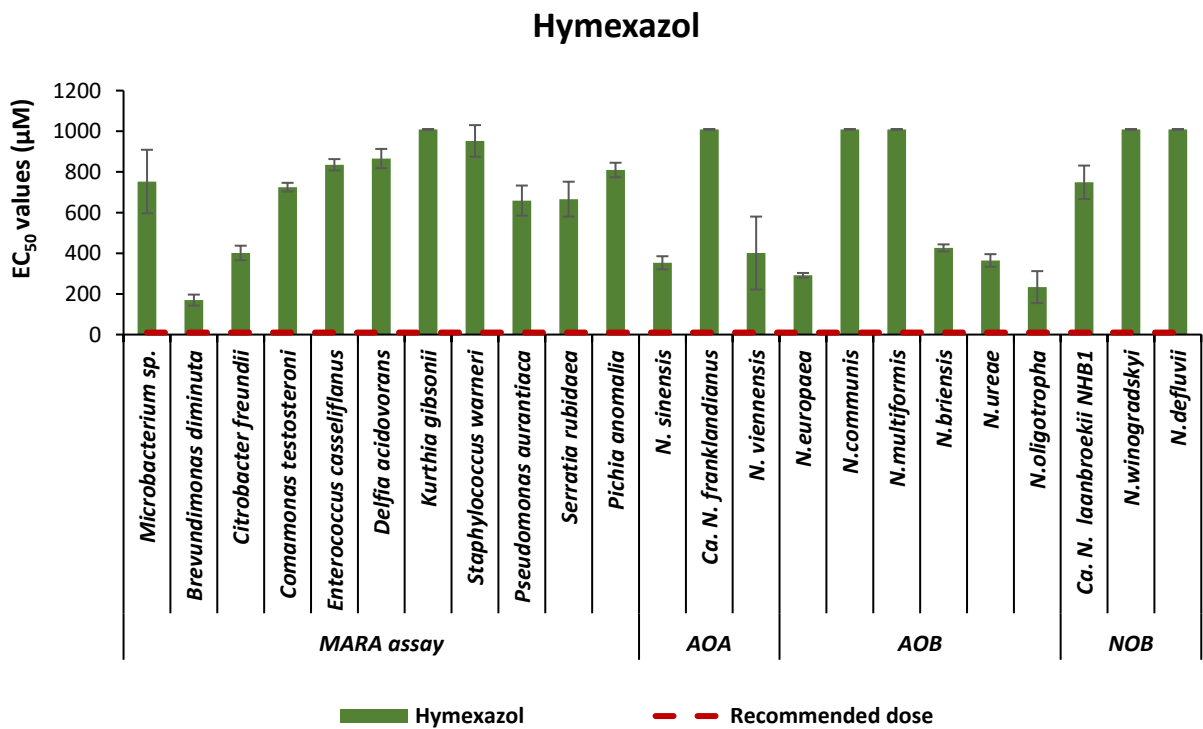


Figure 4.2. Comparison of the sensitivity of MARA strains, ammonia oxidizing archaea (AOA) and bacteria (AOB) and nitrite oxidizing bacteria (NOB), using calculated MTC and EC₅₀ values, respectively for (a) insecticides, (b) herbicides, and (c) fungicides.

4.4 Discussion

The impact of pesticides on soil microorganisms has received considerable attention recently, given the essential role these microorganisms play in maintaining ecosystem health. However, despite this focus, our understanding remains limited, and there is a significant gap in the availability of effective tools, testing methods, and bioindicators. While advanced molecular tools are currently employed to study the toxicity of pesticides on soil microorganisms, an alternative approach could be the use of existing toxicity kits, which have been previously applied in assessing ecotoxicity in aquatic organisms. In this study, we evaluated the toxicity of widely used pesticides utilizing the MARA kit and compared these results with the sensitivity of soil nitrifiers to the same pesticides, drawing on previous findings (refer to Chapter 2). This comparison is particularly relevant as we have previously proposed AOM as key bioindicators for the environmental risk assessment of pesticides on soil microbiota.

The first step in our study was to evaluate the toxicity of pesticides using the MARA kit. The pesticides tested had different effects on the MARA strains. Overall, the pesticides demonstrated a relatively weak effect, inhibiting or reducing the growth of the strains only at the highest tested concentrations, with calculated MTC values exceeding the recommended concentrations. The only exception was pyraclostrobin, where the MTC for *Microbacterium* sp. was equivalent to the recommended dose. This low toxicity observed in MARA strains is consistent with findings from other studies that assessed the toxicity of different compounds. For example, similar sensitivity patterns were observed when MARA strains were exposed to cyanobacterial toxins, which only affected growth at high concentrations that are unlikely to occur in nature (Sieroslawska 2014). In contrast, when assessing the impact of antibiotics, the growth of the MARA strains was significantly inhibited, with calculated MTC values notably low (Fai and Grant 2010). This likely reflects the diverse effects of antibiotics on different bacteria. Nanomaterials showed a broader range of impacts on the MARA strains, depending on the specific compound (Blaise et al. 2008).

The taxonomic diversity offered by the MARA kit likely accounts for the varying toxicity patterns observed, as each strain exhibited distinct sensitivity to the tested pesticides (Wadhia and Thompson 2007). Notably, only glyphosate and its transformation product, AMPA, had a greater impact on the same strain, *Kurthia gibsonii*. Similarly, hymexazol and etridiazole, both belonging to the azole chemical group, showed a higher impact on the same strain, *Brevundimonas diminuta*.

In our previous work, we proposed that AOM are ideal candidates for single species tests for assessing the risk of pesticides to soil microorganisms due to their high sensitivity to these compounds *in vitro* (see Chapter 2). In comparison to MARA strains, AOM exhibited significantly higher sensitivity than MARA strains. Notably, AOA were more sensitive than both the MARA strains and also AOB and NOB. The MARA strains were only affected by the highest concentrations of insecticides with AOB and NOB showing no significant growth inhibition under similar conditions. In contrast, the tested fungicides had greater toxicity to AOB and NOB compared to the MARA strains. The greater sensitivity of soil nitrifiers aligns with findings from previous studies, where environmental sample toxicity was evaluated

using the MARA kit, the Microtox kit, and a nitrification inhibition test. Although the MARA strains exhibited greater sensitivity than those in the Microtox kit, the nitrification inhibition test revealed a much stronger toxic effect, highlighting the greater vulnerability of nitrifiers compared to the MARA strains (Wadhia and Thompson 2007).

Our findings suggest that soil nitrifiers may serve as more effective bioindicators for pesticide risk assessment on soil microorganisms compared to commercially available kits like Microtox and MARA. The MARA kit, recognized for its high sensitivity to a wide range of compounds (Blaise et al. 2008; Fai and Grant 2010), is one of the approved toxicity tests proposed by the UK government for managing produced water discharges from offshore oil and gas installations (“The United Kingdom Risk-Based Approach Programme, A risk-based approach to the management of produced water discharges from offshore installations,” n.d.). However, despite its taxonomic diversity, the MARA kit's strains are isolated from various environments and do not specifically represent soil microbial communities (Wadhia and Thompson, 2007). According to EFSA guidelines, as well as numerous other studies, soil microorganisms involved in nutrient cycling and biochemical processes are more appropriate for evaluating pesticide toxicity. These microorganisms play crucial roles in maintaining soil health and ecosystem functionality, making them superior indicators of pesticide impacts on soil ecosystems. Therefore, incorporating soil nitrifiers into risk assessment frameworks could offer a more accurate and ecologically relevant evaluation of pesticide toxicity, thereby enhancing our ability to protect and manage soil health effectively (European Food Safety Authority 2010; Karpouzas et al. 2016; Karpouzas et al. 2022).

4.5 Conclusion

This study highlights the limitations of the Microbial Assay for Risk Assessment (MARA) in detecting pesticide toxicity, particularly in comparison to soil nitrifiers. Our results show that AOMs, particularly ammonia-oxidizing archaea (AOA), are significantly more sensitive to pesticide exposure than MARA strains. Given their critical role in soil health and their enhanced sensitivity, the use of AOM represents a valuable opportunity to improve environmental risk assessment. The results support the potential development of a specialized test kit, similar to MARA, but focused on soil nitrifiers, which would provide a more accurate and sensitive tool for assessing pesticide toxicity in soil ecosystems. By incorporating AOM, such a kit could facilitate high-throughput testing of pesticides, providing a faster and more accurate method for assessing environmental impact. Future research should focus on refining and validating this approach, with the aim of integrating it into regulatory frameworks to improve soil health protection and support sustainable agricultural practices.

4.5 Acknowledgments

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Chapter 5

Acidotolerant soil nitrite oxidiser '*Candidatus* Nitrobacter laanbroekii' NHB1 alleviates constraints on growth of acidophilic soil ammonia oxidisers

Statement on author contributions

The work presented in Chapter 5 is a manuscript currently under revision for consideration of publication in the journal *ISME Communications* (L. Hink, E. Bachtsevani, Y. Meng, C.J. Sedlacek, S. Lee, H. Daims, M. Wagner, C. Gubry-Rangin, W. de Boer, C. Hazard, J.I. Prosser & G.W. Nicol. In bioRxiv. <https://doi.org/10.1101/2024.07.06.601931>). L. Hink and E. Bachtsevani are joint first authors and performed all growth and physiology work. Y. Meng (University of Aberdeen) isolated strain NHB1 in 2012, C.J. Sedlacek (University of Vienna) performed microrespirometry in 2015, and S. Lee performed genome sequencing and phylogenomic analysis in 2016. All other authors were supervisors or supplied strains/reagents. The manuscript presented here was written (equal contribution) by L. Hink and E. Bachtsevani with comments from all authors.

Abstract

Nitrobacter strain NHB1 is a nitrite-oxidising bacterium previously co-enriched with the neutrophilic ammonia-oxidising bacterium *Nitrosospira* AHB1, a consortium that nitrifies under acidic conditions. Here we characterise the growth of isolated *Nitrobacter* strain NHB1 as a function of pH and nitrite (NO_2^-) concentration, and its influence on the activity of acidophilic soil ammonia-oxidising archaea (AOA) in co-culture. NHB1 is acidotolerant and grows optimally at pH 6.0 (range 5.0 – 7.5) at initial NO_2^- concentrations of 500 μM . However, the optimum decreases to pH 5.0 at lower initial NO_2^- concentrations closer to those found in soil, with detectable growth down to pH 3.5. NHB1 has a comparatively high affinity for NO_2^- with an apparent-half-saturation constant (54 μM) one order of magnitude lower than its closest relative, the neutrophilic strain *Nitrobacter hamburgensis* X14. In co-culture, NHB1 enhances the growth of acidophilic AOA. Specifically, *Nitrosotalea devaniterrae* Nd1 and *Nitrosotalea sinensis* Nd2 are sensitive to NO_2^- -derived compounds and only oxidise ~200–300 μM ammonia (NH_3) in batch cultures. However, in co-culture with NHB1, pH ranges were lowered by ~0.5 pH units and both strains could oxidise up to 2.7–2.9 mM NH_3 , only limited by buffering capacity. NHB1 possesses a cyanase facilitating reciprocal cross-feeding via generating cyanate-derived NH_3 and utilising AOA-derived NO_2^- . Removal of NO_2^- is likely crucial for nitrifier growth in acidic soils and this study highlights the importance of considering substrate and metabolic product concentrations when characterising physiology. Genome analysis reveals that NHB1 is distinct from validated species and the name '*Nitrobacter laanbroekii*' is proposed.

Keywords: *Nitrobacter*, nitrite-oxidising bacteria, ammonia-oxidising archaea, acidophilic, acidic soil, ammonia oxidation, nitrite oxidation, nitrification, cyanase

5.1 Introduction

Microbially-mediated nitrification in soil is typically dominated by chemolithoautotrophs. Canonical ammonia-oxidising archaea (AOA) and bacteria (AOB) can perform the first step whereby ammonia (NH_3) is oxidised to nitrite (NO_2^-) and is coupled to the activity of canonical nitrite-oxidising bacteria (NOB) which subsequently oxidise NO_2^- to nitrate (NO_3^-). In acidic soils, ammonia oxidation activity was considered paradoxical due to the reduced availability of NH_3 (pK_a for $\text{NH}_3:\text{NH}_4^+ = 9.25$). In addition, ammonia oxidisers (AO) are sensitive to NO_2^- -derived nitrous acid (HNO_2) and associated decomposition products formed at low pH (Braida & Ong, 2000), necessitating the use of buffers or neutralising media to sustain growth. The subsequent discovery and cultivation of acidophilic and acidotolerant AO explained this paradox to a large extent (Hayatsu et al., 2017; L. E. Lehtovirta-Morley et al., n.d.; Picone et al., 2021; Wang et al., 2021), but mechanisms including ureolytic activity (Boer et al., 1989; Burton & Prosser, 2001) or growth within biofilms (Allison & Prosser, 1993)

or aggregates (Boer et al., 1991) also facilitate growth of neutrophilic nitrifiers under acidic conditions. As canonical AO are present in environments together with NOB, enabling mutualistic interactions (Boer et al., 1991; Palatinszky et al., 2015), the cooperative activity of NOB in soil is likely crucial for AO activity.

Nitrobacter populations are a major component of soil NOB communities. While most isolates are grown at neutral pH (Bock et al., 1990), acidophilic/acidotolerant strains have been reported (Boer et al., 1991; Hankinson & Schmidt, 1988). These include *Nitrobacter* NHB1 which was originally cultivated from pH 3.8 fertilised heathland soil together with the AOB *Nitrosospira* AHB1 (de Boer & Laanbroek, 1989). Although neutrophilic, AHB1 was active down to pH 4 when grown with NHB1 (Boer et al., 1995) and neutrophilic *Nitrosospira*-like bacteria enriched from acidic forest soil grew at pH 4 when surrounded by *Nitrobacter*-like bacteria in aggregates (Boer et al., 1991). Acidotolerant NOB may therefore have a role in protecting ammonia oxidisers in acidic soils by removing NO_2^- before abiotic conversion to toxic compounds.

In this study we investigated the cell structure, genome content and growth characteristics of the isolate NHB1, including the effect of pH on its growth and a potential influence of NO_2^- concentration in defining its pH range. As isolated acidophilic AOA are particularly sensitive to NO_2^- -derived compounds in culture, we tested the hypothesis that NHB1 will positively impact the growth characteristics of *Nitrosotalea* strains in co-culture via continuous nitrite removal, as typically occurs in soil.

5.2 Material and Methods

5.2.1 Isolation and maintenance of 'Ca. *Nitrobacter laanbroekii*' NHB1 in culture

The co-culture of *Nitrosospira* sp. AHB1 and 'Ca. *N. laanbroekii*' NHB1 was obtained from cultures established in 1988 using pH 3.8 heathland soil (Hoorneboeg; 52°15'N, 5°10'E) under *Calluna vulgaris* (Heather) and *Deschampsia flexuosa* (Wavy hair-grass) vegetation (Boer et al., 1989; de Boer et al., 1988). Cells from a cryostock were resuscitated by growing in 50 ml unbuffered medium with 2.5 mM NH_4^+ adjusted to pH 7. Specifically, the medium contained (L^{-1}): KH_2PO_4 (0.1 g); NaCl (0.5 g); $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.04 g); $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.02 g); $(\text{NH}_4)\text{-SO}_4$ (0.33 g); $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (2.46 mg); $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ (0.1 mg); MnCl_2 (0.2 mg); Na_2EDTA (3.31 mg); $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.1 mg), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (20 μg), and CoCl_2 (2 μg) (de Boer et al., 1988). Cultures were incubated in the dark at 25°C without shaking. The same medium was used to purify 'Ca. *N. laanbroekii*' NHB1, except the pH was reduced to 5.5 and $(\text{NH}_4)_2\text{SO}_4$ replaced with 500 μM NaNO_2 (0.035 g L^{-1}). Isolation was achieved by routine transfer (approximately weekly) of 1 ml exponentially growing culture into 50 ml fresh medium. The purity of 'Ca. *N. laanbroekii*' NHB1 was initially confirmed by the absence of ammonia oxidation activity when inoculated into fresh $(\text{NH}_4)_2\text{SO}_4$ -containing medium, loss of the band corresponding to the 16S rRNA gene amplicon of *Nitrosospira* sp. AHB1 in denaturing gradient gel electrophoresis

(DGGE) analysis, and phase contrast microscopy (data not shown). Purity was confirmed by the absence of any contaminating DNA during genome sequencing and assembly.

The suitability of an acidophilic ammonia-oxidising archaea (AOA) medium for growing '*Ca. N. laanbroekii*' NHB1 in potential co-culture experiments was assessed using NO_2^- instead of NH_4^+ as an energy source. Specifically, acidic 'freshwater medium' (FWM) (Laura E. Lehtovirta-Morley et al., 2011) consisted of NaCl (1 g L^{-1}), MgCl_2 (0.4 g L^{-1}), CaCl_2 (0.1 g L^{-1}), KH_2PO_4 (0.2 g L^{-1}), KCl (0.5 g L^{-1}), 1 ml modified non-chelated trace element solution (Widdel & Bak, 1992), 1 ml 7.5 mM NaFeEDTA , 2 mM NaHCO_3 , and 500 μM NaNO_2 (replacing 500 μM NH_4Cl), was buffered with 10 mM 2-(N-morpholino)ethanesulfonic acid (MES) and adjusted to pH 5.5 before sterilisation by filtration using a GL45 bottle-top 0.2 μm filter unit (Nalgene, Rochester, USA). Successful growth of '*Ca. N. laanbroekii*' NHB1 in this medium resulted in its subsequent use for routine growth and maintenance.

5.2.2 Ammonia, nitrite and nitrate concentration measurements

All inorganic N concentrations were measured colorimetrically in 96-well plates using 50 μl of growth medium or adequate dilutions as described previously (Hink et al., 2018). Briefly, NH_4^+ concentration was estimated using the indophenol method (Kandeler & Gerber, 1988). NO_2^- and NO_3^- concentrations were estimated using a method modified from Shinn (Shinn, 1941) and Doane and Horwath (Doane & Horwath, 2003). NO_2^- was detected by adding 60 μl of diazotising reagent (2.2 mM sulphanilamide in 3.3 M HCl) followed by 20 μl coupling reagent (0.12 mM N-(1-naphthyl)-ethylenediamine in 0.12 M HCl). NO_2^- concentration was estimated immediately before reducing NO_3^- to NO_2^- by adding 20 μl vanadium chloride solution (4.5 mM vanadium(III) chloride in 1 M HCl), incubating for 90 min at 35°C in the dark and measuring $\text{NO}_2^- + \text{NO}_3^-$ concentration.

5.2.3 Characterisation of *Ca. Nitrobacter laanbroekii* NHB1

To determine temperature, pH and NO_2^- concentration range, triplicate 50 ml cultures were statically incubated in sterile 100 ml culture bottles in the dark between 4 and 35°C, in medium adjusted to pH 3.0 to 8.0, and containing initial NO_2^- concentrations between 20 μM and 10 mM, respectively. As NO_2^- is known to degrade abiotically, particularly at pH lower than 5.5, triplicate sterile controls were also incubated with each treatment. All cultures were inoculated with 1% (vol/vol) early stationary phase culture that had consumed 500 μM NO_2^- . The growth of cultures was monitored via regular assessment of NO_2^- concentrations. As NO_2^- was stoichiometrically converted to NO_3^- by '*Ca. N. laanbroekii*' NHB1 (Figure 5.1), measurement of NO_3^- concentrations was not performed on a regular basis, but NO_3^- production was calculated via NO_2^- consumption after calculating the predicted abiotic degradation rate of NO_2^- , particularly under acidic pH (Figure 5.2), enabling calculation of μ_{max} during the exponential growth phase of the cultures (Figure 5.3).

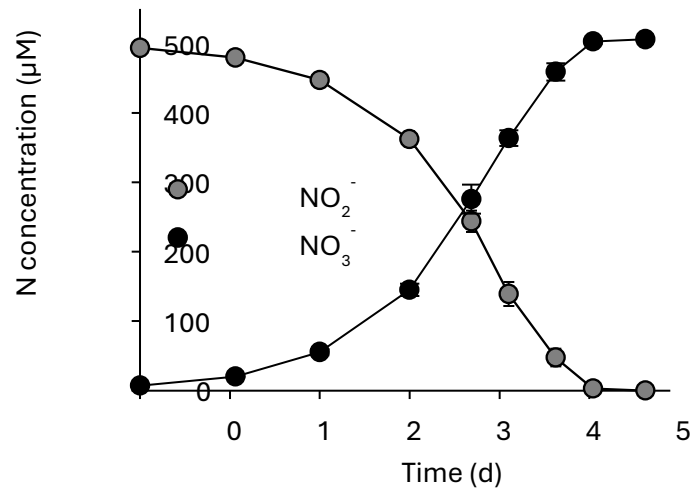


Figure 5.1. Stoichiometric relationship of NO_2^- consumption and NO_3^- production during growth of '*Ca. Nitrobacter laanbroekii*' NHB1. The medium was adjusted to pH 5.5 with an initial NO_2^- concentration of 500 μM and inoculated with 1% early stationary culture (vol/vol) and incubated at 25°C in the dark. Plotted values are the mean and standard errors of NO_2^- and NO_3^- concentrations of triplicate cultures. The confirmed stoichiometry between NO_2^- consumption and NO_3^- production enabled the use of NO_2^- concentrations to infer NO_3^- concentrations in subsequent culture characterisation experiments.

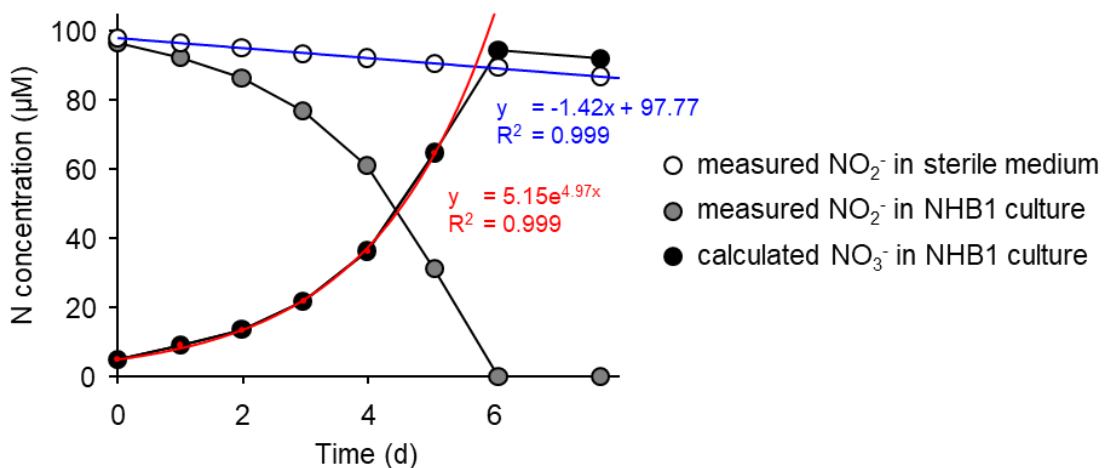


Figure 5.2. An example of assessing μ_{max} of '*Ca. Nitrobacter laanbroekii*' NHB1 when grown at pH 4.5 with an initial 100 μM NO_2^- concentration. Medium was inoculated with a 1% transfer (vol/vol) of an early stationary culture that had consumed 500 μM NO_2^- . Sterile medium served as control to monitor abiotic NO_2^- degradation following a linear decline over time (blue regression and equation). Measured NO_2^- concentrations in the growing culture and the abiotic degradation rate NO_2^- in the sterile control was used to calculate predicted NO_3^- concentrations. An exponential curve was then fitted to the NO_3^- data during exponential growth of the culture (red circles, regression and equation). The parameter in the exponent of the equation corresponds to the μ_{max} value of the culture.

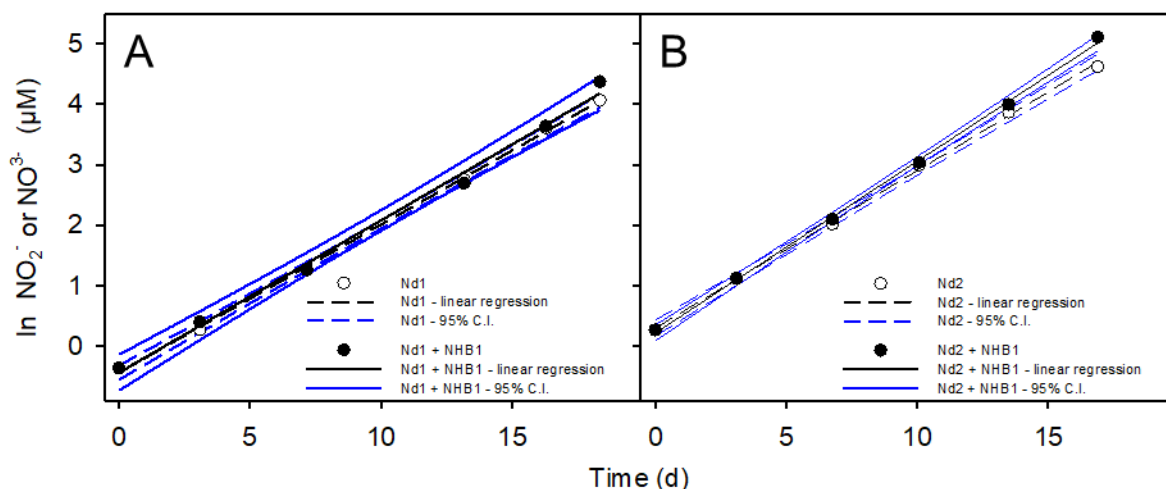


Figure 5.3. Examples of plots used for calculating maximum specific growth rates of *Nitrosotalea devaniterrae* Nd1 and *Nitrosotalea sinensis* Nd2 (assessed via NO_2^- production) or in co-culture with '*Ca. Nitrobacter laanbroekii*' NHB1 (assessed via NO_3^- production) in medium supplied with $500 \mu\text{M NH}_4^+$. Linear regression and 95% confidence intervals (C.I.) were calculated from natural logarithm (\ln) values of NO_2^- concentrations during the exponential phase of growth.

5.2.4 Growth of *Nitrosotalea* strains in isolation or co-culture with '*Ca. Nitrobacter laanbroekii*' NHB1

Cultures of isolated *Nitrosotalea devaniterrae* Nd1 and *Nitrosotalea sinensis* Nd2 or stable co-cultures of *N. devaniterrae* Nd1 or *N. sinensis* Nd2 with '*Ca. N. laanbroekii*' NHB1 were established by inoculating 1 ml of early stationary phase cultures into 50 ml standard FWM medium for acidophilic AOA (pH 5.2) containing $500 \mu\text{M NH}_4\text{Cl}$ in sterile 100 ml culture bottles. While *N. devaniterrae* Nd1 and '*Ca. N. laanbroekii*' NHB1 have similar temperature ranges and growth optima, *N. sinensis* Nd2 grows optimally $\sim 35^\circ\text{C}$ (Laura E. Lehtovirta-Morley et al., 2014) and above the temperature range of '*Ca. N. laanbroekii*' NHB1. A temperature of 25°C was therefore used for isolation vs co-culture experiments. Growth of (co-)cultures was monitored via measurement of NH_4^+ , NO_2^- and NO_3^- concentrations until stationary phase. The effect of pH and substrate concentration was determined in 30 ml sterile plastic Universal bottles (Greiner Bio-One, Les Ulis, France) containing 20 ml of medium. Co-cultures can be maintained indefinitely by transfer (2% vol/vol) every 2-3 weeks into sterile medium.

In co-culture experiments to examine the potential cyanase activity of NHB1, ammonium was substituted with 50 or $500 \mu\text{M}$ cyanate. In experiments examining yields of NO_3^- with higher NH_4^+ concentrations, despite the absence of NO_2^- -derived acids in co-culture, pH decreased with growth and higher concentrations of MES (both 20mM and 50 mM) were required to sustain growth after consumption of $\sim 800 \mu\text{M NH}_4^+$.

5.2.5 Determination of substrate kinetics

Cellular nitrite oxidation kinetics were determined from instantaneous substrate-dependent oxygen uptake measurements as previously described using a multiple injection method (Kits et al., 2017; Martens-Habbena & Stahl, 2011). Briefly, measurements were performed in a water bath at 25°C with a microrespirometry (MR) system, equipped with a PA2000 picoammeter and a 500 µm tip diameter OX-MR oxygen microsensors (Unisense, Aarhus, Denmark), polarized continuously for at least 24 h before use. Active NHB1 cells were taken from early stationary phase cultures soon after substrate depletion or harvested and concentrated (6000 x g, 10 min, 20 °C) from NO₂⁻ replete active cultures using Amicon Ultra-15 10 kDa cut-off centrifugal filter units (Merck Millipore, Darmstadt, Germany). Concentrated cells were washed with and resuspended in substrate-free medium (pH 6) prior to MR measurements. Whole cell activity rates of NHB1 were fit with the Michaelis-Menten model to derive the apparent whole cell reaction half saturation concentration ($K_{m(app)}$; µM NO₂⁻). The $K_{m(app)}$ of '*Ca. N. laanbroekii*' NHB1 was compared with other nitrite oxidiser strains using previously published values (Both et al., 1992; Kitzinger et al., 2018; Nowka et al., 2015; Ushiki et al., 2017).

5.2.6 Transmission electron microscopy

NHB1 cells were recovered from 1 L of late exponential culture by filtering onto a 0.2 µm mixed cellulose ester filter before resuspending cells in 2 ml fresh medium and pelleting by centrifugation at 14,000 x g for 40 min. One volume (500 µl) 4% glutaraldehyde (v/v) was added to ~500 µl medium overlying the pellet and stored at 4°C overnight. Cells were then post-fixed with 1% OsO₄ in 0.3 M cacodylate buffer (pH 7.4) for 1 h at 4°C before ethanol dehydration and transfer to propylene oxide. Cells were embedded in Epon epoxy resin and inclusion obtained by polymerisation at 60°C for 72 h. Ultra-thin sections (100 nm) were cut using a UC7 ultramicrotome (Leica, Nanterre, France), mounted on 200 mesh copper grids (EMS, Hatfield, USA) and contrasted with uranyl acetate and lead citrate. Sections were examined with a JEM-1400 120 kV transmission electron microscope (Jeol, Tokyo, Japan) equipped with a Orius 1000 camera (Gatan, Pleasanton, USA) in the wide-field position and Digital Micrograph software (Gatan) at the Centre d'Imagerie Quantitative Lyon-Est, Université Claude Bernard Lyon 1.

5.2.7 Genome sequencing, assembly and annotation

DNA was extracted from pelleted cells using a standard SDS buffer and phenol:chloroform:isoamyl alcohol chemical lysis method (Laura E. Lehtovirta-Morley et al., 2016) and sequenced using in-house MiSeq (Illumina, San Diego, USA) and MinION (Oxford Nanopore, Oxford, UK) platforms. A library for MiSeq paired-end sequencing was prepared using a Nextera XT kit, according to the manufacturer's instructions (Illumina) and sequencing performed with a V2 kit, producing 20.1 million reads with an average length of 243.4 bp after quality trimming performed using TrimGalore (<https://github.com/FelixKrueger/TrimGalore>). For MinION sequencing, DNA was sheared to

approximately 8 kb using a g-TUBE (Covaris, Brighton, UK) and a library prepared with the SQK-MAP006 kit, according to the manufacturer's instructions (Oxford Nanopore). Sequencing on a MinION flow cell produced 39,064 reads with an average length of 6,739 bp. The genome was assembled using Unicycler (Wick et al., 2017) with default settings for both MinION and Illumina data. Gene prediction was performed using Prodigal version 2.6.3 (Hyatt et al., 2010) and annotation performed using Diamond BLASTp v0.8.36 (e-value $<10^{-5}$) (Buchfink et al., 2015) with the NCBI nr database release 244 (Sayers et al., 2022). Genome quality was assessed using CheckM (Parks et al., 2015), estimating 99.1% completeness and 0.34% contamination, and GToTree (Lee, 2019), detecting 100% of expected single copy genes with 0% redundancy. Average nucleotide identity (ANI) between different *Nitrobacter* strains was assessed using reciprocal best hits (two-way ANI) (<http://enve-omics.ce.gatech.edu/ani/>) (Rodriguez-R & Konstantinidis, 2016).

5.2.3 Phylogenomic analysis

Single copy genes present in all compared genome sequences were identified and aligned using GToTree (Lee, 2019) before manual refinement. Maximum likelihood analysis was performed on unambiguously aligned concatenated protein sequences (11,751 amino acid positions inferred from 63 single copy genes) using PhyML (Guindon et al., 2010) with automatic model selection (Q.plant with FreeRate variation (four rates) across sites) and bootstrap support (100 replicates).

5.3 Results and Discussion

5.3.1 Isolation and characterisation of NHB1

NHB1 was isolated from a cryopreserved co-culture of NHB1 and AHB1 by substituting NH_4Cl in the growth medium with NaNO_2 . NHB1 is rod-shaped with typical stacked intracytoplasmic membranes (Figure 5.4a) and possesses a 3.3 Mb chromosome and two plasmids (0.28 Mb and 0.21 Mb). The closest validated relative is the neutrophile *Nitrobacter hamburgensis* X14 (Bock et al., 1983) (Figure 5.4b), sharing 99, 97, and 98% identity with 16S rRNA, nitrite oxidoreductase sub-unit alpha (NxrA) and beta (NxrB) encoding genes, respectively. High percentage identity of marker genes between *Nitrobacter* species is typically observed (Starkenburger et al., 2008) and contrasts with NHB1 having a 24% smaller genome than *N. hamburgensis* X14 and sharing an average nucleotide identity of 92.4%. We therefore propose the following candidate species: *Nitrobacter laanbroekii* sp. nov. (laan.broek'i.i. N.L. gen. n. *laanbroekii*), named in honour of the Dutch microbiologist Hendrikus J. Laanbroek who was involved in its original cultivation and has made valuable contributions to understanding the ecology of NOB. In addition to expected core genes for carbon and energy metabolism, NHB1 possesses genes for dissimilatory sulphur oxidation, assimilatory nitrite reductase and utilisation of carbon monoxide as found for other *Nitrobacter* species (Starkenburger et al., 2008).

Growth occurred with initial NO_2^- concentrations up to >5 mM (inhibited at ~10 mM) and temperatures ranging 10-30°C, with a maximum specific growth rate (μ_{max}) of 1.17 d⁻¹ (s.e.=0.05) at 25°C and 0.5 mM (Figure 5.4c and 5.4d). As indicated from its previous enrichment, NHB1 is acidotolerant but μ_{max} at different pH varied with initial NO_2^- concentration. At 500 μM , growth occurred at pH 5.0-7.5 with optimal growth at pH 6.0 (Figure 4e), but reduction of initial NO_2^- concentration to 100 μM decreased optimal growth pH to 5.5 (μ_{max} 0.93 d⁻¹ (s.e.=0.01)) with further reductions in pH growth optimum to 5.0 at 50 and 20 μM (μ_{max} 0.77 d⁻¹ (s.e.=0.03) and 0.70 d⁻¹ (s.e.=0.01), respectively). At the lowest NO_2^- concentration, the limit for growth was extended to pH 3.5 (μ_{max} 0.26 d⁻¹ (s.e.=0.01)). Whole cell nitrite oxidation kinetics at 25°C and pH 6 were determined using substrate concentration-dependent oxygen microrespirometry (Figure 5.5). An apparent-half-saturation concentration ($K_{m(\text{app})}$) of 54 μM NO_2^- (s.e.=7) was higher than several canonical nitrite-oxidizing *Nitrospira* but within the lower range of those observed for other *Nitrobacter* strains, and one order of magnitude lower than *N. hamburgensis* X14 (544 μM) (Nowka et al., 2015) (Figure 4f).

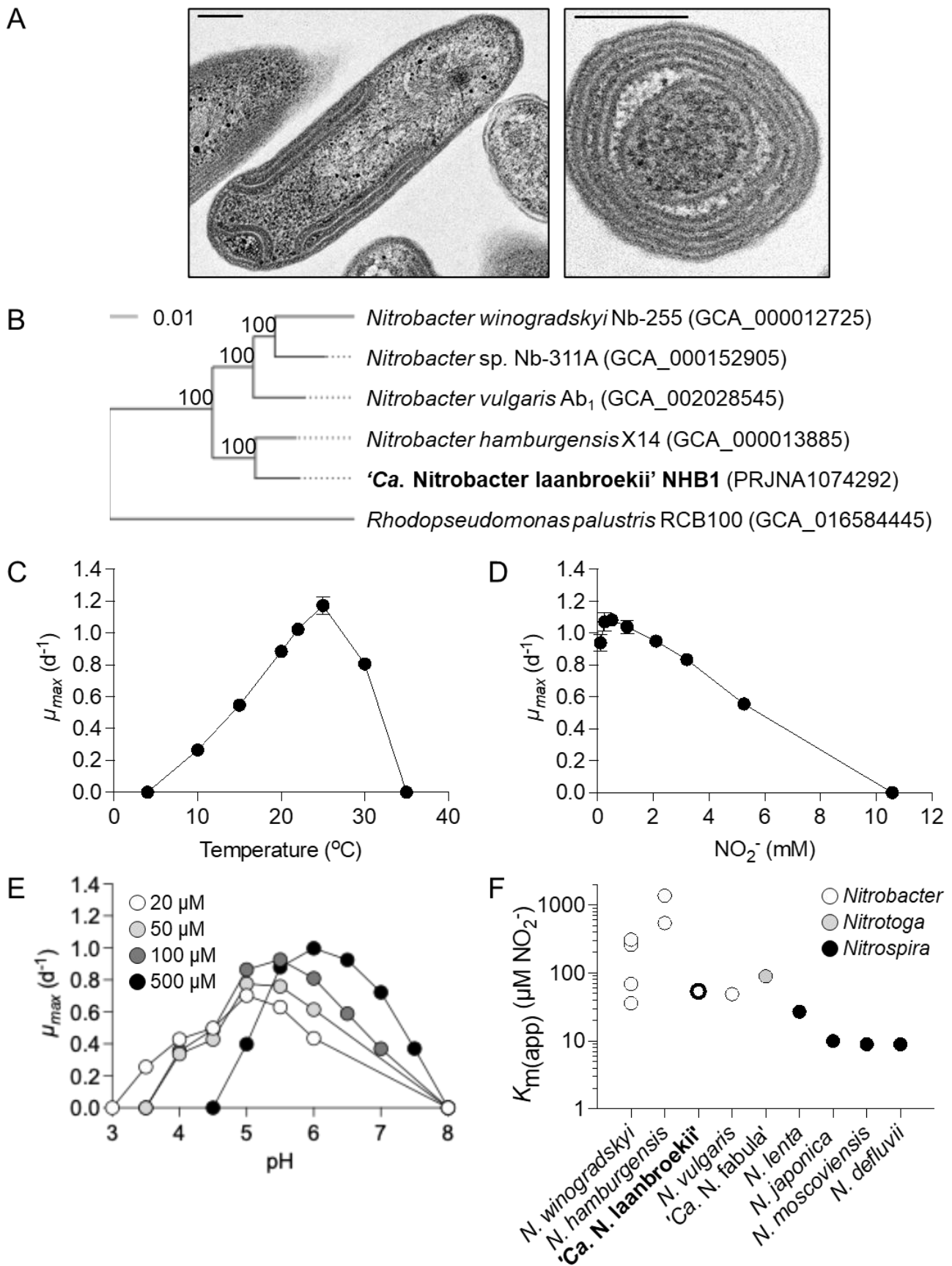


Figure 5.4. Characterisation of the isolated strain '*Ca. Nitrobacter laanbroekii*' NHB1. (A) Transmission electron micrograph of cells in lateral and longitudinal orientation. Scale bars

represent 0.2 μm . (B) Maximum likelihood phylogenomic tree of NHB1, selected *Nitrobacter* isolates and outgroup reference *Rhodopseudomonas palustris* (also of the *Nitrobacteraceae*) using 11,751 unambiguously aligned amino acid positions inferred from 63 single copy genes. The scale bar represents an estimated 0.01 changes per position and values at nodes describe percentage bootstrap support (100 replicates). The influence of (C) temperature, (D) initial nitrite concentration, and (E) pH on maximum specific growth rates (μ_{max}) were determined with mean values and standard errors (mostly smaller than symbol size) from triplicate cultures plotted. (F) Apparent half-saturation constant ($K_{m(app)}$) for NO_2^- of ‘*Ca. Nitrobacter laanbroekii*’ NHB1 (mean value of four replicates) and various *Nitrobacter*, *Nitrotoga* and *Nitrospira* strains.

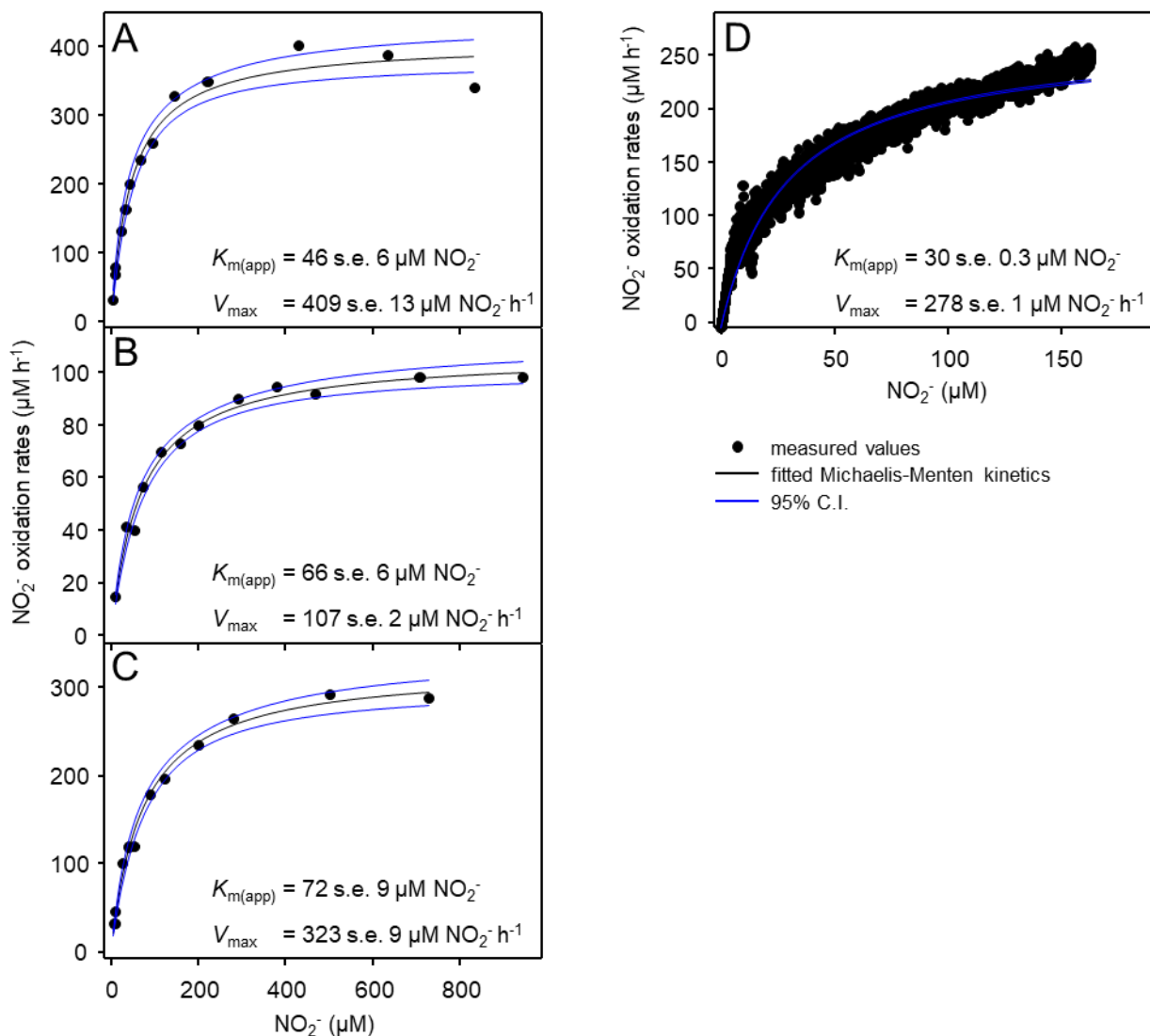


Figure 5.5. Apparent half-saturation ($K_{m(app)}$) and maximum oxidation rates (V_{max}) for NO_2^- calculated using oxygen microrespirometry (MR) after fitting data of oxidation rate vs NO_2^- concentration to the Michaelis–Menten model with 95% confidence intervals (C.I.) plotted.

Three experimental replicates measured oxidation rates after injection varying concentrations of NO_2^- (A,B,C) into the MR chamber and one replicate (D) used a single injection of 250 μM . Biomass was not measured and V_{max} was therefore not normalised to cell numbers or protein content and varies substantially amongst replicates.

5.3.2 Influence of NHB1 on acidophilic AOA physiology

Nitrosotalea devaniterrae Nd1 and *Nitrosotalea sinensis* Nd2 are two AOA strains with pH optima ~ 5 (L. E. Lehtovirta-Morley et al., n.d.). Although obligately acidophilic with adaptations to low pH, both are sensitive to NO_2^- with concentrations as low as 10 μM reducing growth rate (L. E. Lehtovirta-Morley et al., n.d.). In standard batch cultures with 500 μM NH_4^+ and pH 5.2, yields of NO_2^- are typically 200-300 μm (Figure 5.6a) and one to two orders of magnitude lower than neutrophilic soil AOA (L. E. Lehtovirta-Morley et al., 2016; Tourna et al., 2011). However, in contrast to growth in isolation, all 500 μM NH_4^+ was oxidised when grown in co-culture with NHB1 (Figure 5.6b). The amount of NH_4^+ oxidised could be extended to 1.2 and 2.7-2.9 mM for both strains when 2-(N-morpholino)ethanesulfonic acid (MES) buffer concentrations were increased to 20 and 50 mM, respectively (Figure 5.6c, Figure 5.7), with growth inhibited when pH eventually decreased to <3.8 (Figure 5.7). In the presence of NHB1, the pH range of both strains was extended down by ~ 0.5 pH units and μ_{max} increasing significantly at pH ≤ 5.0 ($P < 0.05$) (Figure 5.6d). NHB1 possesses genes encoding enzymes that were previously demonstrated to facilitate mutualistic interactions with AO, including cyanase which produces NH_4^+ from cyanate and enables reciprocal cross-feeding (Palatinszky et al., 2015). While cyanate abiotically degraded rapidly to NH_4^+ in pH 5.2 medium (Figure 5.8), NHB1 cyanase activity was the dominant mechanism generating ammonium at pH 6.0 in cultures supplemented with 0.05 or 0.5 mM cyanate (Figure 5.6e). Although ≥ 0.5 mM cyanate can support other AOA or nitrifying co-cultures (Palatinszky et al., 2015), this concentration inhibited both *Nitrosotalea* strains, with less than 5% of NOB-generated ammonium being oxidised through to NO_3^- and with no accumulation of NO_2^- (Figure 5.9). However, Nd1 and Nd2 used the majority or all cyanate-derived NH_4^+ at 0.05 mM, respectively, a concentration higher than that typically found in soil where cyanase is continuously turned over (Mooshammer et al., 2021).

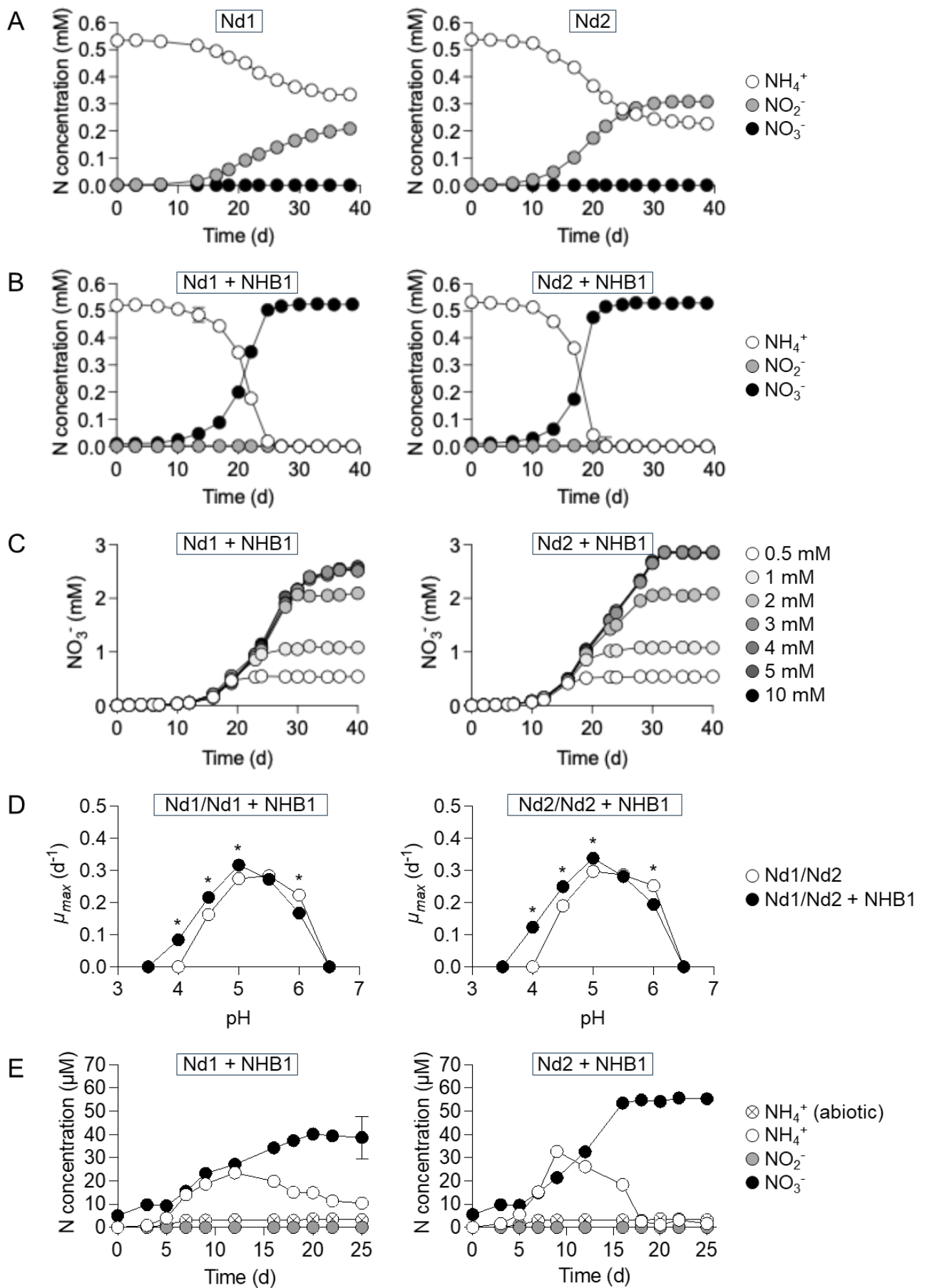


Figure 5.6. Influence of '*Ca. Nitrobacter laanbroekii*' NHB1 on growth characteristics of acidophilic AOA *Nitrosotalea devaniterrae* Nd1 and *Nitrosotalea sinensis* Nd2 in batch culture. Mean values and standard errors (mostly smaller than symbol size) from triplicate cultures are plotted in each panel. (A) Growth of Nd1 and Nd2 grown in isolation and supplied with 0.5 mM NH₄⁺. (B) Growth of Nd1 and Nd2 in co-culture with NHB1 supplied with 0.5 mM NH₄⁺. (C) NO₃⁻ production by co-cultures of Nd1 or Nd2 with NHB1 in medium with MES buffer concentration increased from 10 mM to 50 mM and 0.5, 1, 2, 3, 4, 5 or 10 mM NH₄⁺. (D) The influence of NHB1 on the maximum specific growth rates (μ_{max}) of Nd1 and Nd2 compared to growth in isolation at different pH. An asterisk highlights a significant difference in μ_{max} ($p < 0.05$) for an individual pH (calculated using Student's t-test). (E) Reciprocal cross-feeding at pH 6.0 between NHB1 and Nd1 or Nd2 with 50 μ M cyanate supplied as an NH₄⁺ source. NH₄⁺, NO₂⁻ and NO₃⁻ concentrations were determined for all cultures. Decomposition of cyanate in the abiotic control produced <3.8 μ M NH₄⁺ during the period of incubation.

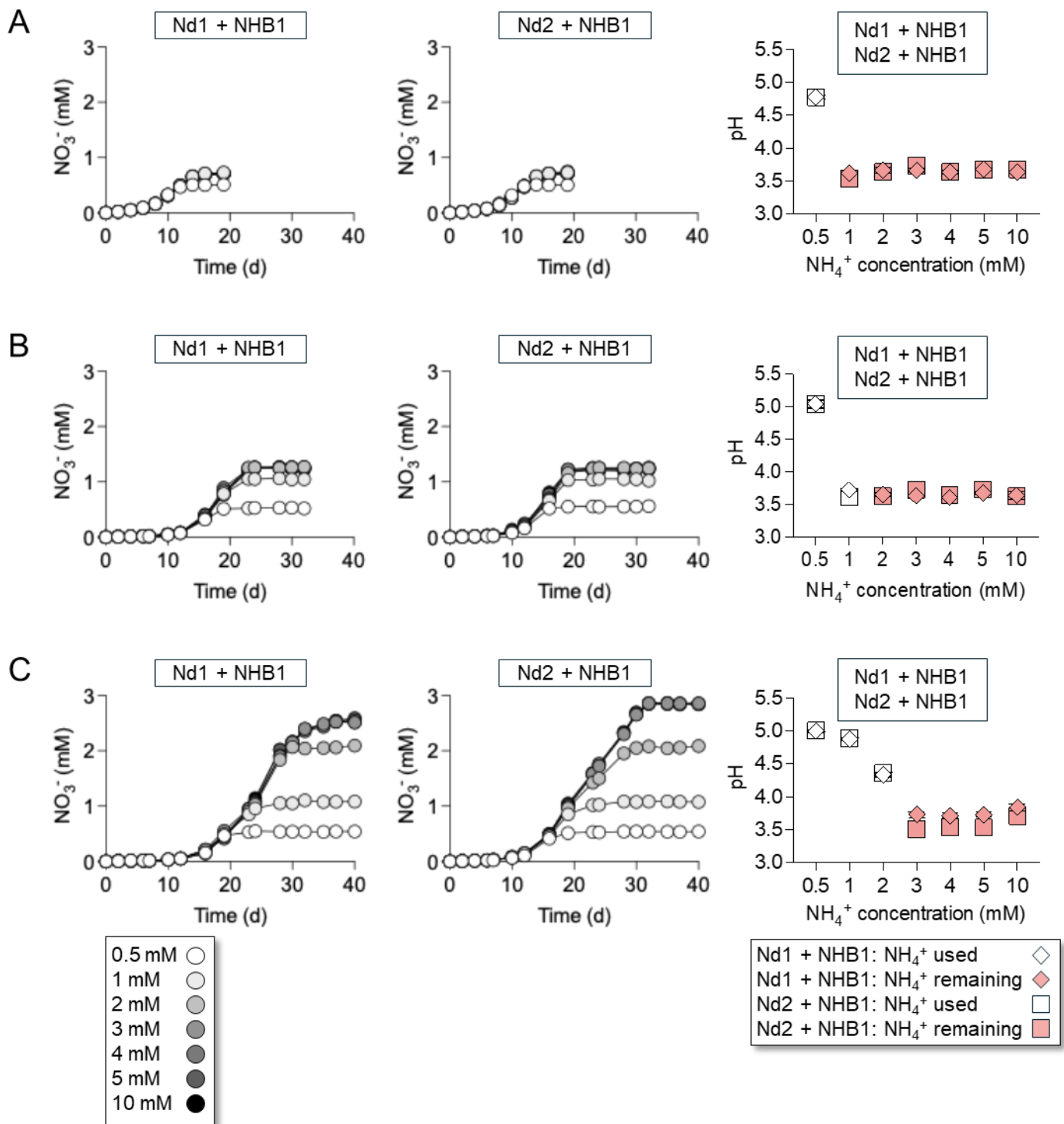


Figure 5.7. Inhibition of acidophilic AOA in 'freshwater medium' batch cultures (pH 6.0) amended with 0.5 mM cyanate. Cultures were inoculated with a previously established co-culture of '*Ca. Nitrobacter laanbroekii*' NHB1 and *Nitrosotalea devaniterrae* Nd1 or *Nitrosotalea sinensis* Nd2. Less than 5% of NOB-generated ammonium was oxidised through to NO₃⁻ with no accumulation of NO₂⁻. Decomposition of cyanate in the abiotic control

produced $<3.5 \mu\text{M}$ NH_4^+ during the period of incubation. Mean values and standard errors (mostly smaller than symbol size) from triplicate cultures are plotted.

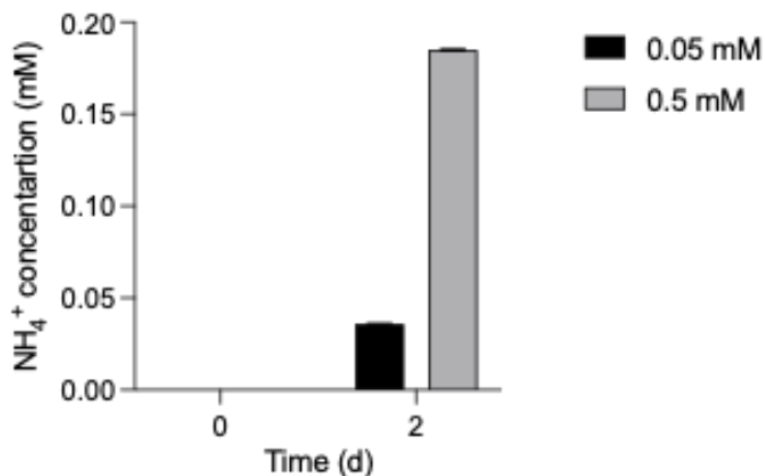


Figure 5.8. Production of NH_4^+ from abiotic decomposition of cyanate in pH 5.2 ‘freshwater medium’ amended with 0.05 mM or 0.5 mM after two days incubation at 25°C.

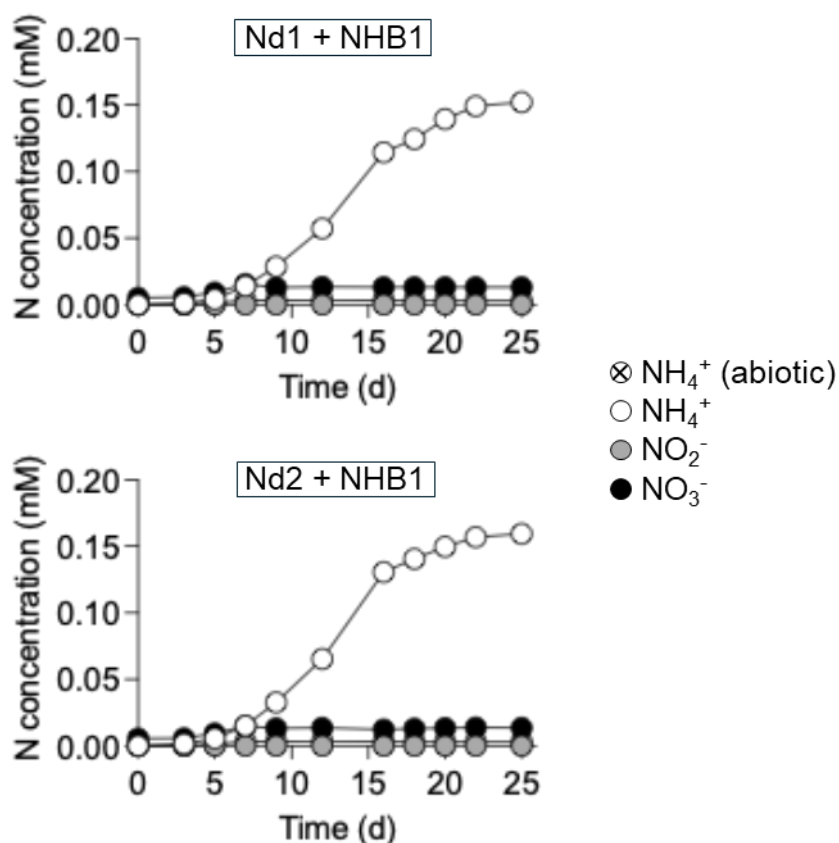


Figure 5.9. Inhibition of acidophilic AOA in ‘freshwater medium’ batch cultures (pH 6.0) amended with 0.5 mM cyanate. Cultures were inoculated with a previously established co-culture of ‘*Ca. Nitrobacter laanbroekii*’ NHB1 and *Nitrosotalea devaniterrae* Nd1 or *Nitrosotalea sinensis* Nd2. Less than 5% of NOB-generated ammonium was oxidised through

to NO_3^- with no accumulation of NO_2^- . Decomposition of cyanate in the abiotic control produced $<3.5 \mu\text{M}$ NH_4^+ during the period of incubation. Mean values and standard errors (mostly smaller than symbol size) from triplicate cultures are plotted.

5.4 Summary

'*Ca. Nitrobacter laanbroekii*' NHB1 is an acidotolerant NOB isolated from acidic soil with a relatively high affinity for NO_2^- . Growth in acidophilic consortia demonstrates that continued removal of NO_2^- in acidic soil is likely crucial for sustained growth of AO and that consideration of substrate and metabolic product concentrations is essential when characterising physiology.

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5.6 Conflict of interest statement

The authors declare no conflicts of interest.

5.7 Data availability

The genome sequence of '*Ca. Nitrobacter laanbroekii*' NHB1 is available under NCBI BioProject accession number PRJNA1074292.

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Chapter 6

General discussion

6.1 Overview

The use of plant protection products (PPP) is a key agricultural practice for achieving high crop yields and meeting global food demands (Tudi et al. 2021). However, only a small proportion of these products reaches the target organisms, with the remainder accumulating in the soil, negatively impacting soil ecosystems and microorganisms (Mitchell et al. 2001). Environmental risk assessments for pesticides typically follow a tiered approach, increasing in complexity and ecological relevance. However, this methodology has not been fully applied

to soil microorganisms, despite their critical role in ecosystem function and soil health. Early research indicated that significant pesticide toxicity observed in laboratory settings warranted further field-scale investigations (Atlas et al. 1978). Building upon this, Karpouzas et al. (2016; Karpouzas et al. 2022) proposed a tiered system specifically to assess the impact of pesticides on soil microbial communities, focusing on key groups like ammonia-oxidizing microorganisms (AOM) and arbuscular mycorrhizal fungi (AMF). In this proposed risk assessment scheme, Tier I involves *in vitro* screening of pesticides against a range of sensitive and representative soil-derived AOM and AMF strains, chosen for their ecological relevance and phylogenetic diversity. If potential toxicity is detected, the assessment proceeds to Tier II, which involves laboratory soil microcosms or planted pot studies (for AMF), and Tier III entails field-scale assessments under agricultural conditions. At these stages, AOM and AMF serve as bioindicators, with advanced biochemical and molecular tools used to monitor microbial activity, abundance, and diversity. Importantly, pesticide dissipation and transformation over time must be tracked, distinguishing between the parent compound and its transformation products (TPs) to ensure that the specific toxicants are identified (Karpouzas et al. 2016; Karpouzas et al. 2022).

The primary aim of this thesis was to develop and validate an *in vitro* bioassay using AOM and functionally related NOB as a standard Tier I assay, related to the single-species tests commonly used in aquatic ecotoxicology (Chapters 2 and 3). These results were compared with an existing toxicity assessment assay, the Microbial Assay for Risk Assessment (MARA) kit from NCIMB, demonstrating the need for a more suitable commercial toxicity kit specifically designed to assess agrochemical impacts on soil ecosystems (Chapter 4). Additionally, we characterized the physiology of *Ca. Nitrobacter laanbroekii* NHB1, one of the most sensitive strains studied, providing insights into the physiology of this isolate and its interactions with acidophilic ammonia-oxidizing archaea (AOA) strains in co-culture, simulating natural soil conditions (Chapter 5).

6.2 Development and validation of an *in vitro* bioassay using nitrifying strains as bioindicators

Chapters 2 and 3 detail the development and validation of single-species tests using nitrifying strains as bioindicators to assess pesticide toxicity on soil microorganisms. This research addresses a critical knowledge gap regarding the potential toxic effects of pesticides on soil microbial communities. By establishing *in vitro* bioassays employing AOA, ammonia-oxidizing bacteria (AOB), and nitrite-oxidizing bacteria (NOB) as bioindicators, the results from this provide a practical tool for evaluating the ecotoxicological impacts of pesticides on soil ecosystems.

These assays, designed as Tier I tests in line with aquatic ecotoxicology practices, identified specific microbial strains that exhibit high sensitivity to pesticides. Among AOA, *N. sinensis* emerged as the most sensitive strain, while *N. briensis* was the most suitable candidate for AOB due to its prevalence in soil ecosystems. For NOB, *Ca. Nitrobacter*

laanbroekii NHB1 demonstrated the highest sensitivity to pesticides, also positioning it as a potential bioindicator for pesticide toxicity.

Work performed for this thesis also revealed pesticide category-specific toxicity patterns. Fungicides, particularly etridiazole and pyraclostrobin, exhibited the highest toxicity to nitrifying microorganisms at concentrations typically used in agriculture. This suggests that regular agricultural use of these fungicides could significantly disrupt the nitrification process, which is crucial for nitrogen cycling and overall soil fertility. NOB showed greater resilience to pesticide toxicity compared to AOM. This resilience may be due to their versatile metabolic pathways, which could enhance resistance to xenobiotics. Some *Nitrobacter* strains, for example, can degrade complex organic compounds (John and Okpokwasili 2012). Similarly, *Nitrospira* exhibit mixotrophy, switching between carbon fixation and the consumption of organic molecules in response to environmental conditions (Koch et al. 2015). Furthermore, genomic studies indicate that NOB can shift from nitrite oxidation to alternative metabolic pathways under certain conditions (Koch et al. 2014).

The study also highlights the critical need to evaluate not only pesticides but also their transformation products, which can be equally or even more harmful to soil ammonia-oxidizing microorganisms (AOM). For example, 3,5-dichloroaniline (3,5-DCA), a metabolite of iprodione, has been shown to be as toxic as, or more toxic than, the original compound (Vasileiadis et al., 2018). Papadopoulou et al. also found that quinone imine—a transformation product of the fruit preservative ethoxyquin—significantly reduced AOM activity, abundance, and diversity in both *in vitro* studies and soil-based experiments (Papadopoulou et al. 2016; Papadopoulou et al. 2020). Field studies showed similar effects of chlorpyrifos which exhibited dose-dependent inhibition of AOA due to both the parent compound and its toxic metabolite, TCP (Karas et al., 2018). Likewise, the pesticide chlorothalonil degrades in soil to form metabolites, such as 4-hydroxy-2,5,6-trichloroisophthalonitrile, 2,4,5-trichloroisophthalonitrile and 2,5,6-trichloro-4-methylthioisophthalonitrile, which were even more toxic than the parent compound, leading to significant reductions in AOA and AOB populations and overall nitrification (Wu et al., 2014; Teng et al., 2017). These findings underscore the importance of a comprehensive pesticide toxicity assessment approach, incorporating both parent compounds and their transformation products, to accurately evaluate long-term impacts on soil health and ecosystem stability.

During the validation of the proposed assay, *N. sinensis*, identified as the most sensitive archaeal strain, consistently demonstrated high sensitivity to additional pesticides tested, particularly fungicides, confirming its potential as an early warning bioindicator of pesticide-induced disturbances in soil microbial communities. In contrast, *N. briensis*, exhibited greater tolerance to pesticides and was significantly affected by a few compounds, such as tebuconazole and chlorothalonil. These results highlight the different sensitivities among AOM, likely due to structural, biochemical, and physiological differences. *Ca. Nitrobacter laanbroekii* NHB1 also displayed low sensitivity to pesticides, indicating its resilience and emphasizing the importance of including a diverse range of microbial responses in toxicity assessments. Overall, fungicides generally posed greater toxicity to AOA than to

AOB and NOB strains, suggesting the need for targeted risk assessments for specific pesticide groups.

This thesis effectively demonstrates the utility of single-species *in vitro* assays using nitrifiers as bioindicators of pesticide toxicity in soil microorganisms. By including both sensitive and tolerant microbial strains, these assays allow for a comprehensive assessment of pesticide effects. Implementation of this approach would enhance early risk assessments, strengthen risk assessment frameworks, and support the preservation of critical ecological functions performed by soil microbiota, contributing to more sustainable agricultural practices that safeguard soil health and essential ecosystem services.

6.3 Evaluating the limitations of a commercial toxicity kit and the critical role of nitrifiers in pesticides ecotoxicity assessments

In Chapter 4, the effectiveness of the MARA kit was evaluated as a high-throughput tool for assessing pesticide ecotoxicity. By comparing the sensitivity of MARA strains to soil nitrifiers, particularly AOM, this work aimed to highlight the need to reassess current methodologies for evaluating pesticide toxicity in soil microbial communities. Our findings demonstrate that soil nitrifiers, especially AOA, exhibit significantly higher sensitivity to various pesticides than those assessed using the MARA kit. While MARA strains showed limited responses at higher pesticide concentrations, AOM experienced substantial growth inhibition at much lower levels, underscoring their potential as effective bioindicators for environmental risk assessments.

The study also revealed variable responses to pesticide exposure between major nitrifier groups, with AOA being more sensitive than both MARA strains and other nitrifiers such as AOB and NOB. This underscores the importance of selecting bioindicators that accurately reflect soil ecosystem dynamics. Given the crucial role of AOM in the nitrogen cycle and their high sensitivity to pesticides, including these microorganisms in regulatory frameworks would allow for a more accurate and ecologically relevant assessment of pesticide impacts.

The limitations of the MARA kit, in the context of assessing the ecotoxicity of agricultural pesticides, highlights the need for new commercial tools that better represent soil microbial communities. The taxonomic diversity of MARA strains derived from different environments fails to capture the specific interactions and functions of soil microorganisms that are critical for key biochemical processes. By focusing on soil nitrifiers, the development of a new commercial toxicity kit would provide a more accurate understanding of soil ecosystem complexity, improving our ability to predict and mitigate the ecological risks posed by pesticide use.

6.4 Ecological importance of *Ca. Nitrobacter laanbroekii* NHB1 in facilitating nitrification in acidic soils

Chapter 5 examined the physiology of *Ca. Nitrobacter laanbroekii* NHB1, an acid-tolerant nitrite-oxidizing bacterial (NOB) strain isolated from acidic soil, highlighting its crucial

role in facilitating ammonia oxidizer (AO) activity under low pH conditions. NHB1 effectively prevents nitrite accumulation and subsequent degradation to toxic intermediates, thereby supporting the survival and metabolic function of acidophilic AOA, particularly obligately acidophilic *Nitrosotalea* strains. Co-culture experiments confirmed that NHB1 extends the pH tolerance of AOA and mitigates nitrite toxicity, underscoring the importance of mutualistic interactions between NOB and AOM in acidic environments.

NHB1 demonstrates remarkable adaptability across a range of nitrite concentrations and pH levels, with optimal growth at pH 6.0. At lower nitrite concentrations, NHB1 can extend its functional pH range, revealing a strong link between its acid tolerance and nitrite oxidation capacity. This phenomenon is likely to occur in acidic soils as well, where NOB plays a key role in removing NO_2^- , reducing the risk of ammonia oxidizers (AOM) being inhibited by toxic forms of nitrite.

However, it is essential to consider that soil systems are more complex, involving multiple factors and mechanisms that can influence AOM survival and function. This adaptability is therefore crucial in acidic environments where nitrite accumulation could otherwise inhibit ammonia oxidizers, underscoring the ecological importance of NOB in sustaining nitrification processes in acidic soils.

The study also revealed physiological interactions between AOA and NOB under acidic conditions. Co-cultures are a powerful tool for studying interactions between different microbial groups, providing a more ecologically relevant and realistic model of natural environments. In nitrification, co-cultures allow to explore the synergistic functions among the microbial groups involved in the process, where each group's survival and efficiency are interdependent. This approach enhances our understanding of the cooperative dynamics essential to nitrification, offering insights into how these microbes thrive collectively in shared ecosystems. In this study, *Nitrosotalea* strains, as obligate acidophiles, exhibited enhanced growth and ammonia oxidation rates when co-cultured with NHB1. These experiments demonstrate that NHB1 not only reduce nitrite toxicity but also indirectly enhances AOA activity through cyanate metabolism, which produces ammonium for oxidation by AOA.

6.5 Future Perspectives

The findings of this thesis provide important insights into the response of nitrifying microorganisms to pesticide exposure in soil ecosystems, paving the way for future research. Key areas of focus include refining high-throughput pesticide toxicity assays, using omics approaches to explore molecular mechanisms of pesticide toxicity, and investigating the regulatory responses of nitrifying strains to environmental stressors like pH. These efforts will help develop more precise environmental risk assessments and sustainable agricultural practices, ultimately preserving soil health and ecosystem functionality.

A promising future direction is the adaptation of current bioassays into high-throughput formats suitable for routine regulatory pesticide testing. Unlike other microbial cultures, nitrifying microorganisms present unique cultivation challenges due to their slow growth rates and extended incubation requirements, necessitating skilled researchers with specialized expertise to maintain these strains. However, advancements are underway to

streamline these assays, particularly in evaluating nitrification inhibitors, which could expand their application in assessing pesticide impacts on soil microorganisms. Developing a specialized test kit focused on soil nitrifiers, similar to the MARA system, could significantly improve sensitivity and specificity in detecting pesticide toxicity within soil ecosystems. By incorporating both ammonia-oxidizing microorganisms (AOM) and nitrite-oxidizing bacteria (NOB), such a kit would provide a more rapid and accurate assessment of pesticide effects, offering a valuable tool for environmental monitoring and regulatory use. This innovation could greatly enhance our ability to protect soil health and foster sustainable agricultural practices.

Future research should focus on refining these assays, validating their efficacy, and integrating them into regulatory frameworks. The proposed single-species test represents one of the first attempts to create a Tier I ecotoxicity test that can complement the widely used Tier II OECD 216 test (OECD 2000) for assessing pesticide risks to soil microorganisms based on nitrogen transformation in soil. Although this single-species test has lower ecological relevance, it offers a promising ecotoxicological tool that, in some cases, may eliminate the need for more complex Tier II and III evaluations. The fast-track approach can save significant time and effort, ultimately enhancing soil health protection and supporting sustainable agricultural practices.

Another important area for future research is an in-depth investigation of the toxicity mechanisms of pesticides on AOA and AOB strains, especially those known to exhibit high toxicity, using advanced omics techniques. Previous studies have employed omics approaches to gain insights into AOM physiology and central metabolism, with transcriptomic and proteomic analyses elucidating AOA and AOB adaptations to conditions such as copper limitation and nutrient starvation, respectively (Kerou et al. 2016; Zorz et al. 2018; Reyes et al. 2020; Hodgskiss et al. 2023). Current work, in collaboration with the University of Vienna, will explore the toxic effects of chlorpyrifos and pyraclostrobin on *Nitrososphaera viennensis* (an AOA strain) and of metsulfuron-methyl and pyraclostrobin on *Nitrosospira multiformis* (an AOB strain) through proteomic and transcriptomic analysis. Proteomics will clarify the metabolic disruptions induced by pesticide exposure, while transcriptomics will reveal the regulatory responses of these microorganisms under stress. Importantly, transcriptomic data will complement proteomics by detecting membrane proteins that are often challenging to analyze, thus providing a comprehensive view of cellular responses to pesticide toxicity. Additional biochemical tests, guided by omics data, will allow for a more targeted analysis of pesticide toxicity. By understanding the mechanisms through which pesticides affect non-target AOM, this research can inform the development of safer, more targeted pesticides that minimize harm to beneficial microbial communities in agricultural ecosystems.

An important extension of the current research involves conducting a transcriptomic analysis of *Ca. Nitrobacter laanbroekii* NHB1 under varying pH conditions. This would provide a deeper understanding of the regulatory responses that enable this acid-tolerant NOB strain to grow in different pH. The analysis of gene expression patterns under different pH conditions, can uncover the molecular mechanisms that govern NHB1's pH tolerance and nitrite oxidation capacity. This knowledge could further elucidate the ecological role of NHB1

in supporting nitrification in acidic environments, offering insights into how these processes might be optimized in agricultural settings facing soil acidification.

6.6 Conclusion

This thesis developed a novel Tier I bioassay using nitrifying microorganisms as bioindicators to assess pesticide toxicity in soil ecosystems. The assays showed that AOA are highly sensitive to pesticides, particularly fungicides, while AOB and NOB demonstrated greater resilience. Key sensitive strains like *N. sinensis* (AOA), *N. briensis* (AOB), and *Ca. Nitrobacter laanbroekii* NHB1 (NOB) were identified, supporting their use in early pesticide risk assessments. The study highlighted limitations in existing assays like MARA, emphasizing the need for soil-specific microbial bioindicators and toxicity kits. Additionally, *Ca. Nitrobacter laanbroekii* NHB1 played a critical role in nitrification in acidic soils, preventing toxic nitrite accumulation and aiding the survival of acidophilic ammonia oxidizers, demonstrating the ecological significance of these interactions. Overall, this thesis provides practical tools for improving pesticide risk assessment and contributes to the development of safer pesticides and sustainable agricultural practices

6.7 References

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Synthèse en français

Résumé

L'utilisation de pesticides est essentielle pour augmenter les rendements agricoles, mais ces produits chimiques peuvent manquer leurs cibles, s'accumuler dans le sol et nuire aux micro-organismes qui sont importants pour la santé de l'écosystème. Les évaluations traditionnelles des risques liés aux pesticides n'ont pas entièrement pris en compte la toxicité des pesticides pour les micro-organismes. Pour remédier à cette situation, un système à plusieurs niveaux a été proposé pour évaluer les effets des pesticides sur un groupe microbien clé, les micro-organismes oxydant l'ammoniac (AOM). Le système comprend des tests de toxicité *in vitro* (niveau I) aux microcosmes de sol puis aux études en pots (niveau II), et enfin aux évaluations à l'échelle du terrain (niveau III). Les principaux objectifs de cette thèse étaient de i) développer et valider un essai biologique *in vitro* utilisant l'AOM et des bactéries

oxydantes des nitrites (NOB) fonctionnellement apparentées comme essai standard de niveau I, lié aux tests monospécifiques couramment utilisés en écotoxicologie aquatique, ii) comparer la toxicité des pesticides sur l'AOM avec un kit d'évaluation de la toxicité disponible dans le commerce, et iii) caractériser la physiologie de la souche de NOB *Ca. Nitrobacter laanbroekii* NHB1, l'une des souches les plus sensibles aux pesticides identifiées dans cette thèse. La toxicité de pesticides représentatifs (insecticides, fongicides et herbicides) a été évaluée sur plusieurs AOM, y compris des bactéries oxydant l'ammoniac (AOB), des archées oxydant l'ammoniac (AOA) et des NOB. Les seuils de toxicité (EC_{50}) ont été déterminés pour chaque combinaison souche-pesticide, *Nitrosotalea sinensis* Nd2 (AOA), *Nitrosospira briensis* (AOB) et *Ca. Nitrobacter laanbroekii* NHB1 (NOB) ont été identifiées comme les souches les plus sensibles et les bioindicateurs appropriés pour l'essai biologique proposé sur une seule espèce. Les fongicides et les insecticides étaient généralement plus toxiques pour AOM que pour NOB, tandis que les herbicides présentaient une toxicité variable pour tous les groupes de nitrifiants. Sur la base de ces résultats, la toxicité d'autres pesticides de différentes catégories sur les trois souches les plus sensibles a été évaluée. En utilisant l'essai *in vitro* proposé pour une seule espèce, les valeurs EC_{50} ont confirmé que ces souches étaient des bioindicateurs efficaces pour les essais de toxicité de niveau I. Les fongicides étaient les plus toxiques pour les AOM que pour les NOB. Les fongicides ont été les plus toxiques pour *N. sinensis*, provoquant une inhibition significative à des niveaux de concentration agronomiques. Les sensibilités différentes de ces souches à diverses classes de pesticides soulignent la nécessité d'une approche globale qui saisisse un large spectre de réponses microbiennes. L'évaluation de la toxicité des pesticides à l'aide du kit MARA (Microbial Assay for Risk Assessment), qui utilise 11 souches microbiennes hétérotrophes pour détecter la toxicité des pesticides, a montré une sensibilité relativement faible, les pesticides n'affectant ces souches qu'à des concentrations élevées, contrairement à l'AOM qui a montré une sensibilité beaucoup plus élevée aux pesticides. La physiologie de *Ca. Nitrobacter laanbroekii* NHB1, l'un des bioindicateurs identifiés, a montré une tolérance à l'acide avec une croissance optimale à un pH de 6,0 et une croissance détectable jusqu'à un pH de 3,5. En co-culture, cette souche a favorisé la croissance d'AOA acidophiles, telles que *Nitrosotalea devaniterrae* Nd1 et *N. sinensis*, en éliminant les nitrites inhibiteurs. Ce mécanisme de mutualisme trophique souligne l'importance des concentrations de substrat dans les interactions microbiennes dans les sols acides.

Introduction Générale

1. Produits phytopharmaceutiques

1.1 Définition, histoire et utilisation au fil des ans

Les pesticides font partie intégrante de l'agriculture moderne et constituent l'un des facteurs les plus importants permettant d'augmenter le rendement des cultures. Selon l'Organisation des Nations Unies pour l'alimentation et l'agriculture (FAO) et l'Organisation mondiale de la santé (OMS) (2014), un pesticide est « toute substance ou mélange de substances à base d'ingrédients chimiques ou biologiques destinés à repousser, détruire ou combattre un organisme nuisible, ou à réguler la croissance des

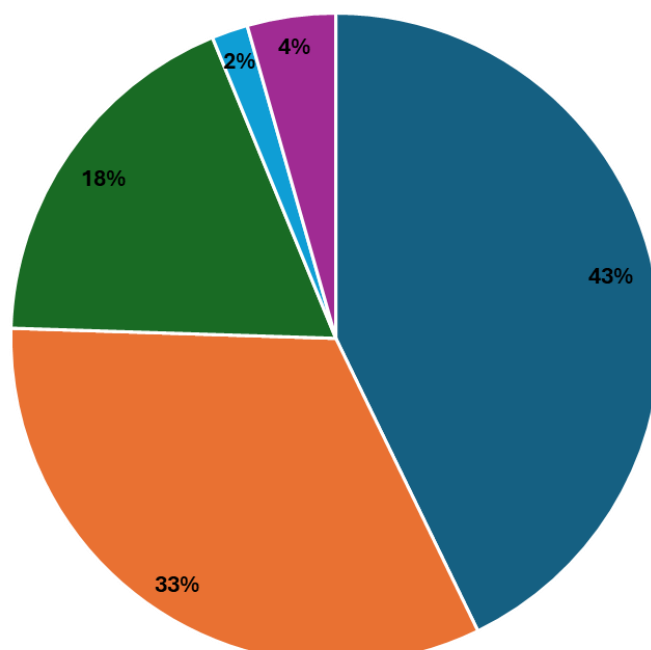
plantes ». Les pesticides englobent deux catégories principales : (i) les produits phytopharmaceutiques (PPP), régis par le règlement (CE) n° 1107/2009 (« Règlement (CE) n° 1107/2009 du Parlement européen et du Conseil en ce qui concerne les principes uniformes d'évaluation et d'autorisation des produits phytopharmaceutiques », 2011), qui vise à améliorer le rendement et la qualité de l'agriculture, et (ii) les biocides, régis par la directive 98/8/CE, utilisés pour gérer les organismes nuisibles dans des contextes non-agricoles. Dans la suite de cette thèse, le terme « pesticide » fait référence aux PPP, et ces termes seront utilisés de manière interchangeable.

L'histoire de l'utilisation des pesticides remonte aux débuts de l'agriculture. Leur première utilisation a été enregistrée il y a environ 4500 ans, lorsque les Sumériens ont utilisé des composés sulfurés comme insecticides pour lutter contre les insectes et les acariens (Unsworth, 2010). Les Grecs et les Romains ont également utilisé de l'huile et de la cendre de soufre pour lutter contre divers parasites, tandis que des composés de mercure et d'arsenic ont été utilisés en Chine contre les poux de corps il y a environ 3200 ans (Hansen, 2013). Dans l'Antiquité, avant l'avènement de l'industrie chimique, les gens s'appuyaient uniquement sur les ressources naturelles pour fabriquer leurs produits. Un exemple typique est le pyrèthre, un insecticide dérivé des fleurs séchées du chrysanthème (*Cineraria folium*). Cet insecticide naturel est utilisé depuis plus de deux millénaires, ce qui souligne son efficacité de longue date dans la lutte contre les parasites. Il s'agit de la première des trois phases de l'histoire de l'utilisation des pesticides. La deuxième période s'étend de 1870 à 1945 et se caractérise par l'utilisation de composés synthétiques inorganiques. En Suède, les composés de cuivre et de soufre ont été utilisés pour la première fois pour lutter contre les maladies fongiques des fruits et des pommes de terre au début du XIXe siècle (Bernardes et al., 2015) et sont toujours utilisés dans la pratique agricole (Sheail, 1991). Après la Seconde Guerre mondiale (1945), la troisième période d'utilisation des pesticides a commencé (Unsworth, 2010), avec un tournant décisif dans la lutte contre les ravageurs grâce à l'apparition de pesticides synthétiques tels que le dichlorodiphényltrichloroéthane (DDT), le captane, le parathion et le β -hexachlorocyclohexane (BHC). Ces composés ont permis une lutte généralisée contre les parasites, une augmentation de la production alimentaire et une réduction des maladies transmises par les insectes. Toutefois, leur utilisation présentait de nombreux inconvénients, notamment la non-sélectivité, les taux d'application élevés et la toxicité. Au cours des décennies suivantes, les pesticides ont connu un développement important. Dans les années 1970 et 1980, l'application de pesticides plus ciblés comme le glyphosate et divers insecticides et fongicides organophosphorés, tels que les triazoles, les imidazoles, les pyrimidines et les dicarboxamides, a été établie. Dans les années 1990, on a assisté à une évolution vers des composés plus sélectifs présentant de meilleurs profils environnementaux et toxicologiques, ce qui a nécessité une diminution des volumes d'application. Cette tendance se poursuit aujourd'hui, les recherches en cours se concentrant sur le développement de pesticides plus sélectifs, sur une meilleure gestion de la résistance et sur une sécurité accrue pour les utilisateurs et l'environnement afin de répondre à la demande mondiale (Unsworth, 2010).

Les pesticides jouent un rôle important dans l'agriculture moderne, en luttant contre diverses menaces telles que les mauvaises herbes et les insectes, ainsi que les

bactéries et les champignons qui provoquent des maladies dans les cultures. Toutefois, leur utilisation généralisée peut nuire à la santé humaine et à l'environnement. Les pesticides peuvent contaminer les sols, les sources d'eau et les organismes non ciblés, ce qui entraîne un déclin de la biodiversité et pose des risques pour les écosystèmes (Bernardes et al., 2015). Le suivi de l'utilisation et du commerce des pesticides est essentiel pour évaluer la durabilité des pratiques agricoles. La base de données FAOSTAT sur l'utilisation des pesticides fournit des informations précieuses sur l'utilisation des pesticides, notamment des données sur les ingrédients actifs, les principales catégories de pesticides et des indicateurs pertinents tels que l'utilisation par hectare de terre cultivée, par habitant et par valeur de production agricole. Ces données permettent d'évaluer l'impact des pesticides sur l'agriculture et l'environnement et d'orienter les efforts vers des pratiques agricoles plus durables (Pesticides use and trade 1990-2021, 2023).

En 2021, l'utilisation mondiale de pesticides a atteint 3,4 millions de tonnes, soit une augmentation de 4 % par rapport à 2020 et une hausse de 11 % au cours de la dernière décennie. Notamment, à l'échelle continentale, l'Amérique (à la fois l'Amérique du Sud et l'Amérique du Nord) a conservé sa position de premier utilisateur de pesticides depuis le milieu des années 1990, consommant environ 1,12 million de tonnes et représentant 43 % de l'utilisation mondiale de PPP chaque année, suivie de l'Asie (33 %) et de l'Europe (18 %) (figure 1.1 a). Cette dernière a connu une augmentation modérée de l'application de pesticides, passant de 468 kt en 2020 à 505 kt en 2021, soit une hausse de 4 % (figure 1.1 b). L'Afrique et l'Océanie affichent les taux d'utilisation de pesticides les plus faibles, avec respectivement 4 % et 2 %. L'Afrique a connu une légère augmentation, passant de 203 kt en 2020 à 204 kt en 2021, tandis que l'utilisation de pesticides en Océanie est restée stable à 72 kt (Figure 1.1) (Pesticides use and trade 1990-2021, 2023).



Parmi les pays européens, l'Espagne et la France se sont révélées être les plus gros consommateurs de pesticides, avec respectivement 76 et 70 kt en 2021, ce qui les place parmi les premiers pays au monde en termes d'utilisation de pesticides (Pesticides use and trade 1990-2021, 2023). Les fongicides et les bactériocides constituent la majorité, soit 51 % de l'utilisation des pesticides en Europe, suivis par les herbicides (36 %) et les insecticides (13 %) (figure 1.2 a). Cette répartition reflète probablement les mesures réglementaires strictes qui régissent l'utilisation des pesticides en Europe. Il est intéressant de noter que les quantités d'herbicides et d'insecticides sont restées relativement stables au cours de la dernière décennie, se situant respectivement autour de 11 kt et 5 kt, tandis que les fongicides et les bactériocides représentent environ 15 kt (figure 1.2 b) (Pesticides use and trade 1990-2021, 2023).



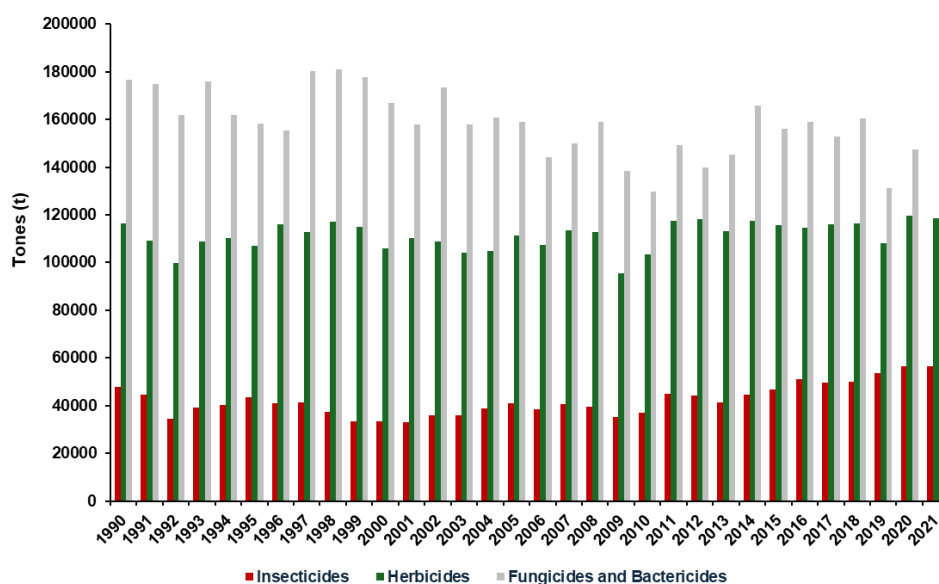


Figure 1.2 (a) Proportion des principales catégories de pesticides dans la pratique agricole en Europe et (b) quantités totales de pesticides (tonnes) par catégorie utilisées dans l'agriculture au cours des 29 dernières années en Europe. Source des données : Organisation des Nations unies pour l'alimentation et l'agriculture (FAO).

1.2 Classification

Les pesticides peuvent être classés de différentes manières. Les approches les plus couramment utilisées sont la classification basée sur le mode d'action, les organismes ciblés ou la structure chimique.

Dans la classification par mode d'action/entrée, les pesticides sont regroupés en fonction de la manière dont ils entrent en contact ou pénètrent dans l'organisme cible (Zacharia & Tano, 2011) et sont classés en pesticides non systémiques (contact externe) et systémiques (pénétration). Les pesticides non systémiques ne pénètrent pas sensiblement dans les tissus et, par conséquent, ne sont pas transportés dans les tissus et sont efficaces au point de contact avec l'organisme nuisible cible. En revanche, les pesticides systémiques pénètrent les tissus et se déplacent vers une zone cible pour produire l'effet désiré. La classification par organisme nuisible permet de classer les pesticides en fonction des rangs taxonomiques élevés de la cible (tableau 1.1) (Tudi et al., 2021). Dans la classification

chimique, les pesticides sont regroupés en fonction de la nature chimique de l'ingrédient actif, établissant une corrélation entre la structure, l'activité, la toxicité et les mécanismes de dégradation. La classification basée sur les structures chimiques concerne à la fois les pesticides organiques et inorganiques. Les pesticides inorganiques comprennent le sulfate de cuivre, le sulfate ferreux, le cuivre, la chaux et le soufre (Hassaan & El Nemr, 2020). Les groupes les plus importants de pesticides organiques sont énumérés dans le tableau 1.2 (Kaur et al., 2019).

Tableau 1.1 Classification des pesticides en fonction de leur cible.

| Nom du pesticide | Organisme nuisible cible |
|-------------------------|---------------------------------|
| Algicides | Algues |
| Bactéricides | Bactéries |
| Fongicides | Champignons |
| Insecticide | Insectes |
| Herbicide | Plantes |
| Nématocides | Nématodes |
| Virucides | Virus |

Tableau 1.2. Les principaux groupes organiques de pesticides.

| Groupe | Organisme nuisible cible |
|--------------------------------|-------------------------------------|
| Organophosphates | Insectes, Nématodes |
| Carbamates | Insectes, Nématodes, Champignons |
| Pyréthroïdes | Insectes |
| Organochlorés | Insectes |
| Néonicotinoïdes | Insectes |
| Strobilurines | Champignons |
| Benzimidazoles | Champignons |
| Dicarboxamides | Champignons |
| Thiocarbamates | Champignons, Plantes |
| Anilinopyrimidines | Champignons |
| Triazoles, Imidazoles | Champignons |
| Phénylurées substituées | Plantes |
| Triazines | Plantes |
| Substituted Phenylureas | Plantes |
| Sulfonylurées | Plantes |
| Tri-cétones | Plantes |
| Diphényléthers | Plantes |
| Chloroacétamides | Plantes |

1.3 Avantages et risques des pesticides

L'utilisation des pesticides est largement reconnue comme faisant partie intégrante des pratiques agricoles modernes afin de réduire les pertes agricoles dues aux ravageurs et d'augmenter les rendements des cultures. On y parvient en réduisant efficacement les

populations de mauvaises herbes, atténuant ainsi leur concurrence avec les cultures pour les nutriments vitaux, en combattant les maladies principalement causées par des champignons et des bactéries pathogènes pour les plantes, et en freinant les insectes nuisibles qui, en tandem avec les pathogènes, constituent des menaces substantielles pour la productivité agricole et peuvent conduire à des pertes économiques importantes en raison de la diminution des récoltes ou des pertes après récolte (Abubakar et al., 2020). Il est important d'augmenter les rendements agricoles pour répondre aux besoins croissants de la population mondiale, qui ne cesse d'augmenter, et l'utilisation des PPP a permis d'accroître les rendements et l'accessibilité des aliments nutritifs tels que les fruits et les légumes. Par conséquent, les PPP atténuent considérablement la faim et garantissent un approvisionnement abondant en nourriture de haute qualité (Tudi et al., 2021), une meilleure nutrition et un niveau de vie plus élevé.

Les pesticides sont également utilisés pour lutter contre les vecteurs de maladies humaines et animales et les organismes nuisibles ; la lutte contre les parasites porteurs de maladies affectant les humains et les animaux est cruciale pour prévenir la propagation de maladies graves telles que le paludisme, la maladie du sommeil et la cécité des rivières. En gérant ces vecteurs de maladies, non seulement nous atténuons les souffrances et réduisons le nombre de décès, mais nous réduisons également le risque de propagation des maladies au-delà des frontières. En outre, dans le secteur de l'élevage, une lutte efficace contre les parasites permet d'augmenter les revenus grâce à des animaux en meilleure santé et de réduire les dépenses liées aux soins vétérinaires et aux médicaments (Cooper & Dobson, 2007).

Malgré ces résultats positifs, l'utilisation de pesticides peut également avoir un impact négatif sur l'environnement et sur la santé de la population. L'utilisation de ces composés chimiques dans les pratiques agricoles conduit à la contamination de diverses niches environnementales et de produits agricoles, en particulier les fruits et légumes frais (Farahy et al., 2021). Le sol, qui soutient la productivité agricole, reçoit les niveaux les plus élevés de résidus (Abubakar et al., 2020). Leurs effets négatifs découlent de la structure chimique et des propriétés physicochimiques essentielles du sol, qui leur permettent de se répandre de manière incontrôlée dans les écosystèmes terrestres et aquatiques (Carvalho, 2017). Il en résulte une détérioration de la qualité des sols et une accumulation ultérieure de produits chimiques dans les eaux souterraines et les réserves d'eau potable. Il est donc urgent de mettre en place des pratiques agricoles durables susceptibles d'atténuer ces effets négatifs sur les écosystèmes et le bien-être humain.

De nombreuses études ont mis en évidence l'impact des PPP sur les organismes non ciblés, couvrant un large éventail d'espèces. Les organismes non cibles sont définis comme des espèces distinctes du groupe d'organismes cibles affectés par l'application de pesticides et comprennent les plantes, les algues, les oiseaux, les poissons, les insectes bénéfiques, les micro-organismes aquatiques et du sol (Iyanwura, 1991). L'effet des PPP sur les micro-organismes du sol, dont l'activité est essentielle au maintien de la santé et de la fonctionnalité du sol, est particulièrement préoccupant. La diminution de leur fonction due à l'exposition aux pesticides peut entraver la décomposition de la matière organique et compromettre

l'équilibre complexe des cycles biogéochimiques, ce qui aggrave encore la dégradation des sols.

En outre, l'accumulation de pesticides dans les écosystèmes amplifie les préoccupations écologiques. Ce phénomène, connu sous le nom de bioaccumulation, implique l'accumulation progressive de résidus de pesticides dans les organismes à différents niveaux trophiques. Cette accumulation se produit par de multiples voies, y compris l'absorption directe à partir de sources d'eau et, surtout, par la consommation de tissus végétaux contaminés, de sédiments et d'autres membres de la chaîne alimentaire (Alexander & Fairbridge, 1999).

1.4 Le devenir des pesticides dans l'environnement

Après leur application, les pesticides sont soumis à de nombreux processus dans l'environnement. Grâce à ces processus, les pesticides pénètrent dans l'environnement avec une influence positive (sur la cible) ou négative (non cible). Les principaux processus qui déterminent le devenir des pesticides dans l'environnement sont l'absorption, la dégradation (biotique, abiotique), le transport vers les eaux de surface (ruissellement, érosion, dérive de pulvérisation) ou les eaux souterraines (lixiviation) et l'air (figure 1.3) (Tudi et al., 2021).

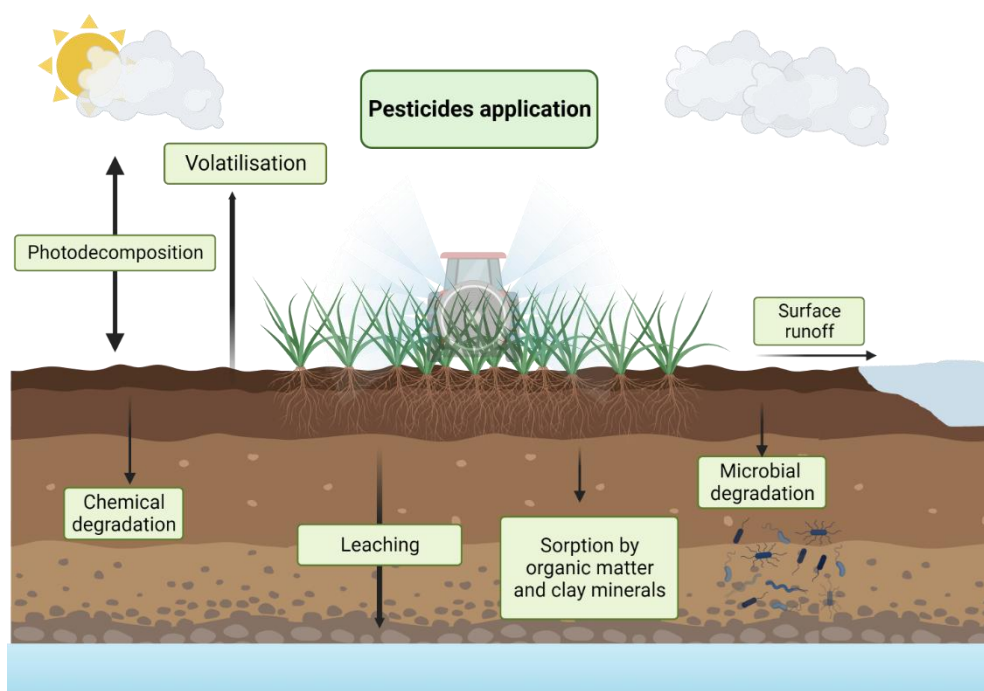


Figure 1.3 Comportement des pesticides dans l'environnement.
1.4.1 Absorption

L'absorption est définie comme la liaison des produits chimiques aux particules du sol (Peña et al., 2020). Les propriétés du sol telles que le pH, l'humidité, la salinité et la matière organique influencent considérablement l'absorption des pesticides, la matière organique étant le facteur le plus crucial. La matière organique sert de surface d'absorption primaire pour les composés non polaires et certains composés polaires (De Wilde et al., 2009 ; Wauchope et al., 2002). En outre, les propriétés chimiques des pesticides, en particulier l'hydrophobie et la solubilité dans l'eau, jouent un rôle essentiel dans leur absorption (Delgado-Moreno et al., 2017).

1.4.2 Dégradation

La dégradation contrôle la persistance des pesticides et le rendement des différents métabolites dans le sol. En outre, les données de dégradation peuvent être utilisées pour déterminer la demi-vie des pesticides, c'est-à-dire le temps nécessaire à la dégradation de 50 % du pesticide appliqué dans l'environnement (Marie et al., 2017). Après leur application, les pesticides peuvent être dégradés de manière abiotique (dégradation chimique, photodégradation) ou biotique (dégradation microbienne) (Abian et al., 1993). Les facteurs environnementaux et les caractéristiques chimiques affectent la dégradation des pesticides, ce qui entraîne la production de nouveaux composés, qui peuvent être plus toxiques que le composé d'origine (Zhao et al., 2017).

La photodégradation a lieu à la surface du sol et des systèmes aquatiques, dans l'air ou sur le feuillage des plantes traitées, car elle dépend fortement de l'intensité de la lumière (Gavrilescu, 2005). La dégradation chimique englobe une série de réactions, notamment l'ionisation, l'hydrolyse et l'oxydo-réduction, et dépend généralement du pH du système environnemental (Tudi et al., 2021).

La dégradation biotique est réalisée par les micro-organismes du sol qui sont capables d'utiliser les composés comme source de nutriments (Han et al., 2013). La capacité des microorganismes à dégrader les produits agrochimiques est due à leur adaptabilité dans les environnements exposés aux pesticides. Cette adaptabilité est facilitée par leur plasticité génétique et leur temps de génération rapide, ce qui leur permet de développer des gènes et des voies cataboliques des pesticides (Russell et al., 2021 ; Upadhyay et al., 2019 ; Zhu et al., 2020). En outre, les micro-organismes peuvent acquérir ces gènes par transfert horizontal d'éléments génétiques mobiles tels que les plasmides et les transposons (French et al., 2020 ; Rios Miguel et al., 2020). La dégradation microbienne est influencée par l'oxygène, la température, l'humidité et le pH (Singh, 2012 ; Su et al., 2017 ; Yue et al., 2017).

1.4.3 Transport des pesticides dans le sol

Les pesticides sont transportés dans l'environnement par volatilisation, lixiviation, ruissellement et érosion. Le processus par lequel un pesticide solide ou liquide est converti en gaz et s'évapore ensuite dans l'atmosphère à partir du sol est appelé volatilisation (Navarro

et al., 2013). Elle est influencée par une série de facteurs tels que la pression de vapeur, la température, l'humidité, le mouvement de l'air et les propriétés du sol telles que la texture, la matière organique et l'humidité (Singh, 2012 ; Tudi et al., 2021). Le mouvement vertical des pesticides dans le sol entraîne le lessivage dans les eaux souterraines et provoque la pollution de l'eau (Fontana et al., 2010 ; Singh, 2012). Le lessivage est influencé par les propriétés du sol, les propriétés chimiques des pesticides, les conditions du site et les pratiques de gestion (Gavrilescu, 2005 ; Peña et al., 2020). La grande solubilité des pesticides et la perméabilité du sol augmentent la possibilité de lessivage des pesticides du sol (Tudi et al., 2021). Le ruissellement est le mouvement des pesticides dans l'eau sur une surface inclinée (Das et al., 2020). Les pesticides peuvent se déplacer avec le ruissellement sous forme de composés dissous dans l'eau ou attachés aux particules du sol. Le ruissellement peut également se produire lorsque l'eau est ajoutée à un champ à un rythme plus rapide que celui auquel elle peut être absorbée par le sol de surface (Singh et al., 2012). Le ruissellement est favorisé par une irrigation excessive, une teneur en eau élevée du sol, la pente du point d'introduction et la quantité et le moment des précipitations (Tudi et al., 2021).

1.5 Impact des pesticides sur les micro-organismes du sol

Le sol est le principal puits des pesticides appliqués en milieu agricole. La dégradation des produits agrochimiques peut conduire à la production de composés métaboliques qui sont plus persistants et toxiques que le composé d'origine (Marie et al., 2017 ; Tudi et al., 2021). La contamination des sols par les pesticides a eu des effets néfastes sur la qualité des aliments et la durabilité de l'agriculture, avec une menace généralisée pour l'eau et la chaîne alimentaire (Tudi et al., 2021).

Les micro-organismes du sol jouent un rôle essentiel dans la santé du sol et participent à des processus biochimiques majeurs tels que les cycles de l'azote et du carbone (Li et al., 2021). Les pratiques agricoles, y compris l'application de pesticides, influencent les micro-organismes du sol (Bedano et al., 2021 ; Sim et al., 2023). Dans le sol, les pesticides interagissent avec les micro-organismes du sol, ce qui entraîne soit une toxicité des pesticides, soit une croissance accrue liée à la dégradation microbienne des pesticides, et est influencé par une série de facteurs biotiques et abiotiques. La toxicité des pesticides sur les micro-organismes du sol est probablement due au niveau élevé de pesticides appliqués qui ne peuvent pas être efficacement dissipés, tandis que la dégradation microbienne liée à la croissance est entraînée par des micro-organismes spécifiques qui possèdent des enzymes pour la dégradation de ces composés (Horemans et al., 2016).

Les pesticides peuvent avoir des impacts à court ou à long terme sur les microorganismes du sol, directement ou indirectement, et avec des résultats positifs ou négatifs, en fonction du type de pesticide et des microorganismes spécifiques impliqués. Les effets directs comprennent la production de composés spécifiques par les micro-organismes du sol après contact avec les pesticides. Ces composés peuvent entraîner la mort des micro-organismes ou modifier considérablement leurs métabolismes. Les effets indirects des pesticides proviennent des modifications du métabolisme des micro-organismes par les pesticides (Jeyaseelan et al., 2024).

L'interaction entre les pesticides et les processus du sol est complexe et multiforme. Plusieurs études ont examiné les effets des pesticides sur le microbiote du sol en laboratoire et sur le terrain (Carpio et al., 2020). Des preuves substantielles indiquent que l'utilisation à long terme de pesticides peut inhiber des fonctions cruciales du sol et modifier la dynamique des ions métalliques. L'ammonification, un processus important dans le cycle de l'azote (N), peut être considérablement réduite, d'environ 70 % à 90 %, en cas d'application de pesticides pendant trois ans ou plus, ce qui a un impact négatif sur la croissance des plantes et la fertilité des sols (Rajak et al., 2023). Au fil du temps, les pesticides peuvent s'accumuler, entraînant une contamination du sol à long terme, ce qui affecte la santé du sol et la productivité des cultures. L'un des processus critiques du sol affecté par les pesticides est la réduction des ions ferriques en ions ferreux. Ce processus d'oxydoréduction est essentiel pour diverses fonctions biochimiques du sol et la disponibilité des nutriments. En outre, les pesticides possèdent des sites de liaison qui leur permettent de former des complexes avec les ions métalliques présents dans les environnements agricoles, ce qui affecte leur disponibilité et leur mobilité. Par exemple, des pesticides tels que le thiophanate-méthyle peuvent se lier à des micronutriments essentiels comme le fer, le cuivre et le zinc, ce qui réduit leur disponibilité pour les plantes et peut entraîner des carences. La formation de complexes de pesticides avec des ions métalliques peut également limiter leur accessibilité aux micro-organismes du sol, ce qui diminue leur contribution aux processus fonctionnels et peut potentiellement nuire à l'intégrité des terres et perturber l'écosystème (Jeyaseelan et al., 2024 ; Rajak et al., 2023).

Plusieurs études ont également identifié des effets négatifs ou positifs des pesticides sur les communautés microbiennes du sol. Walder et al. (2022) ont signalé une augmentation de la taille de la communauté fongique en présence de résidus de pesticides, tandis qu'une corrélation négative a été observée avec l'abondance des bactéries fixatrices d'azote dans plusieurs études, illustrant un risque à long terme pour la fixation biologique de l'azote dans les sols agricoles (Angelini et al., 2013 ; Walder et al., 2022 ; Zhang et al., 2016). Les herbicides couramment utilisés, tels que le glyphosate, le dichlorprop et le napropamide, peuvent inhiber l'activité et la croissance des micro-organismes du sol (Lu et al., 2018), mais aussi modifier la communauté et la diversité microbiennes du sol (Cycoń & Piotrowska-Seget, 2015 ; Tang et al., 2023). Sim et al. ont démontré que l'azoxystrobine et le flutriafol (fongicides), le chlorsulfuron (herbicide) et le fipronil (insecticide) avaient un impact sur les micro-organismes qui participent au cycle de l'azote, tels que les *Nitrososphaeraceae* (AOA), les *Nitrosospora* (AOB) et les *Nitrospira* (NOB), les effets variant selon le type de sol. De même, l'application de l'herbicide mesulfuron-méthyle et de l'insecticide thiaméthoxame a affecté les micro-organismes du sol participant au cycle de l'azote et d'autres organismes non ciblés pendant les 15 jours suivant l'application. Toutefois, les populations microbiennes ont eu tendance à se rétablir après 60 jours. L'impact de ces pesticides sur les communautés microbiennes est fortement influencé par la dose de pesticide et le type de sol (Cao et al., 2023 ; Wu et al., 2021).

1.6 Évaluation des risques environnementaux liés aux pesticides

Les pesticides sont des polluants environnementaux importants et peuvent se disperser dans diverses matrices environnementales (Carvalho, 2017). L'Union européenne (UE) reconnaît le potentiel de pollution des pesticides et régit l'utilisation des PPP par la

directive 2009/128/CE, qui établit un cadre pour l'utilisation durable des pesticides en atténuant les risques et les impacts sur la santé humaine et l'environnement (« Directive 2009/128/CE du Parlement européen : établissant un cadre d'action communautaire pour parvenir à une utilisation durable des pesticides Texte présentant de l'intérêt pour l'EEE », 2009). En outre, l'UE réexamine et réévalue en permanence les substances actives afin de s'assurer qu'elles répondent aux critères d'approbation pour la mise sur le marché conformément au règlement 1107/2009 (« Règlement (CE) n° 1107/2009 du Parlement européen et du Conseil relatif aux principes uniformes d'évaluation et d'autorisation des produits phytopharmaceutiques », 2011) et au règlement 2020/1740 (« Établissant les dispositions nécessaires à la mise en œuvre de la procédure de renouvellement des substances actives, comme prévu par le règlement (CE) n° 1107/2009 du Parlement européen et du Conseil, et abrogeant les mesures d'exécution de la Commission », 2020)

L'UE a élaboré un système d'évaluation des risques bien structuré qui exige que la concentration d'exposition environnementale prévue soit inférieure à la concentration considérée comme sûre pour les organismes non ciblés (Boivin & Poulsen, 2017). L'évaluation des risques environnementaux des pesticides est structurée autour d'un système à plusieurs niveaux, qui comprend des niveaux multiples qui intègrent progressivement des scénarios plus complexes et plus réalistes. L'ensemble des tests d'écotoxicité normalisés qui soutiennent l'évaluation des risques est utilisé pour évaluer la toxicité des pesticides sur les organismes aquatiques et la faune du sol, contrairement aux micro-organismes du sol, dont la réponse écotoxicologique aux pesticides n'a pas été définie comme une priorité dans le cadre actuel d'évaluation des risques. La première étape (premier niveau) consiste en des tests de toxicité standard utilisant un produit chimique contre une espèce sans facteurs de stress supplémentaires en laboratoire. Dans le cas où les pesticides présentent une exposition prédite supérieure à la concentration sûre, une extension des tests de toxicité standard à des scénarios plus complexes est nécessaire (Boivin & Poulsen, 2017 ; Rohr et al., 2016). Tous ces tests de toxicité sont connus sous le nom de tests de l'OCDE (Organisation de coopération et de développement économiques). Les tests de l'OCDE sont des lignes directrices normalisées utilisées à l'échelle mondiale pour évaluer la sécurité et l'impact environnemental des produits chimiques, y compris les pesticides, et sont conçues pour garantir la cohérence, la fiabilité et la reproductibilité des résultats dans différents laboratoires et pays (« ANNEXE 3. Lignes directrices de l'OCDE pour les essais de produits chimiques », 2016).

Les niveaux supérieurs de l'évaluation des risques liés aux pesticides intègrent les processus et les caractéristiques des écosystèmes naturels, comme les systèmes d'essais semi-sur le terrain sur plusieurs espèces utilisés dans l'UE, et réduisent l'exposition grâce à des mesures d'atténuation dans la gestion des risques (« Règlement (CE) n° 1107/2009 : mise en œuvre du Parlement européen et du Conseil en ce qui concerne les principes uniformes d'évaluation et d'autorisation des produits phytopharmaceutiques, texte présentant de l'intérêt pour l'EEE », 2011). Ce cadre à plusieurs niveaux repose sur deux hypothèses clés : (i) le premier niveau fournit une estimation trop protectrice des effets potentiels sur le terrain ; (ii) les niveaux supérieurs offrent des prévisions plus pertinentes sur le plan écologique des effets sur le terrain ou des concentrations qui n'entraînent pas d'impacts inacceptables (Boivin et Poulsen, 2017).

L'EFSA fournit un cadre complet pour l'évaluation des risques liés aux pesticides afin de garantir qu'ils ne présentent pas de risques inacceptables pour l'environnement aquatique. Elle vise à évaluer les impacts potentiels des pesticides sur les écosystèmes aquatiques, notamment les plans d'eau, les sédiments et les organismes aquatiques (Groupe scientifique de l'EFSA sur les produits phytosanitaires et leurs résidus (PPR), 2013). Le premier niveau comprend des tests sur une seule espèce utilisant trois groupes taxonomiques représentant différents niveaux trophiques : i. poissons (poisson zèbre (*Danio rerio*), tête-de-boule (*Pimephales promela*) et truite arc-en-ciel (*Oncorhynchus mykiss*) ; ii. *Daphnia magna* ; et iii. une algue verte (*Pseudokirchneriella subcapitata*). Une série de tests de l'OCDE est incluse dans ce niveau qui évalue la toxicité aiguë et prolongée sur les poissons et leurs embryons (OCDE TG 203, OCDE TG 204, OCDE TG 236 et OCDE TG 210) (OCDE, 1984, 2013b, 2013a, 2019), l'immobilisation aiguë et la reproduction sur *Daphnia magna* (OCDE TG 202, OCDE TG 211) (OCDE, 2004a, 2012a) et l'inhibition de la croissance des algues (OCDE TG 201) (OCDE, 2006). Le résultat de ces tests est la CL50 (concentration létale pour 50 % des organismes testés) ou la CE50 (concentration efficace provoquant une inhibition de 50 % des organismes testés). Le niveau 1 utilise les scénarios les plus pessimistes et des hypothèses prudentes pour garantir que les risques potentiels ne soient pas négligés, et cela inclut les taux d'application maximaux, les conditions qui maximisent le ruissellement et la pénétration des pesticides dans les eaux de surface et les données sur les espèces les plus sensibles disponibles pour assurer la protection des différents organismes aquatiques. Sur la base des points finaux calculés, les pesticides peuvent illustrer soit un faible risque pour les organismes aquatiques et aucune évaluation supplémentaire n'est nécessaire, soit un risque élevé, nécessitant une évaluation des risques plus précise au niveau 2.

Dans l'évaluation de niveau supérieur, des tests standard avec plusieurs espèces sont effectués dans des conditions eau-sédiments simulant une exposition environnementale naturelle. Le niveau 2 étudie l'évaluation initiale des risques en utilisant des données plus réalistes et spécifiques pour une meilleure compréhension de la toxicité des pesticides. L'objectif principal de ce niveau est de réduire le conservatisme et l'incertitude en incorporant des informations plus détaillées sur l'exposition et les effets. Divers tests et lignes directrices de l'OCDE sont couramment utilisés pour générer les données nécessaires. Ces tests fournissent des méthodologies normalisées pour évaluer le devenir environnemental des pesticides en se concentrant sur l'adsorption et la transformation aérobie/anaérobie dans le sol et dans les sédiments aquatiques (OCDE 106, OCDE 307, OCDE 308) (OCDE, 2000a, 2002a, 2002b). De plus, les tests de l'OCDE sur l'effet des pesticides sur les organismes aquatiques sont utilisés (OCDE 210, OCDE 211, OCDE 201 et OCDE 218) (OCDE, 2006, 2012a, 2013a, 2023). Ces tests comprennent des tests de toxicité sur les poissons aux premiers stades de leur vie, des tests de reproduction sur *Daphnia magna*, des tests d'inhibition de la croissance sur les algues fraîches et les cyanobactéries et des tests de toxicité sur le cycle de vie des chironomes sédiments-eau. Français Les essais de bioaccumulation des pesticides sur les poissons et de minéralisation aérobie dans les eaux de surface et les sédiments sont également utilisés au niveau 2 (OCDE 305 et OCDE 309) (OCDE, 2004b, 2012b). Ce niveau est également caractérisé à l'aide d'outils de modélisation comme les modèles FOCUS (par exemple, les scénarios d'eaux de surface FOCUS (référence)) pour des scénarios plus complexes qui simulent diverses conditions agricoles et environnementales pour la prévision

des concentrations de pesticides dans les eaux de surface en bordure des champs et étalonnent à l'aide de données de terrain pour garantir l'exactitude et la fiabilité. Les modèles de niveau supérieur sont également courants pour les prévisions du mouvement des pesticides dans les eaux de surface et les environnements aquatiques. De même, au niveau 1, si les points finaux indiquent des niveaux de risque acceptables, un pesticide est considéré comme sûr à utiliser dans les conditions évaluées. Dans le cas contraire, un affinement supplémentaire ou des évaluations de niveau supérieur (niveau 3) peuvent être nécessaires.

L'objectif principal du niveau 3 est d'affiner et de valider l'évaluation des risques menée au niveau 2 par le biais d'études plus détaillées et souvent spécifiques au site. Ce niveau implique des approches plus complexes et plus gourmandes en ressources pour fournir une caractérisation plus précise des risques environnementaux potentiels. Le composant central de ce niveau comprend des études de terrain avec des mésocosmes aquatiques et des microcosmes de sol afin de stimuler les écosystèmes aquatiques naturels et d'évaluer les effets des pesticides sur des communautés aquatiques complexes sur des périodes prolongées (EFSA Panel on Plant Protection Products and their Residues (PPR), 2013). Une autre approche consiste en des distributions de sensibilité des espèces (SSD) qui sont dérivées des données du niveau 2 ou d'études supplémentaires de niveau supérieur qui peuvent fournir une distribution de sensibilité entre diverses espèces, aidant à affiner les estimations des risques et à évaluer les impacts potentiels sur la biodiversité. En outre, la modélisation spécifique au site est utilisée pour les données de surveillance environnementale de sites spécifiques où les pesticides sont appliqués, fournissant des concentrations d'exposition réelles pour améliorer la précision des évaluations des risques. Des recherches plus approfondies sur les effets sublétaux sur les organismes aquatiques (notamment les paramètres physiologiques, comportementaux et reproductifs) pour comprendre les impacts potentiels à long terme sont réalisées dans ce niveau. Sur la base des résultats des différents tests, une intégration complète de toutes les données disponibles (devenir environnemental, exposition, toxicité) est réalisée pour affiner les estimations des risques et fournir une caractérisation solide des risques potentiels posés par les pesticides. Le niveau 3 représente le niveau de complexité le plus élevé dans le processus d'évaluation des risques pour les PPP, impliquant des méthodologies avancées, des études détaillées et une intégration complète des données. En abordant les incertitudes et en offrant une compréhension différente des risques environnementaux, le niveau 3 garantit que les décisions réglementaires sont fondées sur des preuves scientifiques solides, visant à protéger les écosystèmes aquatiques tout en permettant l'utilisation sûre et durable des pesticides dans l'agriculture (EFSA Panel on Plant Protection Products and their Residues (PPR), 2013).

Contrairement à l'écotoxicologie aquatique et aux bio-essais correspondants, il n'existe pas de système d'évaluation des risques bien structuré pour les micro-organismes du sol. Français Considérant les rôles fonctionnels clés du microbiote du sol dans le fonctionnement des écosystèmes, l'Autorité européenne de sécurité des aliments (EFSA) a identifié les micro-organismes du sol comme un objectif de protection spécifique pour l'évaluation des risques environnementaux des pesticides (Autorité européenne de sécurité des aliments, 2010). Les micro-organismes du sol participent au cycle des nutriments, modulent la fertilité et la santé du sol, favorisent la croissance des plantes et préservent l'équilibre entre les facteurs de stress biotiques et abiotiques. L'évaluation de la toxicité

microbienne des pesticides dans le sol repose sur l'effet des activités enzymatiques microbiennes du sol en termes de transformation de N et de C. Cependant, ces évaluations de toxicité reposent sur des tests microbiens obsolètes de transformation de N et de C (OCDE 216 et 217, respectivement) (OCDE, 2000b, 2000c). L'évolution de la microbiologie du sol a établi une série de normes de l'Organisation internationale de normalisation (ISO) basées sur l'extraction de l'ADN du sol, l'analyse des acides gras phospholipidiques (PLFA), les mesures de l'activité enzymatique du sol et la détermination de l'abondance microbienne par q-PCR. En 2016, l'EFSA a identifié les champignons mycorhiziens à arbuscules comme indicateurs potentiels de la toxicité microbienne des pesticides dans le sol en s'appuyant sur des outils moléculaires avancés (EFSA Panel on Plant Protection Products and their Residues (PPR) et al., 2017). Des études récentes ont également montré que les microbes oxydant l'ammoniac (AOM) étaient des indicateurs microbiens idéaux de la toxicité des produits agrochimiques.

1.7 Nouveau système proposé d'évaluation des risques des pesticides sur les micro-organismes du sol

L'évaluation des risques environnementaux des pesticides utilise un système à plusieurs niveaux qui progresse à travers des niveaux de complexité et de réalisme croissants comme décrit ci-dessus. Cependant, cette approche à plusieurs niveaux n'a pas encore été pleinement appliquée aux micro-organismes du sol. Les premières recherches ont fourni les premières lignes directrices expérimentales pour évaluer la toxicité des pesticides sur les micro-organismes du sol, suggérant que les effets inhibiteurs significatifs observés en laboratoire devraient inciter à d'autres études à l'échelle du terrain. Conformément à cette approche, Karpouzas et al. ont proposé un système à plusieurs niveaux similaire spécifiquement pour évaluer l'impact des pesticides sur les communautés microbiennes du sol. Ce système comprend trois niveaux de complexité croissante, axés sur les réponses écotoxicologiques des principaux groupes fonctionnels du sol, tels que les micro-organismes oxydant l'ammoniac (AOM) et les champignons mycorhiziens à arbuscules (AMF) (Figure 1.4). Le niveau I implique le criblage *in vitro* des pesticides contre une gamme diversifiée de souches d'AOM et d'AMF dérivées du sol les plus sensibles, représentant divers groupes écophysiologicals et phylogénétiques. Si cette évaluation préliminaire suggère des effets toxiques potentiels, l'évaluation passe aux niveaux II et III. Les deuxième et troisième niveaux évaluent la toxicité des pesticides dans des microcosmes de sol en laboratoire ou des études en pots plantés (pour les CMA) et une évaluation à l'échelle du terrain dans des conditions agricoles respectivement, en utilisant l'AOM et les CMA comme bioindicateurs. Si des niveaux inacceptables de toxicité des pesticides sur les micro-organismes du sol sont observés dans des études en microcosme de laboratoire ou en pots, une évaluation plus approfondie au niveau du terrain est réalisée. Dans les deux niveaux, des outils biochimiques et moléculaires avancés peuvent être utilisés pour surveiller l'activité, l'abondance et la diversité des bioindicateurs sélectionnés. À chacune de ces échelles expérimentales, l'évaluation se concentre exclusivement sur les communautés microbiennes naturelles. Que l'étude soit menée dans un microcosme, en pots ou sur le terrain, il est essentiel de surveiller la dissipation et la transformation du pesticide au fil du temps. Cela permet d'identifier le toxique spécifique, en distinguant le composé parent et ses produits de transformation (TP).

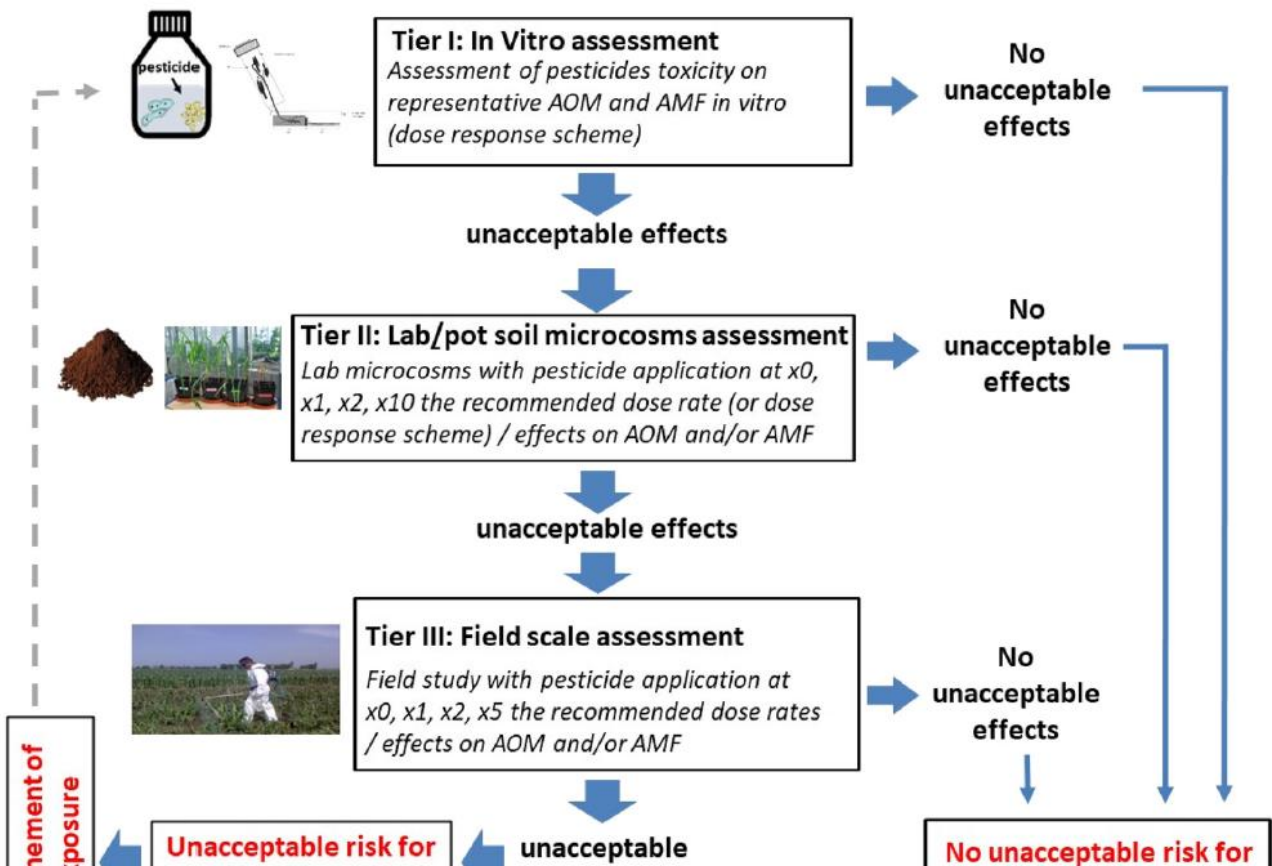


Figure 1.4 Schéma d'évaluation des risques à trois niveaux proposé pour évaluer la toxicité des pesticides sur les micro-organismes du sol (Karpouzas et al., 2022). Micro-organismes oxydant l'ammoniac, AOM ; champignons mycorhiziens à arbuscules, AMF

1.8 Nitrification et micro-organismes nitrifiants

1.8.1 Processus de nitrification

La nitrification joue un rôle essentiel dans le cycle global de l'azote, en convertissant l'ammoniac (NH_3) en nitrate (NO_3^-) via le nitrite (NO_2^-). Ce processus augmente la disponibilité de l'azote pour les plantes et améliore la fertilité du sol (Schleper & Nicol, 2010). La nitrification chimio-litho-autotrophe implique des micro-organismes qui génèrent de l'énergie en oxydant des composés inorganiques, en particulier l'ammoniac ou le nitrite, en utilisant cette énergie pour leurs processus métaboliques. Dans les sols, la concurrence pour l'énergie est intense, ce qui limite souvent l'activité microbienne (Martikainen, 2022). Dans les sols agricoles fertilisés, la forte disponibilité de l'ammoniac favorise des taux élevés de nitrification chimio-litho-autotrophe par les oxydants d'ammoniac. Français Cependant, dans les sols acides et non fertilisés, ce processus devient plus difficile, bien que l'oxydation de l'ammonium et des nitrites puisse toujours se produire (De Boer et Kowalchuk, 2001 ; Faeflen et al., 2016, s.d.). Les micro-organismes et les gènes impliqués dans la nitrification ont été largement étudiés, soulignant l'importance économique et environnementale de ce processus (You et al., 2009). La nitrification est un processus modulaire qui est effectué par une gamme de groupes microbiens spécialisés décrits ci-dessous (Figure 1.5). La nitrification a été considérée comme un processus en deux étapes, l'oxydation de NH_3 et NO_2^- étant considérée comme effectuée par deux groupes fonctionnels indépendants. Français La première étape, généralement considérée comme l'étape limitant la vitesse de nitrification, implique l'oxydation du NH_3 en hydroxylamine (NH_2OH) à l'aide de l'ammoniac monooxygénase (AMO) puis la conversion de ce dernier en oxyde nitrique (NO) par l'hydroxylamine déshydrogénase (HAO), et enfin en nitrite (NO_2^-) (Beeckman et al., 2018 ; Caranto & Lancaster, 2017 ; James I. Prosser et al., 2020 ; Stein, 2019). Les micro-organismes qui réalisent cette étape sont les bactéries oxydantes l'ammoniac (AOB) (Kowalchuk & Stephen, 2001) et plus récemment les archées oxydantes l'ammoniac (AOA) découvertes (Könneke et al., 2005). La monooxygénation de l'ammoniac (AMO) est présente dans les deux groupes microbiens. Cette enzyme est une protéine transmembranaire. Français L'activation de cette enzyme nécessite deux électrons et du cuivre lié dans les sous-unités AmoB et AmoC (Beeckman et al., 2018 ; James I. Prosser et al., 2020). Les sous-unités AmoA, AmoB et AmoC

sont conservées dans l'AOB et l'AOA (Wright & Lehtovirta-Morley, 2023). L'AMO archéenne semble avoir trois sous-unités supplémentaires, AmoX, AmoY et AmoZ (Hodgskiss et al., 2023). La deuxième réaction implique l'oxydation de NH_2OH en NO , par l'hydroxylamine hydrogénase (HAO). Cette enzyme est une protéine multihémique et se trouve principalement dans l'AOB (Arp & Stein, 2003 ; Caranto & Lancaster, 2017). Plusieurs mécanismes ont été proposés pour la conversion du NO en NO_2^- , notamment (a) la transformation abiotique, (b) une nitrite réductase codée par *nirK* car cette enzyme peut également catalyser la réaction inverse et (c) une réductase d'oxyde nitrique non caractérisée avec comme candidat potentiel une protéine Cu rouge, la nitrosocyanine (Caranto & Lancaster, 2017 ; James I. Prosser et al., 2020). La deuxième étape de la nitrification implique la conversion du NO_2^- en NO_3^- par des bactéries oxydant les nitrites (NOB) qui utilisent la nitrite oxydoréductase (NXR) (Beeckman et al., 2018 ; Coskun et al., 2017). Cette enzyme est une protéine transmembranaire qui porte trois sous-unités *NxrA*, *NxrB* et *NxC*. Selon l'emplacement de la sous-unité de liaison au substrat *NxrA*, il existe deux types d'enzymes NXR, périplasmiques et cytoplasmiques (Daims et al., 2016). Des études récentes ont remis en question la théorie de la division obligatoire du travail métabolique dans la nitrification et ont rapporté la découverte d'une bactérie du genre *Nitrospira* sp., auparavant connue sous le nom de NOB, avec la capacité métabolique d'effectuer l'oxydation complète de l'ammoniac en nitrate (« oxydants d'ammoniac complets », comammox) (Daims et al., 2015 ; van Kessel et al., 2015).

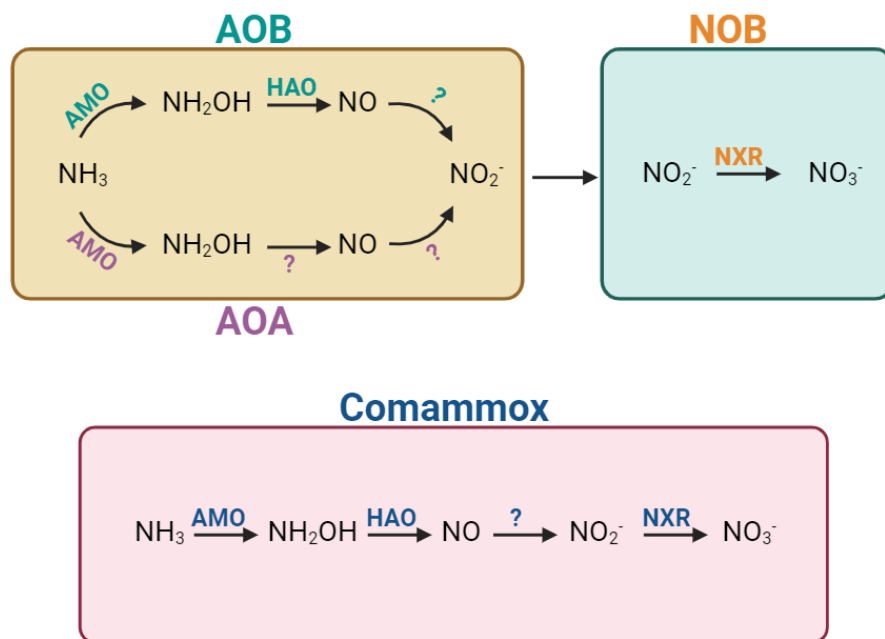


Figure 1.5 Nitrification chimio-lithoautotrophe, réactions et groupes microbiens réalisant chaque étape du processus.

1.8.2 Bactéries oxydant l'ammoniac

Les AOB chimio-litho-autotrophes ont été isolées pour la première fois en 1890 (Winogradsky, 1890) avec des représentants cultivés appartenant aux lignées bêta- et gammaprotéobactériennes. Les bêta-protéobactéries AOB appartiennent à deux genres de la même famille, *Nitrosomonas* et *Nitrospira* des *Nitrosomonadaceae*, et les gammaprotéobactéries AOB appartiennent aux genres *Nitrosococcus*, *Nitrosoglobus* et *Nitrosacidococcus* (Dang et al., 2018 ; Di et al., 2010 ; Kowalchuk & Stephen, 2001 ; Purkhold et al., 2000). Dans le sol, les AOB dominants sont *Nitrospira* (Aigle et al., 2019 ; Purkhold et al., 2000, 2003) tandis que *Nitrosoglobus* et *Nitrosacidococcus* sont communément présents dans les sols acides (Hayatsu et al., 2017 ; Picone et al., 2021). *Nitrosomonas* a été trouvé dans le sol mais domine principalement dans les écosystèmes artificiels (Mobarry et al., 1996 ; A. Schramm et al., 1996) tandis que *Nitrosococcus* se trouve principalement dans les environnements marins (Campbell et al., 2011).

La structure membranaire, la taille et la forme des AOB varient selon les différents groupes microbiens impliqués dans la nitrification. Les AOB sont en forme de bâtonnet (*Nitrosomonas*), en forme de spirale ou lobées pléomorphes (*Nitrospira*) et sphériques/ellipsoïdales (*Nitrosococcus*, *Nitrosoglobus* et *Nitrosacidococcus*). La membrane cellulaire des AOB est caractérisée par la présence de lipides liés à l'éther qui ont des acides gras comme chaînes latérales. La structure de la membrane détermine la perméabilité de la cellule et par conséquent sa sensibilité aux substances xénobiotiques telles que les pesticides (Shen et al., 2013).

Les AOB sont omniprésents dans les habitats terrestres tels que les zones agricoles, les prairies et les forêts (Gubry-Rangin et al., 2010) ainsi que dans les habitats aquatiques (Kowalchuk & Stephen, 2001). Leur abondance dans le sol varie d'environ 10⁴-10⁶ cellules AOB g⁻¹ de sol. Les facteurs environnementaux tels que la teneur en métaux lourds, la présence ou l'absence de plantes et le pH peuvent affecter l'abondance et l'activité des AOB (Gubry-Rangin et al., 2010). Des études antérieures ont montré qu'une augmentation du pH du sol conduit à une augmentation des transcrits du gène *amoA* des AOB (Nicol et al., 2008). La température est un autre facteur qui semble affecter l'activité des AOB. La température optimale pour la croissance des AOB est de 25 à 30 °C, tandis qu'une diminution de la température peut réduire la production de NO₂⁻ (Tourna et al., 2008). De plus, la température est un facteur qui peut provoquer des changements dans la structure et la diversité de la communauté microbienne des AOB dans les écosystèmes végétaux et artificiels (You et al., 2009).

Les AOB sont caractérisés comme des micro-organismes autotrophes, chimiolithotrophes avec un inventaire génétique pour l'oxydation de l'ammoniac pour générer de l'énergie pour la fixation du CO₂ en utilisant le cycle de Calvin (Kowalchuk & Stephen, 2001). Français L'ammoniac (NH₃) est le principal substrat de l'AMO (Suzuki et al., 1974). L'azote peut pénétrer dans les cellules par diffusion à travers les membranes biologiques sous forme de NH₃ ou à travers des protéines membranaires dont les transporteurs d'ammoniac et d'ammonium (NH₄⁺) (Andrade et al., 2005 ; Ellerbeck et al., 2013 ; Tobias Wacker et al., 2014). Les transporteurs d'ammonium ou transporteurs Amt dépendent de l'énergie et présentent une forte affinité se produisant dans la survie des micro-organismes dans des conditions de faible pH et de concentration limitée en substrat (Wright & Lehtovirta-Morley, 2023). Les protéines de type Rh servent de transporteurs d'ammoniac, fonctionnant comme des canaux de faible affinité qui permettent le mouvement bidirectionnel de NH₃. Ces protéines sont efficaces à des niveaux de pH élevés et à des concentrations de substrat élevées (Ellerbeck et al., 2013 ; T. Wacker et al., 2014 ; Wright et Lehtovirta-Morley, 2023). Selon les génomes AOB disponibles, certains AOB contiennent des protéines Rh tandis que l'absence de transporteurs d'ammoniac reconnaissables illustre probablement qu'ils dépendent de la diffusion de l'ammoniac (Lehtovirta-Morley, Sayavedra-Soto et al., 2016a ; Wright et Lehtovirta-Morley, 2023).

L'oxydation de l'ammoniac est un métabolisme spécialisé de l'AOB, malgré les produits intermédiaires toxiques, tels que NH₂OH, NO et NO₂⁻. Les AOB oxydent NH₃ en NH₂OH en utilisant AMO (Beeckman et al., 2018 ; James I. Prosser et al., 2020 ; Stein, 2019). Cette enzyme est un complexe transmembranaire hétérotrimérique dépendant du cuivre, composé de trois sous-unités (AmoA, AmoB et AmoC) dont le site actif n'est pas défini (Tolar et al., 2017 ; Wright & Lehtovirta-Morley, 2023). Outre le cuivre, l'AMO a besoin d'O₂ et de deux électrons (e⁻) pour oxyder NH₃ en NH₂OH, qui est ensuite transformé en NO par l'hydroxylamine hydrogénase. Au cours de cette étape, 3 e⁻ sont produits et tous deux répondent aux besoins de l'AMO tandis que le troisième se retrouve dans la chaîne de transport d'électrons (Stein, 2019). L'intermédiaire NO indique l'existence d'une oxydoréductase d'oxyde nitrique qui catalyse la transformation de NO en NO₂⁻. Deux complexes enzymatiques différents ont été proposés pour l'AOB : (a) la nitrosocyanine, codée par le gène *ncyA* et (b) la nitrite réductase contenant du cuivre à fonctionnement inverse codée par *nirK* régulée par une exposition à une

concentration élevée en NH_3 . (Caranto & Lancaster, 2017 ; James I. Prosser et al., 2020 ; Stein, 2019 ; Zorz et al., 2018). *NcyA* est le candidat le plus probable de cette étape car il est présent dans tous les génomes AOB et est un gène fortement exprimé contrairement à *nirK* qui est absent dans certains AOB et n'est pas systématiquement exprimé (Zorz et al., 2018) (Figure 1.6).

De nombreuses études ont rapporté l'isolement de souches AOB en laboratoire appartenant aux genres *Nitrosomonas* et *Nitrospira* et qui poussent à un pH neutre et à une température de croissance optimale de 25-30°C. Récemment, l'isolement de souches AOB acidophiles, qui poussent à pH 3,5, a été rapporté (Hayatsu et al., 2017 ; Picone et al., 2021). Dans les études présentées dans cette thèse, seules des souches de bêtaprotéobactéries ont été utilisées (*Nitrosomonas europaea* ATCC25978, *Nitrospira multififormis* ATCC25196, *Nitrosomonas communis* DSM 28436, *Nitrospira briensis* C-128, *Nitrosomonas ureae* Nm 10 et *Nitrosomonas oligotropha* Nm 45). Toutes ces souches AOB sont Gram négatives et sont isolées du sol.

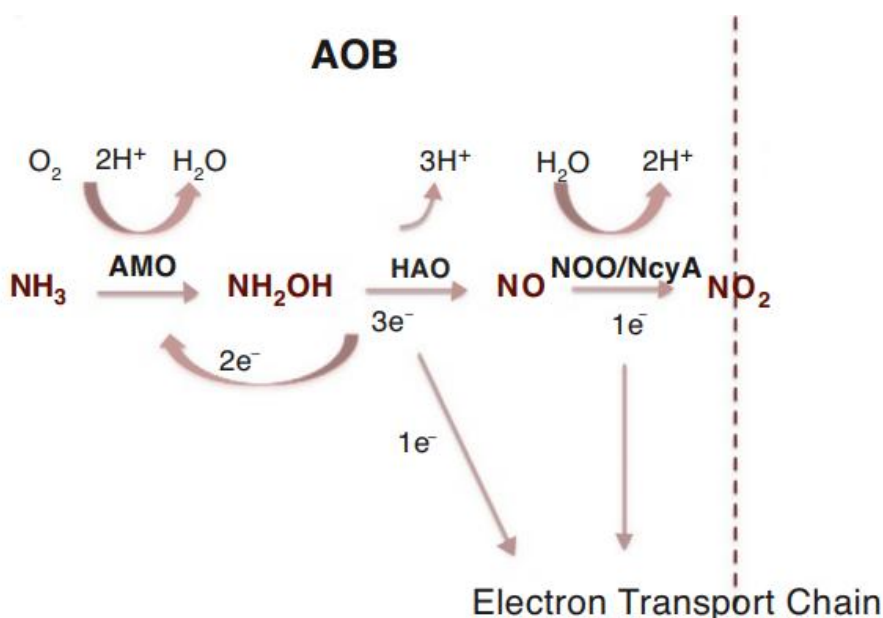


Figure 1.6 Oxydation de l'ammoniac par des bactéries oxydant l'ammoniac (AOB) (modifiée d'après Stein et al., 2019).

1.8.3 Archées oxydant l'ammoniac

Les AOA sont le deuxième groupe microbien qui participe à l'oxydation de l'ammoniac en NO_2^- . En 2005, le premier isolement d'une AOA marine, *Nitrosopumilus maritimus*, a été décrit (Könneke et al., 2005). Les AOA sont omniprésentes et très diversifiées dans les écosystèmes terrestres et marins (Alves et al., 2018 ; Gubry-Rangin et al., 2010). Elles appartiennent à la classe *Nitrososphaeria* au sein du phylum *Nitrososphaerota* (ou *Thermoproteota* dans la taxonomie GTDB) et sont synonymes du *Thaumarchaeota* précédemment utilisé. Toute la diversité connue des AOA est représentée par quatre lignées phylogénétiques : *Nitrososphaerales*, *Nitrosopumilales*, *Nitrosotaleales* et thermophile. *Nitrosocaldales* (Brochier-Armanet et al., 2008 ; Kerou et al., 2016 ; Stieglmeier et al., 2014) avec *Nitrososphaerales* et *Nitrosotaleales* étant les deux lignées dominantes dans les écosystèmes du sol. Les AOA dominent l'oxydation de l'ammoniac dans les sols acides

(environ 30 % des sols à l'échelle mondiale), les *Nitrosotaleales* étant les principaux micro-organismes qui oxydent le NH_3 à faible pH. Dans les sols acides, cette activité était considérée comme paradoxale en raison de la faible disponibilité du NH_3 et de la sensibilité de l'AOM à l'acide nitreux (HNO_2) et à ses produits de décomposition, qui sont formés à partir du NO_2^- à de faibles niveaux de pH (Braidà & Ong, 2000). La découverte et la culture ultérieures de *Nitrosotaleales* acidophiles et acidotolérantes ont expliqué en grande partie ce paradoxe (Hayatsu et al., 2017 ; Lehtovirta-Morley et al., 2014 ; Picone et al., 2021). Cependant, des mécanismes tels que l'activité uréolytique (Burton & Prosser, 2001 ; De Boer et al., 1988), la croissance au sein de biofilms (Allison & Prosser, 1993) et les agrégats (De Boer et al., 1991) favorisent également la croissance des nitrificateurs neutrophiles dans des environnements acides.

Comme pour les AOB, la structure membranaire, la taille et la forme des AOA les distinguent des autres groupes microbiens impliqués dans la nitrification. Les AOA sont en forme de bâtonnet ou de sphère (Hatzenpichler, 2012 ; Lehtovirta-Morley, Ross, et al., 2016). La membrane cellulaire de l'AOA se distingue par la présence de lipides glycérol-éther, où les chaînes latérales isoprénoïdes sont attachées via une liaison éther à un squelette glycérol, contrairement aux acides gras de l'AOB. De plus, ces chaînes latérales isoprénoïdes sont souvent liées entre elles, ce qui entraîne la formation d'une monocouche lipidique avec des chaînes C40, plutôt que la structure bicouche typique observée chez d'autres organismes. Par conséquent, le composant hydrophobe central des lipides de la membrane cellulaire des archées est principalement composé de tétraéthers de glycérol dialkyl glycérol (Schleper & Nicol, 2010).

Les AOA sont systématiquement plus abondants dans les écosystèmes terrestres et marins que les AOB. Le sol contient généralement plus de 107 cellules AOA g^{-1} de sol et on les trouve dans de nombreux écosystèmes terrestres tels que les zones agricoles, les prairies, les forêts, les sols alpins (Gubry-Rangin et al., 2010), mais aussi dans de nombreux habitats aquatiques, et sont considérés comme l'AOM dominant dans les systèmes marins (référence). Les AOA ont également été trouvés dans les stations d'épuration des eaux usées (You et al., 2009). Les facteurs qui affectent l'abondance et la fonction des AOB dans différents environnements affectent également les AOA dans la même mesure (Gubry-Rangin et al., 2010).

Les AOA sont également des micro-organismes chimiolithoautotrophes et utilisent l'ammoniac monooxygénase (AMO) pour oxyder l'ammoniac pour les besoins énergétiques. Cependant, ils utilisent l'acide 3-hydroxypropionique/acide 4-hydroxybutyrique au lieu du cycle de Calvin (Walker et al., 2010) pour la fixation du carbone. Comme pour l'AOB, le NH_3 est le substrat principal de l'AMO (Jung et al., 2022). L'ammoniac pénètre dans les cellules archéennes par diffusion à travers les membranes biologiques. Cependant, en raison de la composition différente des membranes archéennes et bactériennes, la perméabilité membranaire et la vitesse à laquelle l'ammoniac peut diffuser varient. La plupart des souches d'AOA codent des transporteurs de type Amt pour l'absorption de NH_4^+ (Lehtovirta-Morley, Sayavedra-Soto et al., 2016b ; Wright et Lehtovirta-Morley, 2023). La réponse transcriptionnelle des transporteurs Amt archaïques à différentes concentrations d'ammoniac suggère que ces transporteurs fonctionnent à la fois comme des systèmes à

haute et à faible affinité (Nakagawa et Stahl, 2013 ; Qin et al., 2018 ; Santoro et Casciotti, 2011).

Les AOA oxyde le NH_3 en NH_2OH en utilisant l'AMO (Beeckman et al., 2018 ; James I. Prosser et al., 2020 ; Stein, 2019). Cette enzyme est similaire à l'AOA et à l'AOB, la principale différence étant la présence de trois sous-unités supplémentaires, AmoX, AmoY et AmoZ (Hodgskiss et al., 2023). L'AMO nécessite de l'oxygène et deux e^- pour oxyder le NH_3 en NH_2OH . L'oxydation ultérieure de l'hydroxylamine n'est pas encore caractérisée et aucune enzyme candidate n'a été identifiée. Kozłowski et al. (2016) ont proposé qu'un complexe enzymatique à base de cuivre co-oxyde NH_2OH et NO pour former deux molécules de NO_2^- (avec la libération de cinq e^-) avec une molécule de NO_2^- réduite en NO par la nitrite oxydoréductase (NirK) et renvoyée au complexe enzymatique à base de cuivre (Kozłowski et al., 2016 ; Stein, 2019). Une deuxième voie a été proposée sur la base de données de métagénome, de transcriptome et de métatranscriptome par laquelle NirK oxyde soit NH_2OH en NO avec la libération de 3 e^- ou, dans une fonction inverse (rNIR), oxyde NO en NO_2^- avec la production de 1 e^- , et un complexe enzymatique à base de cuivre qui agit soit comme hydroxylamine-oxydoréductase soit comme NO-oxydoréductase, selon le rôle de NirK ou d'un autre Cu-ME capable de ces réactions (Carini et al., 2018) (Figure 1.7).

Les souches d'AOA du sol peuvent être acidophiles ou neutrophiles, et se développent dans des cultures liquides avec une température de croissance optimale de 30 à 40 °C. Les AOA appartenant au genre *Nitrosotalea* poussent dans des milieux dont le pH est compris entre 4 et 6 (Lehtovirta-Morley et al., 2014, 2011) et toutes les autres souches poussent à pH neutre. Dans les études présentées dans cette thèse, des souches d'AOA acidophiles (*Nitrosotalea sinensis* Nd2) et neutrophiles (*Candidatus Nitrosocosmicus franklandianus* C13, *Nitrososphaera viennensis* EN76) ont été utilisées.

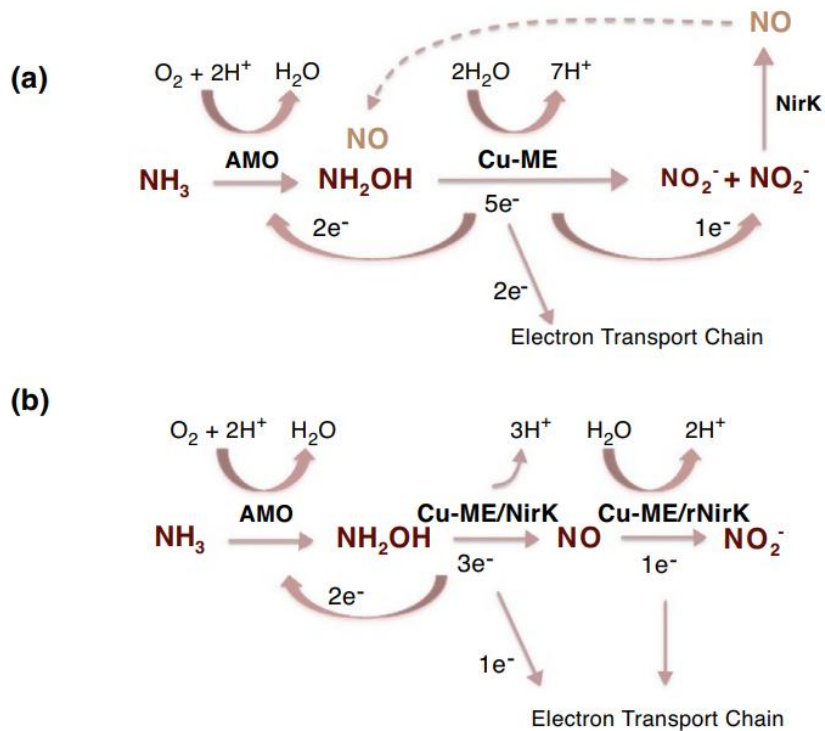


Figure 1.7 Oxydation de l'ammoniac par les archées oxydant l'ammoniac (AOA). Les panneaux (a) et (b) montrent les voies proposées pour l'oxydation de l'hydroxylamine (Stein et al., 2019).

1.8.4 Bactéries oxydantes les nitrites

La deuxième étape du processus de nitrification modulaire implique la transformation du NO_2^- en NO_3^- , le produit final de la nitrification. Ce processus est réalisé par des bactéries oxydantes les nitrites (NOB) qui appartiennent à sept genres dans quatre embranchements bactériens. Plus précisément, *Nitrobacter*, *Nitrococcus* et *Nitrotoga* sont placés dans le phylum Proteobacteria, *Nitrospira* dans le phylum Nitrospirae, *Nitrospina* et *Candidatus Nitromaritima* dans le phylum Nitrospinaea, et *Nitrolancetus* dans le phylum Chloroflexi (Daims et al., 2016 ; Liu et al., 2010). Dans les écosystèmes terrestres, *Nitrospira* et *Nitrobacter* sont les NOB dominants, *Nitrobacter* étant généralement plus actif et avec une croissance plus rapide que *Nitrospira*, sous un apport élevé en azote (Daims et al., 2016 ; Nowka, Off, et al., 2015 ; Xia et al., 2011). En tant que tels, *Nitrobacter* est considéré comme des stratégies r tandis que *Nitrospira* est des stratégies k et atteint une abondance élevée sous limitation en nitrite (Daims et al., 2016 ; Andreas Schramm et al., 1999). En plus des associations avec différentes lignées phylogénétiques, il existe des différences structurelles et biochimiques entre les NOB (Daims et al., 2016 ; Lücker et al., 2010).

Tous les NOB sont Gram-négatifs. Les souches de *Nitrobacter* sont en forme de bâtonnet ou de poire. Français Les souches de ce genre possèdent une membrane intracytoplasmique caractéristique (Pillay et al., 1989 ; Jim I. Prosser, 2007). Les souches *Nitrospira* se développent sous forme de cellules hélicoïdales ou de vibrions (Ehrich et al., 1995). L'oxydation du NO_2^- contribue aux besoins énergétiques du NOB et elles sont considérées comme chimioautolithotrophes (Daims et al., 2016 ; Lücker et al., 2010). Les NOB

fixent le CO₂ via le cycle de Calvin pour répondre à leurs besoins en carbone (Daims et al., 2016 ; Lücker et al., 2015, 2010). Cependant, les *Nitrospira* sont également capables de mixotrophie et peuvent basculer entre la fixation du carbone et la consommation de molécules organiques telles que le glycérol, le pyruvate ou le formate, selon les conditions environnementales (Koch et al., 2015). Des études récentes ont également indiqué que certains *Nitrospira* de différents écosystèmes terrestres et aquatiques peuvent utiliser l'urée comme source de nutriments (Koch et al., 2015). Leurs génomes peuvent coder l'uréase ou la cyanase, qui transforment l'urée ou le cyanate en CO₂ et en ammoniac (Fujitani et al., 2014 ; Koch et al., 2015), facilitant l'alimentation croisée réciproque des micro-organismes oxydant l'ammoniac et l'utilisation du NO₂⁻ généré (Daims et al., 2016). L'analyse du génome a également indiqué que le NOB peut basculer entre l'oxydation des nitrites et des métabolismes alternatifs tels que l'oxydation du H₂ ou du formate (Koch et al., 2014).

Le NOB catalyse l'oxydation du NO₂⁻ en NO₃⁻ à l'aide de la nitrite oxydoréductase (NXR). Au cours de cette étape, deux e⁻ sont transférés dans la chaîne respiratoire. Cette enzyme appartient à la famille des enzymes de liaison à la molybdoptérine de type DMSO réductase de type II (Lücker et al., 2013). NXR est généralement associée à la membrane cytoplasmique et comprend probablement trois sous-unités : NxrA, NxrB et NxrC (Lücker et al., 2013, 2010). Chez des espèces telles que *Nitrospira*, *Nitrospina* et '*Candidatus Nitromaritima*', la sous-unité de liaison au substrat NxrA est située dans l'espace périplasmique tandis que chez *Nitrobacter*, *Nitrococcus* et *Nitrolancea*, elle se trouve dans le cytoplasme (Daims et al., 2016). Les NOB avec NXR cytoplasmique utilisent des transporteurs pour le transfert de nitrite et de nitrate à travers la membrane cytoplasmique, créant potentiellement un goulot d'étranglement physiologique en fonction de l'affinité du substrat et du taux de renouvellement du transporteur (Daims et al., 2016) (Figure 1.8).

Des cultures pures de souches de *Nitrobacter* issues du sol sont disponibles avec différents représentants poussant à pH acide ou neutre (De Boer et al., 1988 ; Hink et al., 2024 ; K. D. Kits et al., 2017 ; Nowka, Daims, et al., 2015) tandis que des souches de *Nitrospira* ont été isolées à partir de traitements des eaux usées (Keuter et al., 2023 ; Nowka, Off, et al., 2015). Dans les études présentées dans cette thèse, *Candidatus Nitrobacter laanbroekii* NHB1, *Nitrobacter winogradskyi* DSM 10237 et *Nitrospira defluvii* A17 ont été utilisés. Toutes les souches NOB sont isolées du sol à l'exception de *N. defluvii* qui est isolée des boues activées.

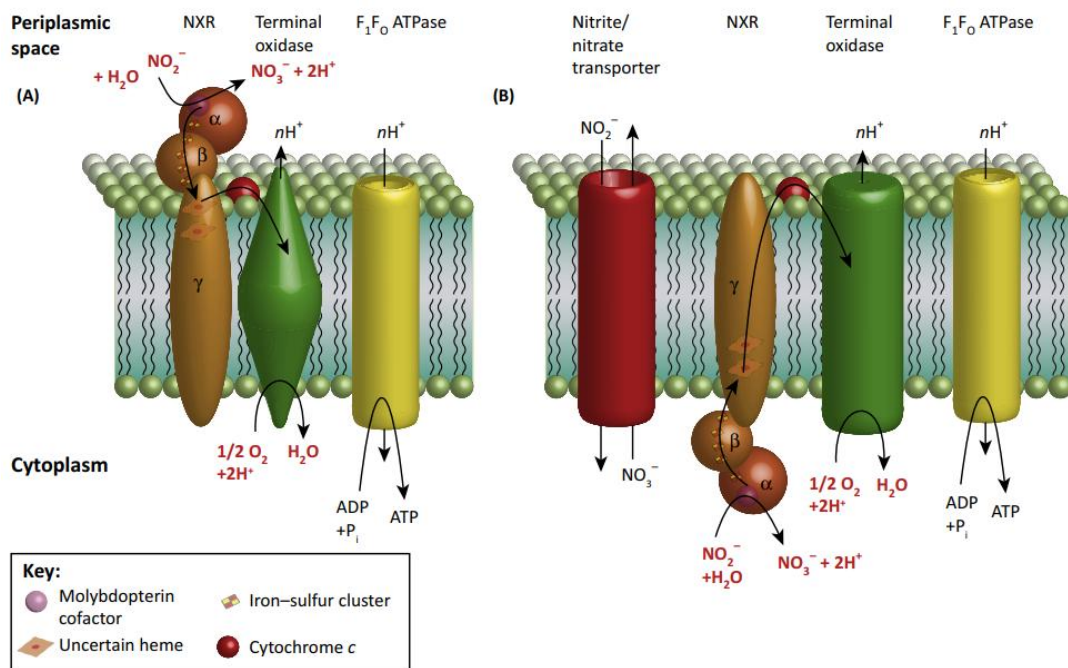


Figure 1.8 Le mécanisme d'oxydation du nitrite en nitrate par la nitrite oxydoréductase (NXR) (A) cytoplasmique et (B) périplasmique (Daims et al., 2016).

1.8.5 Bactéries oxydantes complètes de l'ammoniac (comammox)

Le dogme d'une division obligatoire du travail métabolique dans la nitrification a été remis en cause à partir de 2015 avec la culture de souches de *Nitrospira* capables d'oxyder l'ammoniac en nitrate dans la même cellule, *Candidatus Nitrospira inopinata*, *Candidatus Nitrospira nitrificans*, *Candidatus Nitrospira nitrosa* et *Candidatus Nitrospira kreftii* qui sont capables de réaliser les deux étapes de la nitrification. Ces micro-organismes sont appelés comammox (oxydants complets de l'ammoniac). Les bactéries Comammox appartiennent au genre *Nitrospira* qui comprend également le NOB canonique (Daims et al., 2015), et ont été détectées dans une gamme d'environnements naturels et artificiels (Fowler et al., 2018 ; Palomo et al., 2018 ; Pjevac et al., 2017 ; Wang et al., 2019 ; Zheng et al., 2019). L'analyse des séquences du gène *amoA* suggère deux groupes phylogénétiques (clade A et B), tous deux présents dans le sol (Pjevac et al., 2017).

Les Comammox *Nitrospira* sont des bactéries Gram-négatives de forme spiralée (Daims et al., 2015 ; van Kessel et al., 2015). Les Comammox ont une affinité relativement élevée pour le substrat ammoniacal, ce qui indique qu'à de faibles concentrations, ils obtiennent un avantage concurrentiel sur les AOB et les AOA. Français En revanche, leur affinité pour le nitrite est bien inférieure à celle du NOB canonique (K. Dimitri Kits et al., 2017). Les différents clades de comammox ont des mécanismes d'absorption d'ammoniac/ammonium différents. Le clade A de comammox code des transporteurs de type Rh avec une forte similitude avec ceux de l'AOB tandis que le clade B de comammox code des transporteurs de type Amt (Palomo et al., 2018).

Les analyses génomiques des souches cultivées de *Nitrospira* ont montré la présence de gènes nécessaires à l'oxydation de NH_3 en NO_3^- . L'analyse génomique comparative des comammox *Nitrospira* révèle que leurs gènes d'oxydation de l'ammoniac sont plus similaires à ceux de l'AOB qu'à ceux de l'AOA (Daims et al., 2015 ; Sakoula et al., 2021 ; van Kessel et al., 2015). Français Des homologues de tous les gènes amo sont présents dans le comammox, ainsi que des HAO de type AOB et des cytochromes de type c, qui facilitent le transfert d'électrons vers le pool de quinones (Daims et al., 2015). Comme pour d'autres AOM, le complexe enzymatique qui catalyse la transformation de NO en NO_2^- n'est pas encore caractérisé. NirK est un candidat possible pour cette étape car les génomes du comammox sont dépourvus de ncyA (Kits et al., 2019). Ces micro-organismes peuvent également utiliser l'urée comme source alternative d'azote car ils codent les gènes nécessaires à l'hydrolyse de l'urée et à la haute affinité des transporteurs d'urée (Daims et al., 2015 ; Sakoula et al., 2021 ; van Kessel et al., 2015).

La culture du comammox est difficile. Jusqu'à présent, une seule souche de comammox, *Ca. Nitrospira inopinata*, appartenant au clade A, a été purement isolé, dérivé d'un biofilm microbien dans une conduite d'eau chaude et se développe à pH neutre (Daims et al., 2015). Des cultures d'enrichissement de comammox du clade A, *Ca. Nitrospira nitrificans*, *Ca. Nitrospira nitrosa* et *Ca. Nitrospira kreftii* provenant d'un système d'aquaculture ont également été signalées (Sakoula et al., 2021 ; van Kessel et al., 2015). Actuellement, aucune souche de comammox dérivée du sol n'est disponible malgré leur présence dans les écosystèmes terrestres.

1.9 Les microorganismes oxydant l'ammoniac comme indicateurs microbiens idéaux de la toxicité des pesticides

Les microbes du sol et les complexes enzymatiques impliqués dans la nitrification ont été largement étudiés en raison de la grande importance économique et écologique du processus (Brochier-Armanet et al., 2008 ; Francis et al., 2007 ; You et al., 2009). Plusieurs études utilisant des outils moléculaires et biochimiques avancés ont évalué la toxicité des pesticides et de leurs produits métaboliques sur l'AOM dans le sol (Karas et al., 2015 ; Papadopoulou et al., 2016 ; Vasileiadis et al., 2018) et au niveau in vitro (Papadopoulou et al., 2020 ; Vasileiadis et al., 2018). Au-delà de leur rôle écologique clé, les AOM ont des caractéristiques spécifiques qui en font des candidats idéaux comme bioindicateurs de la toxicité des pesticides : i. ils présentent une sensibilité aux composés xénobiotiques, dont les pesticides (Wessén & Hallin, 2011), ii. ils participent à l'étape limitante du cycle de l'azote (Beeckman et al., 2018 ; James I. Prosser & Nicol, 2008), et iii. il existe des connaissances sur leur écologie, leur physiologie et leur cycle biologique ainsi que des outils bien établis pour mesurer leur activité et leur abondance in vitro (Lehtovirta-Morley et al., 2013) et dans le sol (Junier et al., 2010).

L'étude de l'impact des pesticides sur le groupe NOB est importante pour élargir le spectre de toxicité non ciblée des pesticides sur un groupe microbien fonctionnellement associé à l'AOM et pour prédire une éventuelle contribution indirecte aux émissions de N_2O . La réduction de l'activité du NOB ou son inhibition après l'application de produits

agrochimiques pourrait augmenter la perte d'azote des écosystèmes agricoles perturbés et aggraver le problème de la pollution atmosphérique (James I. Prosser et al., 2020).

1.10 Objectifs

L'objectif principal de cette thèse était le développement et la normalisation de tests *in vitro* pionniers pour un nouveau schéma d'évaluation des risques visant à évaluer la toxicité des pesticides sur des AOM sensibles, phylogénétiquement et écophysiologiquement distincts. Étant donné le nombre limité de cultures disponibles d'AOA et d'AOB dérivées du sol, notre objectif principal était de sélectionner les AOA, AOB et NOB qui sont systématiquement plus sensibles et présentent des réponses écotoxicologiquement pertinentes claires à l'exposition aux pesticides (chapitre 2).

Ces tests sont destinés à servir d'étape de test de premier niveau, analogue aux tests sur une seule espèce effectués pour les organismes aquatiques dans l'évaluation des risques liés aux pesticides, dans un cadre révisé d'évaluation des risques à plusieurs niveaux pour évaluer la toxicité des pesticides sur les micro-organismes du sol. Plus précisément, l'objectif était de tester la toxicité de pesticides sélectionnés sur les niveaux d'activité d'une gamme de souches AOA, AOB et NOB présentes dans les écosystèmes du sol, et de calculer des paramètres de toxicité tels que les valeurs CE50 (concentration efficace 50 %). À titre de preuve de concept, un deuxième ensemble de pesticides a été utilisé pour tester la sensibilité des souches sélectionnées, validant ainsi davantage la robustesse et la fiabilité des résultats initiaux (chapitre 3).

Pour les composés qui ont montré des effets de toxicité significatifs *in vitro* sur l'AOA et l'AOB, l'exploration des mécanismes de toxicité est en cours en utilisant la transcriptomique et la protéomique pour obtenir des informations sur les réponses moléculaires et les voies affectées par l'exposition aux pesticides. Cette approche globale permet d'identifier des biomarqueurs de la toxicité des pesticides et de comprendre les impacts écologiques plus larges sur les communautés microbiennes du sol. La compréhension de ces mécanismes peut aider au développement de pesticides plus sûrs et plus ciblés qui minimisent les impacts négatifs sur les communautés microbiennes dans les écosystèmes agricoles et l'environnement.

Les résultats du chapitre 2 ont été comparés à ceux d'un kit d'évaluation de la toxicité microbienne (MARA) disponible dans le commerce et fourni par la société de biotechnologie NCIMB afin de valider et de comparer les résultats (chapitre 4). Ce travail pourrait contribuer au développement d'un format rapide et à haut débit qui faciliterait les pratiques de test de toxicité des pesticides, fournissant un outil pratique aux organismes de réglementation et aux programmes de surveillance environnementale.

Au chapitre 5, *Ca. Nitrobacter laanbroekii* NHB1, l'une des souches les plus sensibles étudiées, a été caractérisée pour obtenir des informations plus approfondies sur ses caractéristiques physiologiques et ses interactions avec les archées acidophiles oxydantes de l'ammoniac (AOA), telles que les souches *Nitrosotalea*. Ce chapitre met en évidence le rôle essentiel de *Ca. Nitrobacter laanbroekii* NHB1 dans la promotion de l'activité des oxydants d'ammoniac (AO) dans des conditions de faible pH et souligne l'importance des interactions

mutualistes entre les bactéries oxydant les nitrites (NOB) et les micro-organismes oxydant l'ammoniac (AOM) dans les environnements acide.

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(N.d.).

Discussion Générale

2.1 Vue d'ensemble

L'utilisation de produits phytosanitaires (PP) est une pratique agricole essentielle pour obtenir des rendements élevés des cultures et répondre à la demande alimentaire mondiale (Tudi et al. 2021). Cependant, seule une petite proportion de ces produits atteint les organismes ciblés, le reste s'accumulant dans le sol, ce qui a un impact négatif sur les écosystèmes et les micro-organismes du sol (Mitchell et al. 2001). Les évaluations des risques environnementaux liés aux pesticides suivent généralement une approche à plusieurs niveaux, dont la complexité et la pertinence écologique augmentent. Cependant, cette méthodologie n'a pas été pleinement appliquée aux micro-organismes du sol, malgré leur rôle essentiel dans le fonctionnement des écosystèmes et la santé des sols. Les premières recherches ont indiqué que la toxicité importante des pesticides observée en laboratoire justifiait des études plus poussées à l'échelle du terrain (Atlas et al. 1978). Sur cette base, Karpouzas et al. (2016 ; Karpouzas et al. 2022) ont proposé un système à plusieurs niveaux spécifiquement pour évaluer l'impact des pesticides sur les communautés microbiennes du sol, en se concentrant sur des groupes clés comme les micro-organismes oxydant l'ammoniac (AOM) et les champignons mycorhiziens à arbuscules (AMF). Dans ce système d'évaluation des risques proposé, le niveau I implique un criblage *in vitro* des pesticides contre une gamme de souches AOM et AMF sensibles et représentatives dérivées du sol, choisies pour leur pertinence écologique et leur diversité phylogénétique. Si une toxicité potentielle est détectée, l'évaluation passe au niveau II, qui implique des microcosmes de sol en laboratoire ou des études en pots plantés (pour les AMF), et le niveau III implique des évaluations à l'échelle du terrain dans des conditions agricoles. À ces stades, les AOM et les AMF servent de bioindicateurs, avec des outils biochimiques et moléculaires avancés utilisés pour surveiller l'activité microbienne, l'abondance et la diversité. Français II est important de suivre la dissipation et la transformation des pesticides au fil du temps, en distinguant le composé parent de ses produits de transformation (TP) pour garantir l'identification des toxiques spécifiques (Karpouzas et al. 2016 ; Karpouzas et al. 2022).

L'objectif principal de cette thèse était de développer et de valider un bio-essai *in vitro* utilisant l'AOM et le NOB fonctionnellement apparenté comme essai standard de niveau I, lié aux tests monospécifiques couramment utilisés en écotoxicologie aquatique (chapitres 2 et 3). Ces résultats ont été comparés à un essai d'évaluation de la toxicité existant, le kit Microbial Assay for Risk Assessment (MARA) du NCIMB, démontrant la nécessité d'un kit de toxicité commercial plus adapté, spécialement conçu pour évaluer les impacts des produits agrochimiques sur les écosystèmes du sol (chapitre 4). De plus, nous avons caractérisé la physiologie du *Ca. Nitrobacter laanbroekii* NHB1, l'une des souches les plus sensibles étudiées, fournit des informations sur la physiologie de cet isolat et ses interactions avec les souches d'archées acidophiles oxydant l'ammoniac (AOA) en co-culture, simulant les conditions naturelles du sol (chapitre 5).

2.2 Développement et validation d'un essai biologique in vitro utilisant des souches nitrifiantes comme bioindicateurs

Les chapitres 2 et 3 détaillent le développement et la validation de tests monospécifiques utilisant des souches nitrifiantes comme bioindicateurs pour évaluer la toxicité des pesticides sur les microorganismes du sol. Cette recherche comble une lacune critique dans les connaissances concernant les effets toxiques potentiels des pesticides sur les communautés microbiennes du sol. En établissant des essais biologiques in vitro utilisant des AOA, des bactéries oxydant l'ammoniac (AOB) et des bactéries oxydant les nitrites (NOB) comme bioindicateurs, les résultats de ces essais fournissent un outil pratique pour évaluer les impacts écotoxicologiques des pesticides sur les écosystèmes du sol.

Ces essais, conçus comme des tests de niveau I conformément aux pratiques d'écotoxicologie aquatique, ont permis d'identifier des souches microbiennes spécifiques qui présentent une sensibilité élevée aux pesticides. Parmi les AOA, *N. sinensis* est apparue comme la souche la plus sensible, tandis que *N. briensis* était le candidat le plus approprié pour les AOB en raison de sa prévalence dans les écosystèmes du sol. Pour les NOB, *Ca. Nitrobacter laanbroekii* NHB1 a montré la plus grande sensibilité aux pesticides, ce qui en fait également un bioindicateur potentiel de la toxicité des pesticides.

Les travaux réalisés pour cette thèse ont également révélé des schémas de toxicité spécifiques à chaque catégorie de pesticides. Les fongicides, en particulier l'étridiazole et la pyraclostrobine, ont montré la plus forte toxicité pour les micro-organismes nitrifiants aux concentrations généralement utilisées en agriculture. Cela suggère que l'utilisation agricole régulière de ces fongicides pourrait perturber de manière significative le processus de nitrification, qui est crucial pour le cycle de l'azote et la fertilité globale du sol. Le NOB a montré une plus grande résilience à la toxicité des pesticides que l'AOM. Cette résilience peut être due à leurs voies métaboliques polyvalentes, qui pourraient améliorer la résistance aux xénobiotiques. Certaines souches de *Nitrobacter*, par exemple, peuvent dégrader des composés organiques complexes (John et Okpokwasili 2012). De même, *Nitrospira* présente une mixotrophie, alternant entre la fixation du carbone et la consommation de molécules organiques en réponse aux conditions environnementales (Koch et al. 2015). De plus, des études génomiques indiquent que le NOB peut passer de l'oxydation des nitrites à des voies métaboliques alternatives dans certaines conditions (Koch et al. 2014).

L'étude souligne également la nécessité cruciale d'évaluer non seulement les pesticides mais aussi leurs produits de transformation, qui peuvent être tout aussi nocifs, voire plus, pour les micro-organismes oxydant l'ammoniac (AOM) du sol. Par exemple, la 3,5-dichloroaniline (3,5-DCA), un métabolite de l'iprodione, s'est avérée aussi toxique, voire plus toxique, que le composé d'origine (Vasileiadis et al., 2018). Papadopoulou et al. ont également constaté que la quinone imine, un produit de transformation de l'éthoxyquine, un conservateur de fruits, réduisait de manière significative l'activité, l'abondance et la diversité des AOM dans les études in vitro et les expériences sur le sol (Papadopoulou et al. 2016 ; Papadopoulou et al. 2020). Français Des études sur le terrain ont montré des effets similaires du chlorpyrifos qui présentait une inhibition dose-dépendante de l'AOA due à la fois au composé parent et à son métabolite toxique, le TCP (Karas et al., 2018). De même, le pesticide chlorothalonil se dégrade dans le sol pour former des métabolites, tels que le 4-hydroxy-2,5,6-

trichloroisophtalonitrile, le 2,4,5-trichloroisophtalonitrile et le 2,5,6-trichloro-4-méthylthioisophtalonitrile, qui étaient encore plus toxiques que le composé parent, entraînant des réductions significatives des populations d'AOA et d'AOB et de la nitrification globale (Wu et al., 2014 ; Teng et al., 2017). Ces résultats soulignent l'importance d'une approche complète d'évaluation de la toxicité des pesticides, intégrant à la fois les composés parents et leurs produits de transformation, pour évaluer avec précision les impacts à long terme sur la santé des sols et la stabilité de l'écosystème.

Pendant la validation de l'essai proposé, *N. sinensis*, identifiée comme la souche d'archées la plus sensible, a constamment démontré une sensibilité élevée aux pesticides supplémentaires testés, en particulier aux fongicides, confirmant son potentiel en tant que bioindicateur d'alerte précoce des perturbations induites par les pesticides dans les communautés microbiennes du sol. En revanche, *N. briensis* a montré une plus grande tolérance aux pesticides et a été significativement affectée par quelques composés, tels que le tébuconazole et le chlorothalonil. Ces résultats mettent en évidence les différentes sensibilités des AOM, probablement dues à des différences structurelles, biochimiques et physiologiques. *Ca. Nitrobacter laanbroekii* NHB1 a également affiché une faible sensibilité aux pesticides, indiquant sa résilience et soulignant l'importance d'inclure une gamme diversifiée de réponses microbiennes dans les évaluations de toxicité. Dans l'ensemble, les fongicides ont généralement présenté une toxicité plus élevée pour les souches AOA que pour les souches AOB et NOB, ce qui suggère la nécessité d'évaluations ciblées des risques pour des groupes de pesticides spécifiques.

Cette thèse démontre efficacement l'utilité des tests *in vitro* monospécifiques utilisant des nitrifiants comme bioindicateurs de la toxicité des pesticides dans les microorganismes du sol. En incluant à la fois des souches microbiennes sensibles et tolérantes, ces tests permettent une évaluation complète des effets des pesticides. La mise en œuvre de cette approche améliorerait les évaluations précoces des risques, renforcerait les cadres d'évaluation des risques et soutiendrait la préservation des fonctions écologiques essentielles assurées par le microbiote du sol, contribuant ainsi à des pratiques agricoles plus durables qui préservent la santé des sols et les services écosystémiques essentiels.

2.3 Évaluation des limites d'un kit de toxicité commercial et du rôle essentiel des nitrifiants dans les évaluations de l'écotoxicité des pesticides

Au chapitre 4, l'efficacité du kit MARA a été évaluée en tant qu'outil à haut débit pour évaluer l'écotoxicité des pesticides. En comparant la sensibilité des souches MARA aux nitrifiants du sol, en particulier l'AOM, ce travail visait à souligner la nécessité de réévaluer les méthodologies actuelles d'évaluation de la toxicité des pesticides dans les communautés microbiennes du sol. Nos résultats démontrent que les nitrifiants du sol, en particulier l'AOA, présentent une sensibilité significativement plus élevée à divers pesticides que ceux évalués à l'aide du kit MARA. Alors que les souches MARA ont montré des réponses limitées à des concentrations de pesticides plus élevées, l'AOM a connu une inhibition de croissance substantielle à des niveaux beaucoup plus faibles, soulignant leur potentiel en tant que bioindicateurs efficaces pour les évaluations des risques environnementaux.

L'étude a également révélé des réponses variables à l'exposition aux pesticides entre les principaux groupes de nitrifiants, l'AOA étant plus sensible que les souches MARA et d'autres nitrifiants tels que l'AOB et le NOB. Cela souligne l'importance de sélectionner des bioindicateurs qui reflètent avec précision la dynamique de l'écosystème du sol. Étant donné le rôle crucial des AOM dans le cycle de l'azote et leur grande sensibilité aux pesticides, l'inclusion de ces micro-organismes dans les cadres réglementaires permettrait une évaluation plus précise et plus pertinente sur le plan écologique des impacts des pesticides.

Les limites du kit MARA, dans le contexte de l'évaluation de l'écotoxicité des pesticides agricoles, soulignent la nécessité de nouveaux outils commerciaux qui représentent mieux les communautés microbiennes du sol. La diversité taxonomique des souches MARA dérivées de différents environnements ne parvient pas à capturer les interactions et les fonctions spécifiques des micro-organismes du sol qui sont essentiels aux processus biochimiques clés. En se concentrant sur les nitrificateurs du sol, le développement d'un nouveau kit de toxicité commercial permettrait de mieux comprendre la complexité de l'écosystème du sol, améliorant ainsi notre capacité à prédire et à atténuer les risques écologiques posés par l'utilisation des pesticides.

2.4 Importance écologique de *Ca. Nitrobacter laanbroekii* NHB1 pour faciliter la nitrification dans les sols acides

Le chapitre 5 a examiné la physiologie de *Ca. Nitrobacter laanbroekii* NHB1, une souche bactérienne oxydant les nitrites (NOB) tolérante aux acides isolée d'un sol acide, soulignant son rôle crucial dans la facilitation de l'activité d'oxydant d'ammoniac (AO) dans des conditions de faible pH. NHB1 empêche efficacement l'accumulation de nitrites et la dégradation ultérieure en intermédiaires toxiques, soutenant ainsi la survie et la fonction métabolique des AOA acidophiles, en particulier les souches *Nitrosotalea* obligatoirement acidophiles. Des expériences de co-culture ont confirmé que NHB1 étend la tolérance au pH de l'AOA et atténue la toxicité des nitrites, soulignant l'importance des interactions mutualistes entre NOB et AOM dans les environnements acides.

NHB1 démontre une adaptabilité remarquable sur une gamme de concentrations de nitrites et de niveaux de pH, avec une croissance optimale à pH 6,0. À des concentrations plus faibles en nitrites, NHB1 peut étendre sa plage de pH fonctionnelle, révélant un lien étroit entre sa tolérance à l'acide et sa capacité d'oxydation des nitrites. Ce phénomène est également susceptible de se produire dans les sols acides, où le NOB joue un rôle clé dans l'élimination du NO_2^- , réduisant ainsi le risque que les oxydants d'ammoniac (AOM) soient inhibés par des formes toxiques de nitrites.

Cependant, il est essentiel de considérer que les systèmes de sol sont plus complexes, impliquant de multiples facteurs et mécanismes qui peuvent influencer la survie et la fonction des AOM. Cette adaptabilité est donc cruciale dans les environnements acides où l'accumulation de nitrites pourrait autrement inhiber les oxydants d'ammoniac, soulignant l'importance écologique du NOB dans le maintien des processus de nitrification dans les sols acides.

L'étude a également révélé des interactions physiologiques entre l'AOA et le NOB dans des conditions acides. Les co-cultures sont un outil puissant pour étudier les interactions entre différents groupes microbiens, fournissant un modèle écologiquement plus pertinent et plus réaliste des environnements naturels. En nitrification, les co-cultures permettent d'explorer les fonctions synergétiques entre les groupes microbiens impliqués dans le processus, où la survie et l'efficacité de chaque groupe sont interdépendantes. Cette approche améliore notre compréhension de la dynamique coopérative essentielle à la nitrification, offrant un aperçu de la façon dont ces microbes prospèrent collectivement dans des écosystèmes partagés. Dans cette étude, les souches de *Nitrosotalea*, en tant qu'acidophiles obligatoires, ont montré des taux de croissance et d'oxydation de l'ammoniac améliorés lorsqu'elles étaient co-cultivées avec NHB1. Ces expériences démontrent que NHB1 réduit non seulement la toxicité des nitrites, mais améliore également indirectement l'activité de l'AOA par le biais du métabolisme du cyanate, qui produit de l'ammonium pour l'oxydation par l'AOA.

2.5 Perspectives d'avenir

Les résultats de cette thèse apportent des informations importantes sur la réponse des micro-organismes nitrifiants à l'exposition aux pesticides dans les écosystèmes du sol, ouvrant la voie à de futures recherches. Les principaux domaines d'intérêt comprennent le perfectionnement des tests de toxicité des pesticides à haut débit, l'utilisation d'approches omiques pour explorer les mécanismes moléculaires de la toxicité des pesticides et l'étude des réponses régulatrices des souches nitrifiantes aux facteurs de stress environnementaux comme le pH. Ces efforts contribueront à développer des évaluations des risques environnementaux plus précises et des pratiques agricoles durables, préservant ainsi la santé des sols et la fonctionnalité des écosystèmes.

Une orientation prometteuse pour l'avenir est l'adaptation des bio-essais actuels dans des formats à haut débit adaptés aux tests réglementaires de routine des pesticides. Contrairement à d'autres cultures microbiennes, les micro-organismes nitrifiants présentent des défis de culture uniques en raison de leurs taux de croissance lents et de leurs exigences d'incubation prolongées, ce qui nécessite des chercheurs qualifiés dotés d'une expertise spécialisée pour maintenir ces souches. Cependant, des progrès sont en cours pour rationaliser ces tests, en particulier dans l'évaluation des inhibiteurs de nitrification, ce qui pourrait étendre leur application à l'évaluation des impacts des pesticides sur les micro-organismes du sol. Le développement d'un kit de test spécialisé axé sur les nitrifiants du sol, similaire au système MARA, pourrait améliorer considérablement la sensibilité et la spécificité de la détection de la toxicité des pesticides dans les écosystèmes du sol. En incorporant à la fois des micro-organismes oxydant l'ammoniac (AOM) et des bactéries oxydant les nitrites (NOB), un tel kit permettrait une évaluation plus rapide et plus précise des effets des pesticides, offrant un outil précieux pour la surveillance environnementale et l'utilisation réglementaire. Cette innovation pourrait grandement améliorer notre capacité à protéger la santé des sols et à favoriser des pratiques agricoles durables.

Les recherches futures devraient se concentrer sur le perfectionnement de ces tests, la validation de leur efficacité et leur intégration dans les cadres réglementaires. Le test mono-

espèce proposé représente l'une des premières tentatives de création d'un test d'écotoxicité de niveau I qui peut compléter le test OCDE 216 de niveau II largement utilisé (OCDE 2000) pour évaluer les risques des pesticides pour les micro-organismes du sol en fonction de la transformation de l'azote dans le sol. Bien que ce test mono-espèce ait une pertinence écologique moindre, il offre un outil écotoxicologique prometteur qui, dans certains cas, peut éliminer le besoin d'évaluations de niveau II et III plus complexes. L'approche accélérée peut permettre d'économiser beaucoup de temps et d'efforts, améliorant ainsi la protection de la santé des sols et soutenant des pratiques agricoles durables.

Un autre domaine important de recherche future est une étude approfondie des mécanismes de toxicité des pesticides sur les souches AOA et AOB, en particulier celles connues pour présenter une toxicité élevée, en utilisant des techniques omiques avancées. Des études antérieures ont utilisé des approches omiques pour mieux comprendre la physiologie et le métabolisme central de l'AOM, avec des analyses transcriptomiques et protéomiques élucidant les adaptations de l'AOA et de l'AOB à des conditions telles que la limitation du cuivre et la privation de nutriments, respectivement (Kerou et al. 2016 ; Zorz et al. 2018 ; Reyes et al. 2020 ; Hodgskiss et al. 2023). Français Les travaux actuels, en collaboration avec l'Université de Vienne, exploreront les effets toxiques du chlorpyrifos et de la pyraclostrobine sur *Nitrososphaera viennensis* (une souche AOA) et du metsulfuron-méthyl et de la pyraclostrobine sur *Nitrosospira multiformis* (une souche AOB) par le biais d'analyses protéomiques et transcriptomiques. La protéomique clarifiera les perturbations métaboliques induites par l'exposition aux pesticides, tandis que la transcriptomique révélera les réponses régulatrices de ces micro-organismes sous stress. Il est important de noter que les données transcriptomiques complèteront la protéomique en détectant des protéines membranaires souvent difficiles à analyser, offrant ainsi une vue complète des réponses cellulaires à la toxicité des pesticides. Des tests biochimiques supplémentaires, guidés par des données omiques, permettront une analyse plus ciblée de la toxicité des pesticides. En comprenant les mécanismes par lesquels les pesticides affectent les AOM non ciblés, cette recherche peut éclairer le développement de pesticides plus sûrs et plus ciblés qui minimisent les dommages causés aux communautés microbiennes bénéfiques dans les écosystèmes agricoles.

Une extension importante de la recherche actuelle consiste à effectuer une analyse transcriptomique du *Ca. Nitrobacter laanbroekii* NHB1 dans des conditions de pH variables. Cela permettrait de mieux comprendre les réponses régulatrices qui permettent à cette souche NOB tolérante à l'acide de se développer dans différents pH. L'analyse des profils d'expression génétique dans différentes conditions de pH peut révéler les mécanismes moléculaires qui régissent la tolérance au pH et la capacité d'oxydation des nitrites de NHB1. Ces connaissances pourraient mieux élucider le rôle écologique de NHB1 dans le soutien de la nitrification dans les environnements acides, offrant ainsi des informations sur la manière dont ces processus pourraient être optimisés dans les environnements agricoles confrontés à l'acidification des sols.

2.6 Conclusion

Cette thèse a développé un nouveau bio-essai de niveau I utilisant des micro-organismes nitrifiants comme bio-indicateurs pour évaluer la toxicité des pesticides dans les écosystèmes du sol. Les essais ont montré que les AOA sont très sensibles aux pesticides, en particulier aux fongicides, tandis que les bactéries oxydant l'ammoniac AOB et NOB ont démontré une plus grande résilience. Des souches sensibles clés comme *N. sinensis* (AOA), *N. briensis* (AOB) et *Ca. Nitrobacter laanbroekii* NHB1 (NOB) ont été identifiées, ce qui étaye leur utilisation dans les premières évaluations des risques liés aux pesticides. L'étude a mis en évidence les limites des essais existants comme MARA, soulignant la nécessité de bio-indicateurs microbiens et de kits de toxicité spécifiques au sol. De plus, *Ca. Nitrobacter laanbroekii* NHB1 a joué un rôle essentiel dans la nitrification dans les sols acides, empêchant l'accumulation de nitrites toxiques et favorisant la survie des oxydants d'ammoniac acidophiles, démontrant ainsi l'importance écologique de ces interactions. Dans l'ensemble, cette thèse fournit des outils pratiques pour améliorer l'évaluation des risques liés aux pesticides et contribue au développement de pesticides plus sûrs et de pratiques agricoles durables.

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Annex I-Supplemental Material of Chapter 2

Single species *in vitro* assays with nitrifying bacteria and archaea as a first-tier tool for assessing the toxicity of pesticides on soil microorganisms

Statement on author contributions

The work presented in Chapter 2 is a manuscript currently under revision for consideration of publication in the journal *Environmental Science and Technology*. This work was carried out in collaboration with the University of Thessaly. The work examining the toxicity of pesticides on two AOA strains (*Nitrosotalea sinensis*, *Nitrososphaera viennensis*), four AOB strains (*Nitrosomonas europaea*, *Nitrosomonas oligotropha*, *Nitrosomonas ureae*, *Nitrospira briensis*), and one NOB strain (*Nitrospira defluvii*) was performed at ECL by E. Bachtsevani, and in parallel, PhD candidate M. Kolovou conducted similar experiments at UTH, testing the toxicity on one AOA strain (*Candidatus Nitrosocosmicus franklandianus*), two AOB strains (*Nitrosomonas communis*, *Nitrospira multiformis*), and two NOB strains (*Nitrobacter winogradskyi*, *Candidatus Nitrobacter laanbroekii* NHB1). The study of pesticide stability in liquid cultures was also performed at UTH. Both Ph.D. candidates collaborated on the analysis of the results. The manuscript was written entirely by E. Bachtsevani, with all authors providing comments only.

1. Material and Methods

1.1 Culture conditions

All strains were grown aerobically in the dark without shaking. Five AOB strains (*N. europaea* ATCC25978, *N. multiformis* ATCC25196, *N. briensis* C-128, *N. ureae* Nm 10 and *N. oligotropha* Nm 45) were grown at 28°C in Skinner and Walker's medium (Skinner & Walker, 1961) containing 1 mM NH₄⁺ (NH₄)₂SO₄ and phenol red (0.5 mg L⁻¹) as a pH indicator. The AOB *N. communis* Nm 2 (DSM 28436), provided by the DSMZ (German Collection of Microorganisms and Cell Cultures), was grown at 28°C in 1583 DSMZ medium with 10 mM NH₄⁺ (NH₄Cl) and phenol red (0.5 mg L⁻¹) as a pH indicator. AOA, *Ca. N. franklandianus* C13 and *N. sinensis* Nd2, were incubated at 35°C in a medium supplemented with 1 mM NH₄⁺ and 0.5 mM NH₄⁺ (NH₄Cl), respectively. The former was cultured in HEPES-buffered modified freshwater medium (pH 7.5) (Lehtovirta-Morley et al., 2016), while the latter was grown in MES-buffered freshwater medium (pH 5.2) (Lehtovirta-Morley et al., 2011). *N. viennensis* EN76 was grown at 35°C in HEPES-buffered freshwater medium (pH 7.5) supplemented with 2 mM NH₄⁺ (NH₄Cl) (Tourna et al., 2011). *Ca. Nitrobacter laanbroekii* NHB1 and *N. winogradskyi* DSM 10237 (also provided by DSMZ) were grown at 28°C in freshwater medium (pH 5.2) (Lehtovirta-Morley et al., 2011) and modified Skinner and Walker medium (pH 7.5) (Skinner & Walker, 1961) supplemented with 0.5 mM NO₂⁻ and 20 mM NO₂⁻ (NaNO₂) and trace element solution, respectively. *N. defluvii* A17, which was isolated from activated sludge, was incubated at 28°C in a medium supplemented with 0.3 mM NO₂⁻ (NaNO₂) (Nowka et al., 2015).

1.2 Mode of calculations for pesticides concentration

The concentration levels of the pesticides used in the *in vitro* tests (Supplementary Table S1) were calculated, considering different factors which could influence the amount reaching soil after the application of the recommended dose rate. The tested pesticides are applied either through foliage (chlorpyrifos, glyphosate, clethodim, metsulfuron-methyl, pyraclostrobin) or soil drenching (etridiazole, hymexazol). For foliar-applied pesticides the foliage interception factors derived from the FOCUS working group (FOCUS 2021) were used to calculate the amount of pesticide reaching the soil. For all pesticides we considered an application at the top 5 cm of soil except for compounds which are specifically incorporated in the top 20 cm of soil. We further assumed that all pesticide reaching the soil would end up in the soil solution and considered an average gravimetric soil moisture content of 20% and a bulk density of 1.3 g mL⁻¹. Finally, for pesticides applied more than once per season (chlorpyrifos, pyraclostrobin, etridiazole), we took into consideration their soil DT₅₀ value (as derived from EFSA documents) and the time interval between two successive applications. Hence for chemicals whose soil DT₅₀ value is longer than the time interval between two successive applications, no degradation was assumed and the amount of pesticide from each application was multiplied by the number of times applied. Alternatively, for chemicals with soil DT₅₀ values shorter than the time intervals between two successive applications, pesticide degradation was considered in our calculations. Also, some pesticides are registered for use

in many different crops at different dose rates. For the purpose of this study, we have selected for our calculations the highest dose rate from all registered uses, to represent a worst-case scenario exposure regime.

Below we provide two examples of the assumptions and calculations for the derivation of the concentrations of pesticides in the soil solution expected to occur after the application of the agronomic recommended dose rate of the tested pesticides. The first example is the application (only foliar) of the insecticide chlorpyrifos. The recommended dose rate of chlorpyrifos for a potato crop (worst-case scenario) according to the EFSA database is 1.2 kg of the active substance per hectare (ha) and the growth stage-season of application is at the beginning of the attack when the first symptoms are visible. We therefore considered that the minimum foliar interception for a foliar application of chlorpyrifos is 15% (FOCUS 2021), hence 1.02 kg of the active substance reaches the soil (1.2 kg of active substance \times 0.85). The weight of 500 m³ of soil (1 ha = 10,000 m² \times 0.05 m (pesticides applied at the soil surface and its residues are distributed in the top 5 cm)) with a soil bulk density of 1.3 t m⁻³ equals 650 t. Hence, after a single chlorpyrifos application, the final concentration of pesticide in soil will be 1.57 mg kg⁻¹ (1020 g active substance in 650 t of soil). For a potato crop, two applications per year are recommended with the minimum interval between these applications being 15 days. Considering that the geometric mean DT₅₀ for chlorpyrifos in soil is 40.2 days (according to EFSA database), it is assumed that no degradation occurs during sequential applications. Therefore, the final recommended dose rate (1.57 mg kg⁻¹) is multiplied by a factor of two to account for two sequential applications with no degradation of the compound in between the 15-day interval. This leads to an estimated worst-case concentration of chlorpyrifos in the soil of 3.14 mg kg⁻¹. For *in vitro* tests it is assumed that all pesticide reaching the soil will end up in the soil solution. Thus, if 3.14 mg of active ingredient are present in 1000 g of soil and considering the soil moisture content of 20% (200 g water in 1000 g soil) then 3.14 mg will be present at a concentration of 15.69 mg L⁻¹ in water. Hence, 15.69 mg L⁻¹ or 29 μ M will be the concentration of the pesticide in the soil water solution to which microorganisms will be exposed if the recommended dose of chlorpyrifos will be applied.

Hymexazol is a soil applied fungicide with a recommended dose rate in sugar beet 0.07245 kg of the active substance per hectare and the growth stage-season of application is at the seed level (EFSA database). Hymexazol is applied to soil directly thus there is no interception and full dispersal of the pesticide in the top 5 cm of the soil is assumed. After a single hymexazol application, the final concentration of pesticide in soil will be 0.11 mg kg⁻¹ (72.45 g active substance in 650 t of soil). For *in vitro* tests it is assumed that all pesticide reaching the soil will end up in the soil solution. Thus, if 0.11 mg of active ingredient is present in 1000 g of soil with a soil moisture content of 20% then 0.11 mg will be present in 200 ml of water at 0.56 mg L⁻¹. Hence, 0.56 mg L⁻¹ or 10 μ M is the assumed concentration of the pesticide in the soil water solution to which microorganisms will be exposed if the recommended dose of hymexazol is applied.

1.2 Pesticides extraction from liquid cultures

The extraction of TCP from liquid cultures was performed by mixing 0.05 mL liquid culture with 0.45 mL of methanol and 0.1% H₃PO₄ (1:10 vol/vol). The herbicide, metsulfuron-methyl, was extracted from liquid media by mixing 0.1 mL liquid culture with 0.4 mL of methanol (1:5 vol/vol), while clethodim was extracted by mixing 0.1 mL liquid culture with 0.4 mL of acetonitrile (1:5 vol/vol). Fungicides, pyraclostrobin, etridiazole and 3,5-DCA were extracted from liquid media by mixing 0.05 mL of liquid culture with 0.45 mL of acetonitrile (1:10 vol/vol), whereas hymexazol was extracted by mixing 0.1 mL liquid culture with 0.4 mL of methanol (1:5 vol/vol). Chlorpyrifos, glyphosate and AMPA residues were not determined. The derived mixtures were vortexed for 1 min, sonicated for 10 min and stored at -20°C until chromatographic analysis.

1.3 Recovery tests and validation of the extraction methods

Analysis of fortified samples of the different growth media was performed to verify the efficiency of the extraction methods applied. For this purpose, two growth media (i) 1583 medium (pH 7.8) and (ii) freshwater medium (FW) (pH 5.2; Lehtovirta-Morley et al., 2011), differing in chemical composition and pH, were used. Triplicate samples (5 mL) of each growth medium were spiked with pesticide solution in DMSO (0.1% vol/vol) to achieve the target concentration. Recovery tests were performed in triplicate for both media at three concentration levels. Samples were stored at laboratory temperature (25- 28°C) for at least two hours before extraction and analysis to ensure interaction of analyte and medium components.

The summarized results of the recovery tests (%) in the 1583 or to FW media and relative standard deviations (RSDs) (%) are shown in Table S2. The accuracy of the methods was considered acceptable with the average recovery for at least at two fortification levels being in the range of 70-120%, except for metsulfuron-methyl, 3,5-DCA and hymexazol at the lowest concentration levels. The recoveries for metsulfuron-methyl and 3,5-DCA in FW medium were approx. 50-65% while the lowest concentration of hymexazol in both media could not be measured due to the higher limit of detection for this compound in HPLC. The RSDs, which illustrate the precision of the developed procedures, were less than 20%, in all cases except for metsulfuron-methyl and 3,5-DCA, at the lowest tested concentration in the FW medium.

1.4. Chromatography analyses

High performance liquid chromatography (HPLC) analysis of the tested pesticides was performed with a Shimadzu LC-20ADHPLC system equipped with an UV/VIS PDA detector. A Shimadzu GVP-ODs (4.6 mm by 150 mm, 5 mm) pre-column and connected to a RP Shimadzu VPODs (4.6 mm x 150 mm, 5 mm) column was used for pesticide separation. The injection volume was 20 µL and the flow rate of the mobile phase was set at 1 mL min⁻¹ for all pesticides.

The column temperature was set at 25 °C for all tested compounds except for metsulfuron-methyl which was set at 40°C. Chromatographic separation of all compounds was achieved at isocratic conditions. For the insecticides, chlorpyrifos and TCP, a mixture of methanol and orthophosphoric acid [0.1% (vol/vol)] was used at ratio of 80:20 (vol/vol) for mobile phase with detection at 230nm. Chromatographic separation of metsulfuron-methyl was achieved in a mobile phase of methanol and orthophosphoric acid [0.1% (vol/vol)] at a ratio of 55:45 (vol/vol), for clethodim with a mixture of acetonitrile and orthophosphoric acid [0.1% (vol/vol)] (90:10 [(vol/vol)], and detection was performed at 224 and 255 nm for metsulfuron-methyl and clethodim, respectively. Mixtures of acetonitrile and distilled water were used at a ratio of 65:35 (vol/vol) and 80:20 (vol/vol) as mobile phases for the chromatographic elution of 3,5-DCA and etridiazole, respectively, and detection of these compounds was achieved at 220nm. Mixtures of methanol and acetonitrile with orthophosphoric acid [0.1% (vol/vol)] were used for the chromatographic separation of hymexazol and pyraclostrobin at a ratio of 40:60 and 80:20 (vol/vol) and detection at 205 and 275nm, respectively. Table S3 summarizes the conditions used for the chromatographic separation of each compound. Calibration curves obtained by the injection of standard solutions of pesticides in methanol or acetonitrile (ranging from 0.05 to 20 mg L⁻¹) were used for quantification. The linearity of the calibration curve was assessed by calculating the determination coefficient (r^2).

2. Results

2.1 Stability of pesticides on AOM and NOB cultures

2.1.1. Insecticides

TCP was generally stable in liquid cultures of AOM and NOB strains with no significant degradation observed throughout the incubation period. However, in liquid cultures of *N. europaea*, *N. multiformis* and *N. winogradskyi* TCP at a concentration of 25 µM showed up to 25% degradation (Supplementary Figure S4).

2.1.2. Herbicides

Metsulfuron-methyl was significantly degraded in liquid cultures of *Ca. N. sinensis* from ~15 to 30 % of the initial recovered amount. The same degradation patterns were observed in liquid cultures of *Nitrobacter* sp. NHB1 at the lowest concentrations while approximately 50% degradation was recorded at a concentration 131 µM. In cultures of three AOB strains (*N. europaea*, *N. multiformis* and *N. communis*) and *N. viennensis* no significant degradation ($p > 0.05$) of metsulfuron-methyl was observed throughout the incubation period. In liquid cultures of *Ca. N. franklandianus* significant degradation of the pesticide ($p < 0.05$) was observed only at the lowest tested concentration of 1.31 µM representing approximately 30%. Finally, in liquid cultures of *N. winogradskyi* the degradation of metsulfuron-methyl varied from 25 to 100% (Supplementary Figure S5a). Clethodim was stable in AOA cultures (*Ca. N. sinensis*, *N. viennensis* and *Ca. N. franklandianus*) and only approximately 15% degradation was observed in *Ca. N. sinensis* and *Ca. N. franklandianus* at tested concentration of 83 and

8.3 μM , respectively. In *N. europaea*, *N. multiformis* and *N. communis* liquid cultures up to 40% degradation at a concentration 8.3 μM clethodim was observed. In *Nitrobacter* sp. NHB1 liquid cultures, clethodim was significantly degraded at the highest tested concentrations of 83 and 415 μM only with approximately 30 to 60% degradation of the initially amount recovered. In liquid cultures of *N. winogradskyi*, the degradation of clethodim varied from 30 to 100% (Supplementary Figure S5b).

2.1.3. Fungicides

Pyraclostrobin showed degradation from 30 to 95% in liquid cultures of *Ca. N. sinensis* and *Nitrobacter* sp. NHB1, with the highest proportion degraded at the highest tested concentration due to precipitation of the pesticide in liquid cultures. For *N. viennensis*, approximately 50% was degraded at an initial measured concentration of 77 μM . In contrast, no significant degradation ($p > 0.05$) was observed in liquid cultures of *Ca. N. franklandianus* throughout the incubation period. In *N. europaea* and *N. multiformis* liquid cultures pyraclostrobin degradation varied from 30 to 70% at the highest tested concentrations of 77 and 385 μM , while in *N. communis* cultures degradation from 20 to 50% was observed at all tested concentrations. Finally, in *N. winogradskyi* liquid cultures, significant degradation was observed only at the lowest tested concentration 7.7 μM with approximately 25% degradation (Supplementary Figure S6a). Etridiazole was significantly degraded in liquid cultures of *Ca. N. sinensis* and *Nitrobacter* sp. NHB1, from approximately 25 to 80% and with the highest degradation proportion observed at the highest tested concentration but which may have also been due to precipitation at the highest concentrations. In liquid cultures of *N. viennensis* and *Ca. N. franklandianus*, degradation of etridiazole ranged from 25 to 70% at concentrations of 40.4 and 404 μM . In AOB cultures no significant degradation ($p > 0.05$) was observed, with the only exception being approximately 60% degradation of etridiazole in *N. communis* cultures at 40.4 μM . In liquid cultures of *N. winogradskyi*, the degradation of etridiazole varied from 20 to 90% (Supplementary Figure S6b). For hymexazol, approximately 40 to 90% was degraded in liquid cultures of *N. viennensis* and *Ca. N. franklandianus* at 10 μM . In contrast, no significant degradation ($p > 0.05$) of the pesticide was observed in the liquid cultures of *Ca. N. sinensis* throughout the incubation period. In *Nitrobacter* sp. NHB1 liquid cultures, up to 20% of hymexazol degradation occurred at all tested concentrations from 10 to 1009 μM . Finally, 30 to 100% of hymexazol was degraded in liquid cultures of *N. winogradskyi* (Supplementary Figure S6c). 3,5 DCA was generally stable and degradation ranging from 15 to 20% was only observed in the liquid cultures of *N. communis* and *Ca. N. franklandianus* (Supplementary Figure S6d).

3. References

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Table S1. The concentration levels used in the *in vitro* bioassays per pesticide (and transformation product) tested. The concentration of pesticides expected to be found in the soil solution upon application of the recommended agronomic dose, are indicated in bold.

| Group of pesticides | Pesticide | Concentration level (μM) |
|---------------------|-----------------------------------|---------------------------------------|
| Insecticides | Chlorpyrifos | 2.9, 29 , 114, 228 |
| | 3,5,6-trichloro-2-pyridinol (TCP) | 5, 25 , 75, 164 |
| Herbicides | Glyphosate | 5.9, 59 , 590, 2957 |
| | Aminomethylphosphonic acid (AMPA) | 9, 90 , 900, 4500 |
| | Metsulfuron-methyl | 0.26, 2.62 , 26.22, 262.2 |
| | Metsulfuron-methyl ^a | 0.13, 1.31 , 13.11, 131.1 |
| | Clethodim | 0.83, 8.3 , 83, 415 |
| Fungicides | Pyraclostrobin | 0.77, 7.7 , 77, 385 |
| | Etridiazole | 4, 40.4 , 404, 1010 |
| | Hymexazol | 1, 10 , 100, 1009 |
| | 3,5-Dichloroaniline (3,5-DCA) | 0.31, 3.1 , 31, 310 |

^a Concentrations of metsulfuron-methyl which was calculated via HPLC after addition in liquid cultures of *Ca. N. franklandianus*, *N. communis*, *Nitrobacter* sp. NHB1 and *N. winogradskyi*.

Table S2. Recovery levels obtained for the extraction of each of the pesticides tested in the different growth media used (1583 medium; FW: MES-buffered freshwater medium). The recovery value at each concentration level is the mean of triplicates.

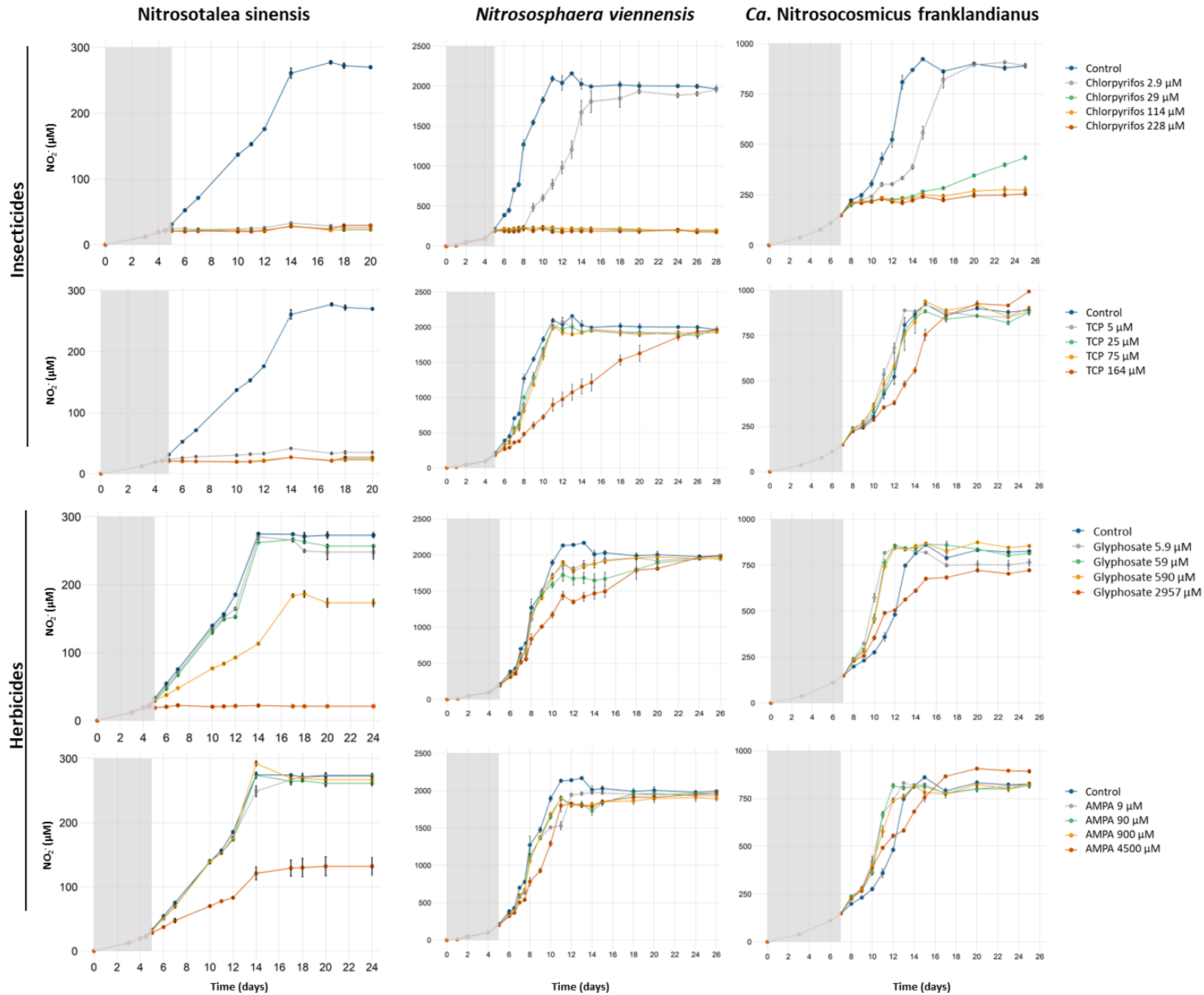
| Group of pesticides | Pesticide | Medium | Extraction method | Fortification level (μM) | Recovery (%) (Mean, n=3) | Standard deviation | Relative standard deviation | Sensitivity (%) |
|---------------------|--------------------|--------|---------------------|---------------------------------------|--------------------------|--------------------|-----------------------------|-----------------|
| Insecticides | TCP | 1583 | 1:10 MeOH (vol/vol) | 164 | 129.18 | 26.6 | 20.55 | 121.21 |
| | | | | 75 | 130.31 | 0.5 | 0.35 | |
| | | | | 5 | 104.14 | 1.4 | 1.30 | |
| | | FW | | 164 | 137.02 | 0.6 | 0.46 | 127.82 |
| | | | | 75 | 128.87 | 2.5 | 1.98 | |
| | | | | 5 | 117.58 | 4.9 | 4.14 | |
| Herbicides | Metsulfurol-methyl | 1583 | 1:5 MeOH (vol/vol) | 26 | 146.24 | 10.7 | 7.34 | 128.33 |
| | | | | 2.62 | 138.12 | 11.7 | 8.47 | |
| | | | | 0.262 | 100.63 | 21.8 | 21.65 | |
| | | FW | | 26 | 127.56 | 2.9 | 2.25 | 98.39 |
| | | | | 2.62 | 117.28 | 3.6 | 3.03 | |
| | | | | 0.262 | 50.31 | 174.3 | 346.41 | |
| | Clethodim | 1583 | 1:10 MeCN (vol/vol) | 83 | 97.89 | 1.1 | 1.07 | 108.54 |
| | | | | 8.3 | 113.61 | 1.3 | 1.13 | |
| | | | | 0.83 | 114.12 | 19.0 | 16.64 | |
| | | FW | | 83 | 91.67 | 0.9 | 0.96 | 91.92 |
| | | | | 8.3 | 93.19 | 2.8 | 3.01 | |
| | | | | 0.83 | 90.92 | 14.3 | 15.71 | |
| Fungicides | Pyraclostrobin | 1583 | 1:10 MeCN (vol/vol) | 77 | 109.14 | 12.3 | 11.25 | 108.49 |
| | | | | 7.7 | 93.37 | 1.5 | 1.57 | |
| | | | | 0.77 | 122.95 | 5.4 | 4.40 | |
| | | FW | | 77 | 72.10 | 5.2 | 7.25 | 88.81 |
| | | | | 7.7 | 91.42 | 3.3 | 3.59 | |
| | | | | 0.77 | 102.91 | 4.1 | 4.01 | |

Table S2. Continued.

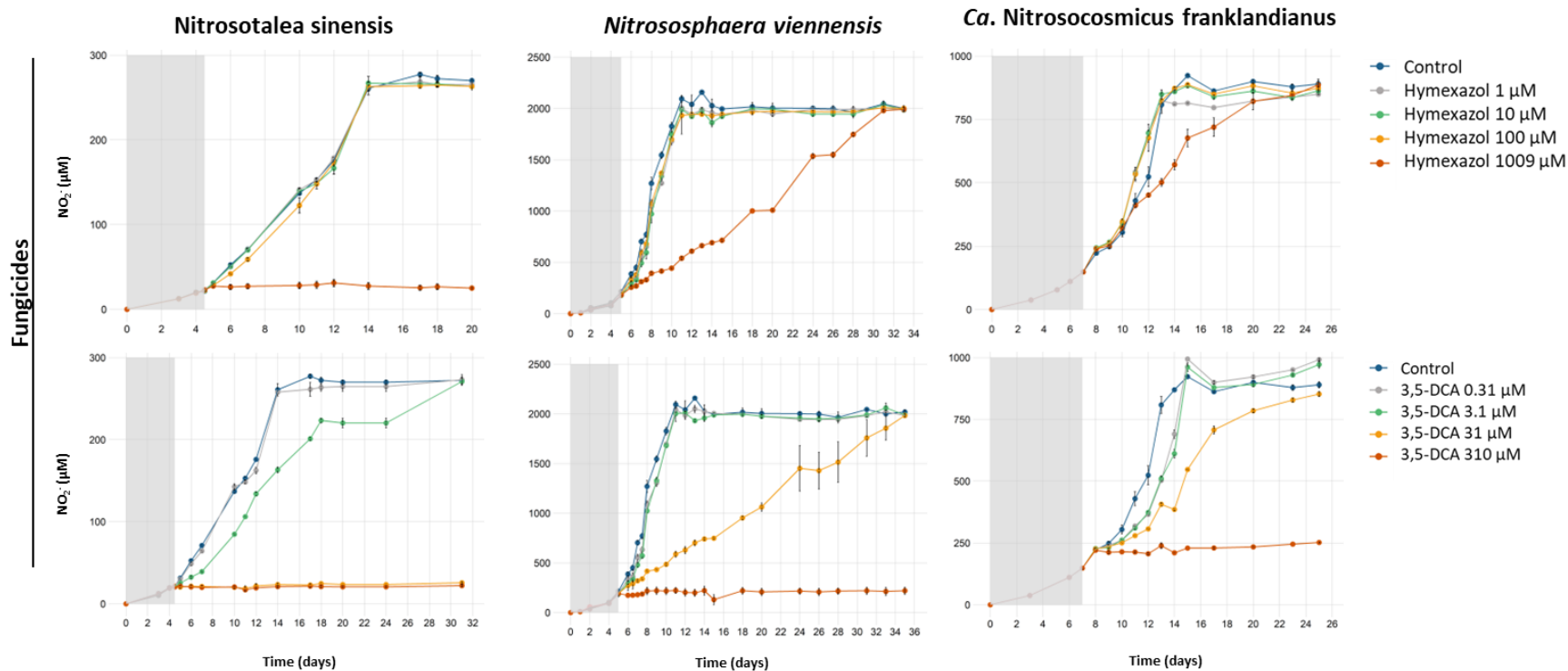
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|------------|-------------|------|------------------------|------|--------|-------|--------|--------|
| Fungicides | Etridiazole | 1583 | 1:10 MeCN (vol/vol) | 404 | 86.47 | 1.9 | 2.24 | 106.61 |
| | | | | 40.4 | 109.82 | 3.8 | 3.50 | |
| | | | | 4 | 123.53 | 12.8 | 10.38 | |
| | | FW | | 404 | 107.44 | 1.8 | 1.67 | 113.65 |
| | | | | 40.4 | 112.93 | 1.6 | 1.43 | |
| | | | | 4 | 120.59 | 7.4 | 6.10 | |
| | Hymexazol | 1583 | 1:5 MeOH (vol/vol) | 101 | 125.53 | 6.2 | 4.96 | 85.11 |
| | | | | 10 | 129.79 | 8.4 | 6.48 | |
| | | | | 1 | 0.00 | 0.0 | 0.00 | |
| | | FW | | 101 | 125.52 | 8.0 | 6.37 | 83.39 |
| | | | | 10 | 124.64 | 15.6 | 12.48 | |
| | | | | 1 | 0.00 | 0.0 | 0.00 | |
| | 3,5-DCA | 1583 | 1:10 MeCN (vol/vol) | 310 | 130.43 | 5.9 | 4.54 | 110.31 |
| | | | | 3.1 | 105.24 | 36.9 | 35.07 | |
| | | | | 0.31 | 95.26 | 26.5 | 27.77 | |
| | | FW | | 310 | 109.16 | 3.6 | 3.32 | 95.50 |
| | | | | 3.1 | 110.53 | 18.3 | 16.55 | |
| | | | | 0.31 | 66.81 | 112.4 | 168.23 | |

Table S3. The chromatographic conditions used for the analysis of the residues of the tested pesticides.

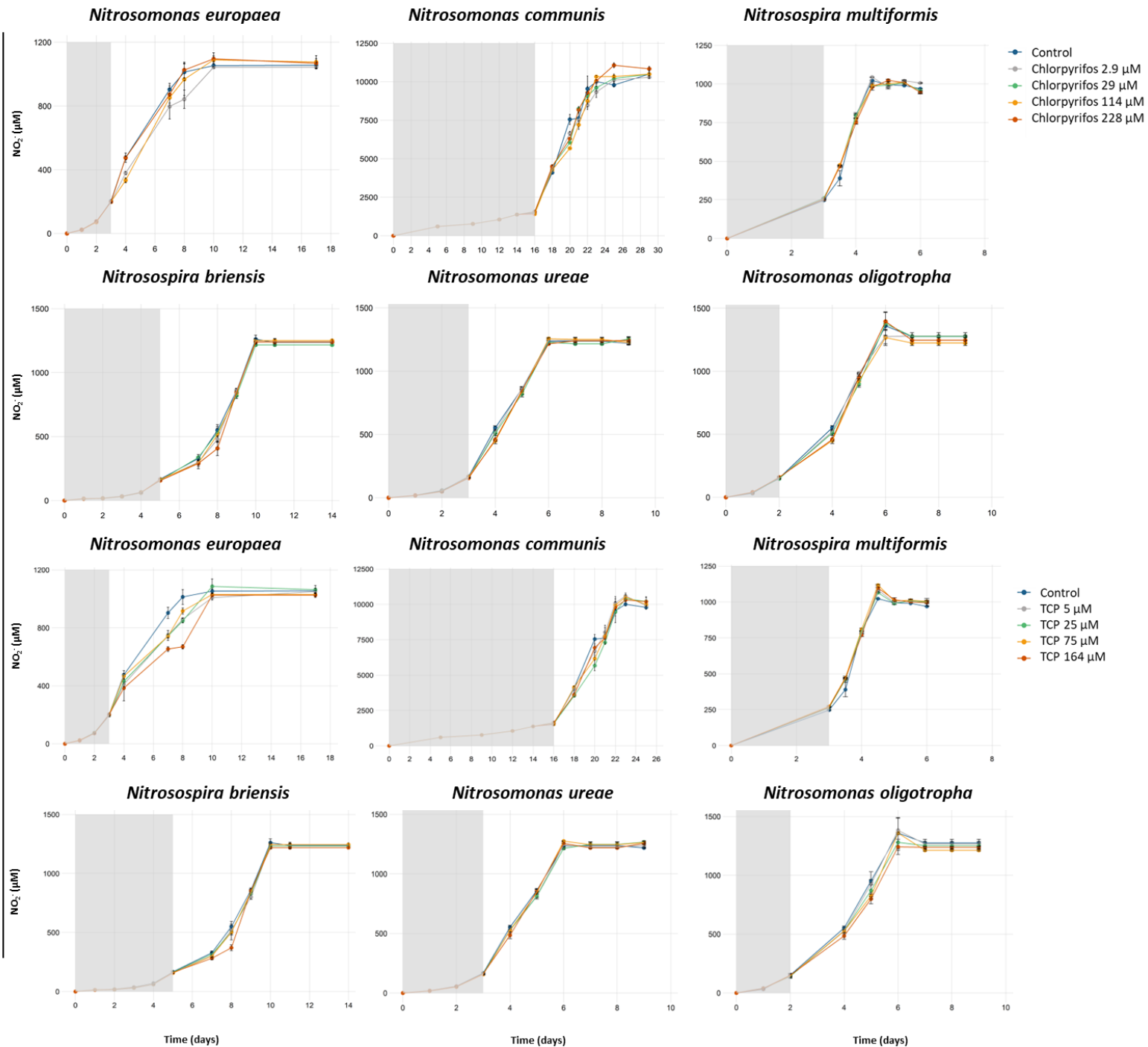
| Group of pesticides | Pesticide | Mobile phase | Detection (nm) | Retention time (min) | Column Temperature (°C) | Flow rate (mL/min) | Injection volume (µL) |
|---------------------|--------------------|--|----------------|----------------------|-------------------------|--------------------|-----------------------|
| Insecticides | Chlorpyrifos | 80 MeOH : 20 H ₂ O + 0.1(%) H ₃ PO ₄ | 230 | 11.3 | 25 | 1.0 | 20 |
| | TCP | | | 4.1 | | 1.0 | 20 |
| Herbicides | Metsulfuron-methyl | 55 MeOH : 45 H ₂ O + 0.1(%) H ₃ PO ₄ | 224 | 5.1 | 40 | 1.0 | 20 |
| | Clethodim | 90 MeCN : 10 H ₂ O + 0.1 (%) H ₃ PO ₄ | 255 | 3.8 | 25 | 1.0 | 20 |
| Fungicides | Pyraclostrobin | 80 MeCN : 20 H ₂ O + 0.1 (%) H ₃ PO ₄ | 275 | 4.2 | 25 | 1.0 | 20 |
| | Etridiazole | 80 MeCN : 20 H ₂ O | 220 | 4.3 | 25 | 1.0 | 20 |
| | Hymexazol | 4 MeOH : 60 H ₂ O + 0.1 (%) H ₃ PO ₄ | 205 | 3 | 25 | 1.0 | 20 |
| | 3,5-DCA | 65 MeCN : 35 H ₂ O | 220 | 4.6 | 25 | 1.0 | 20 |



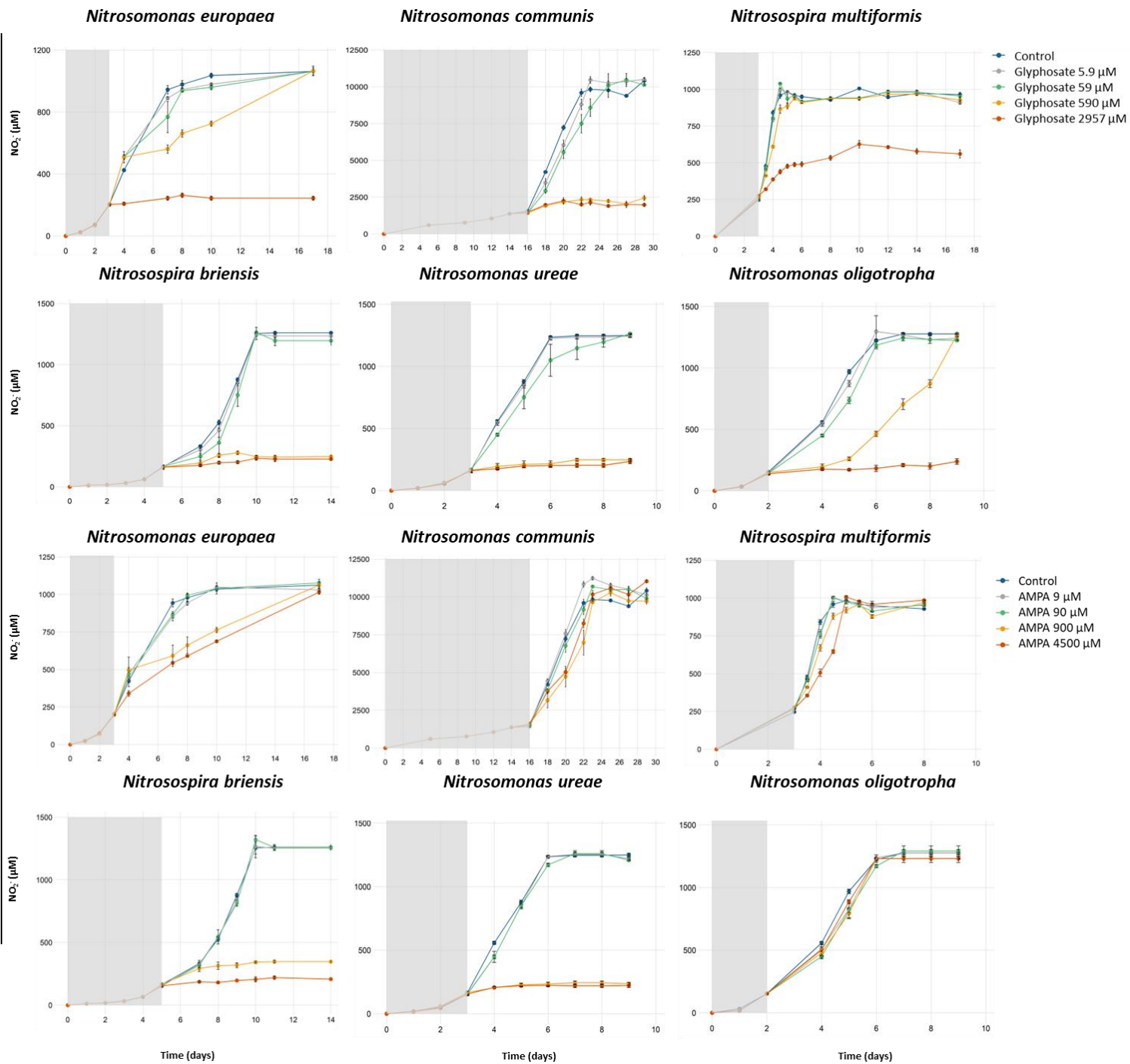
Supplementary Figure S1. The effect of different concentrations of pesticides on nitrite production by the ammonia-oxidizing archaea (AOA) *N. sinensis*, *Ca. N. franklandianus* and *N. viennensis*. Error bars represent the standard error of the mean from triplicate cultures. Grey colour indicate the time when the pesticides were added.



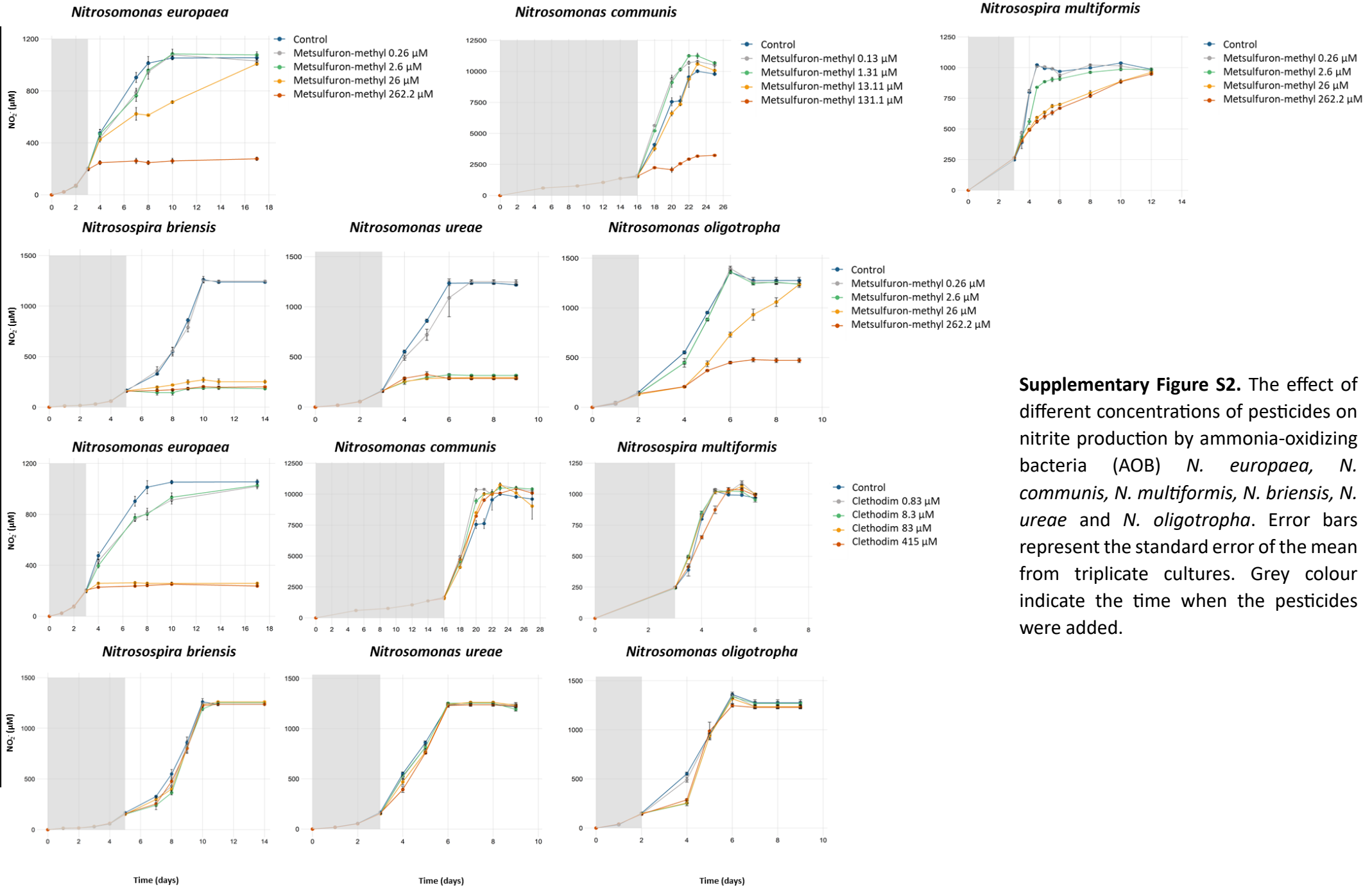
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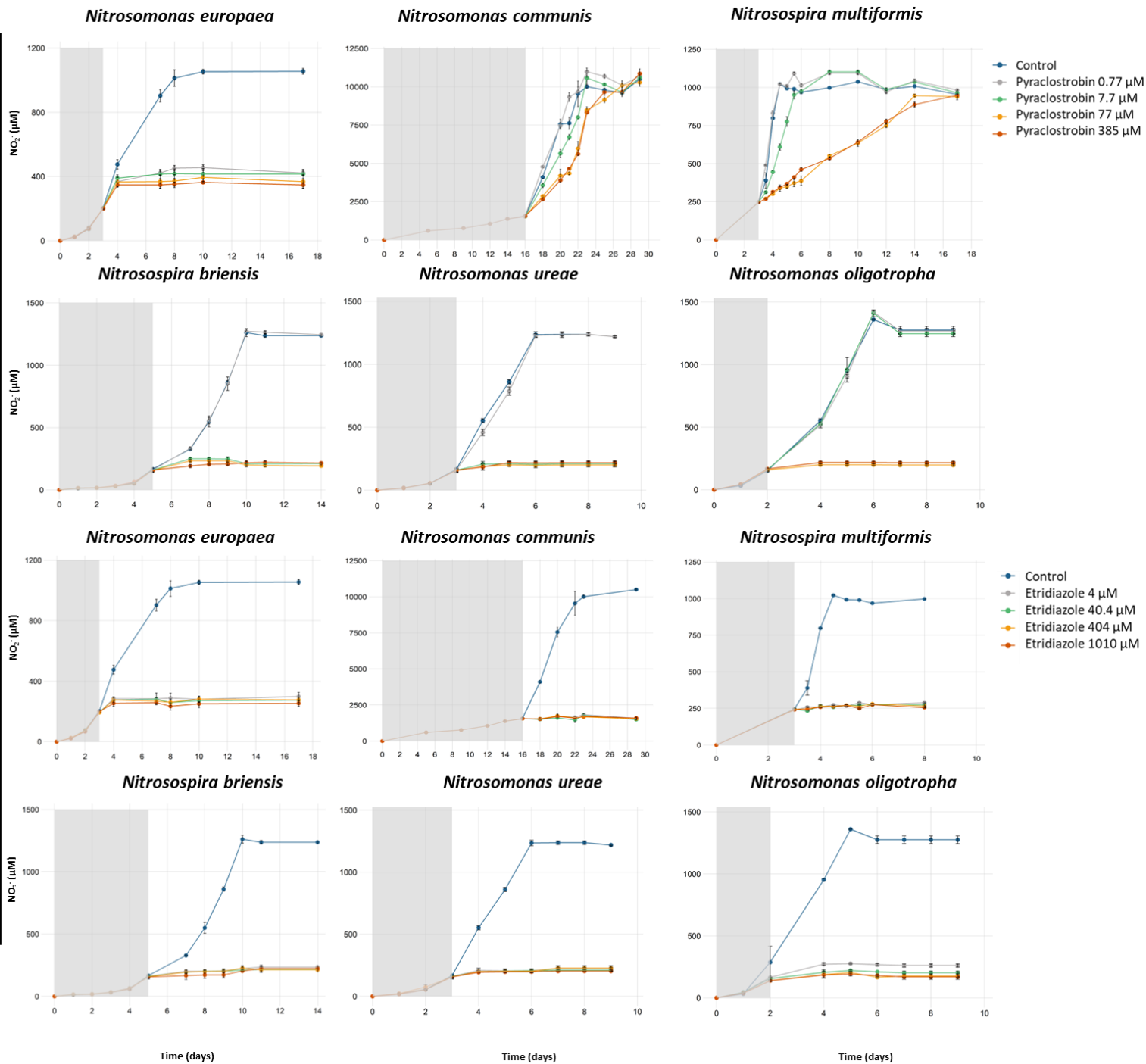
Supplementary Figure S2. The effect of different concentrations of pesticides on nitrite production by ammonia-oxidizing bacteria (AOB) *N. europaea*, *N. communis*, *N. multiformis*, *N. briensis*, *N. ureae* and *N. oligotropha*. Error bars represent the standard error of the mean from triplicate cultures. Grey colour indicate the time when the pesticides were added.



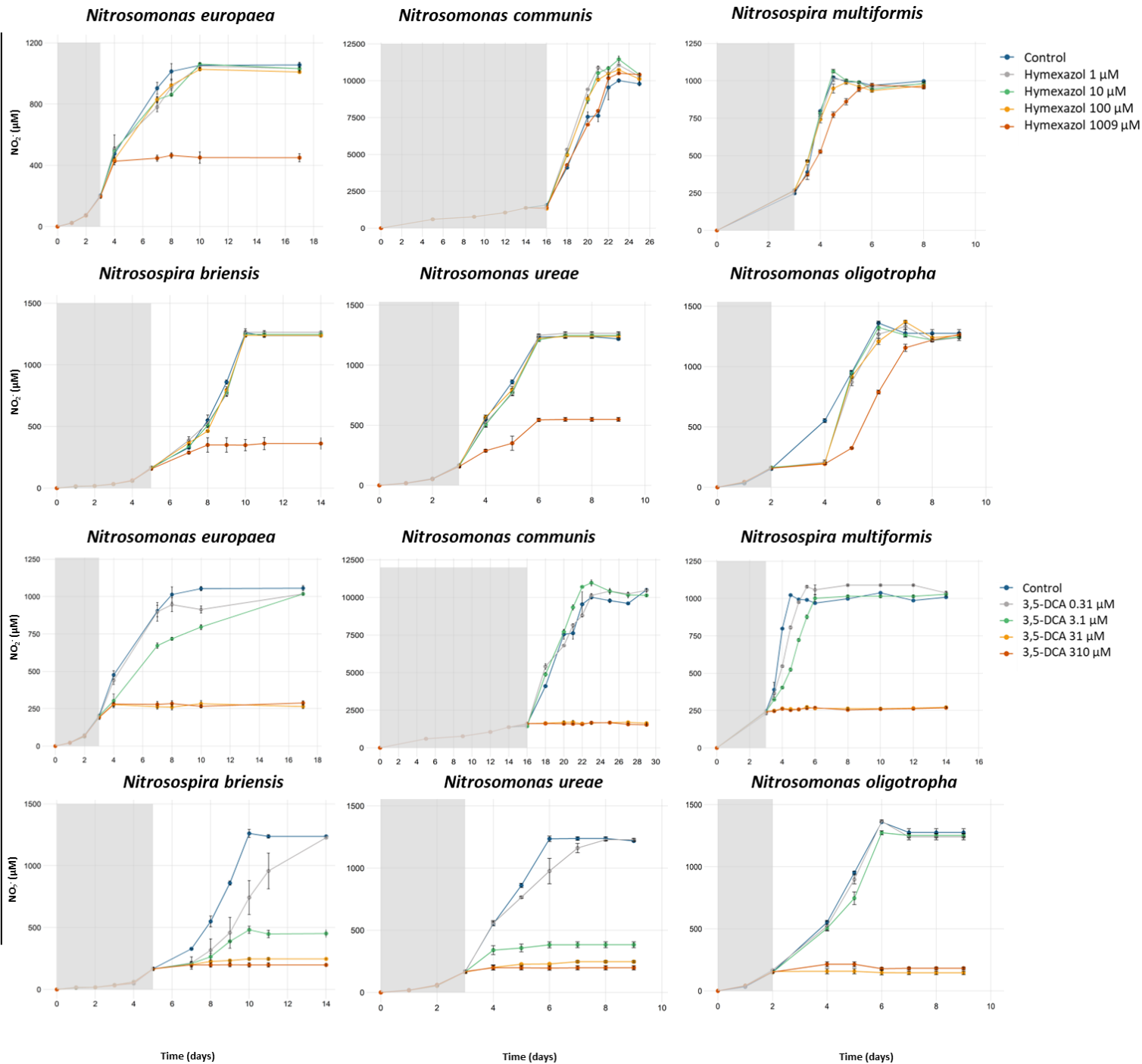
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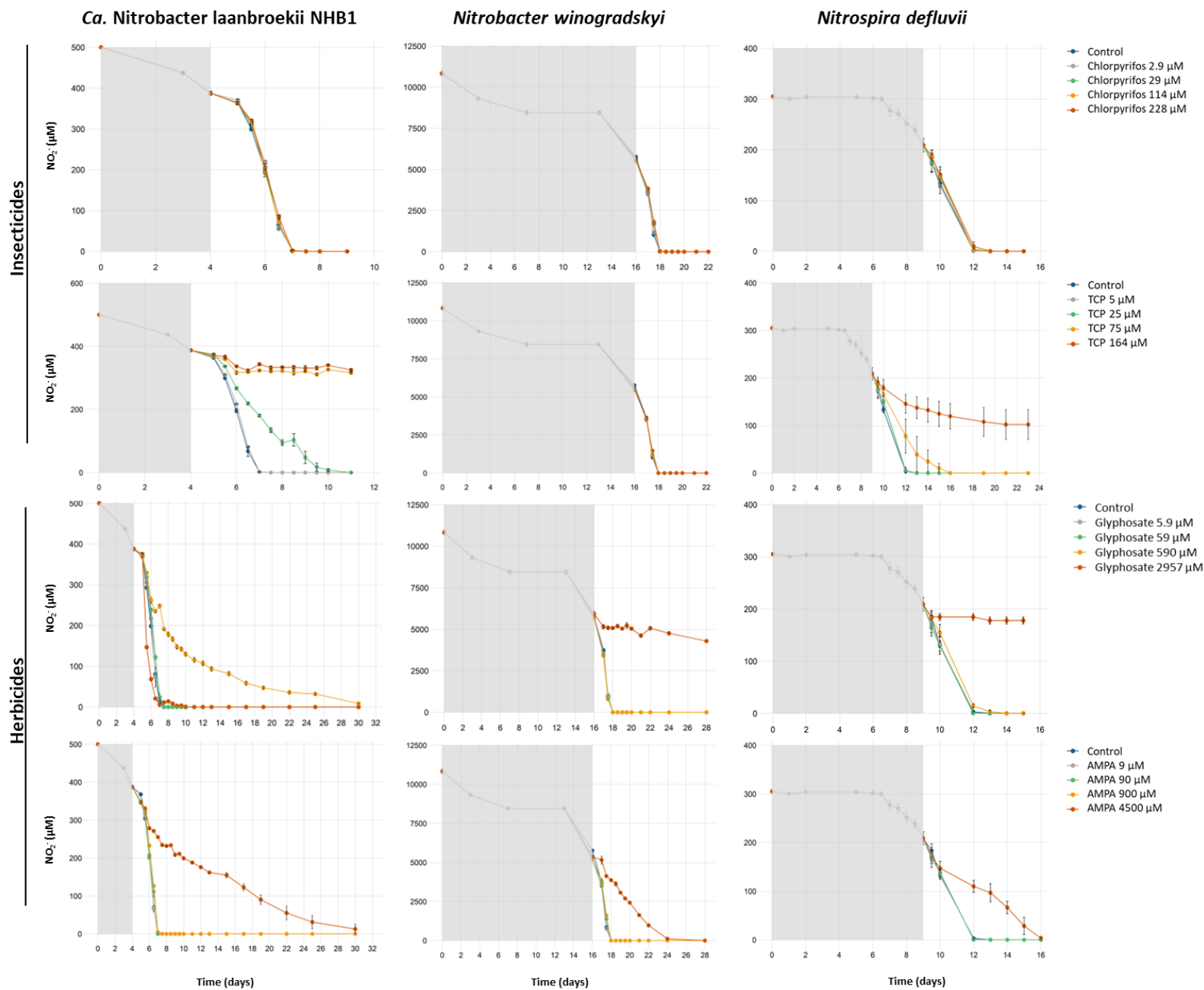
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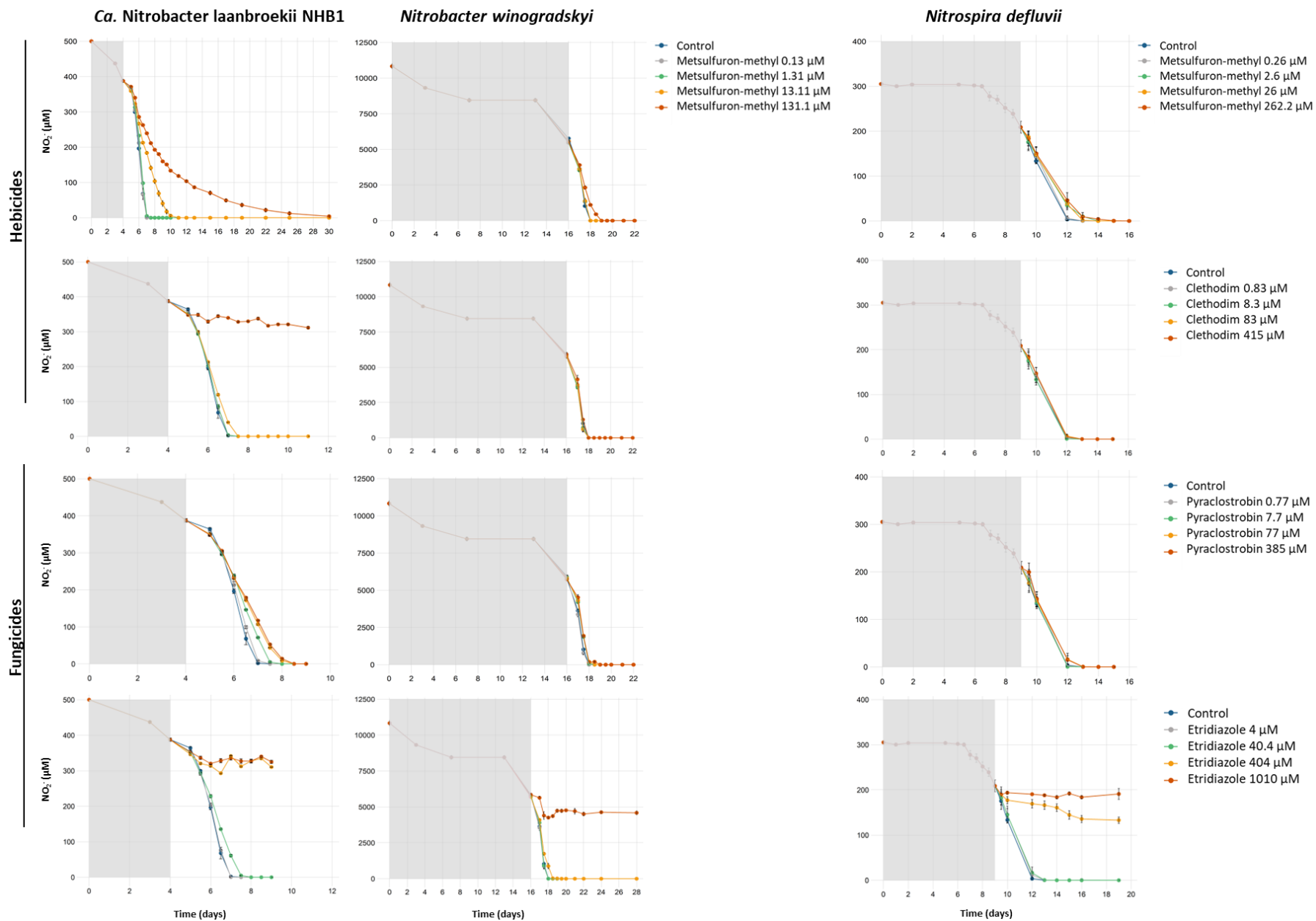
Supplementary Figure S2. The effect of different concentrations of pesticides on nitrite production by ammonia-oxidizing bacteria (AOB) *N. europaea*, *N. communis*, *N. multiformis*, *N. briensis*, *N. ureae* and *N. oligotropha*. Error bars represent the standard error of the mean from triplicate cultures. Grey colour indicate the time when the pesticides were added.



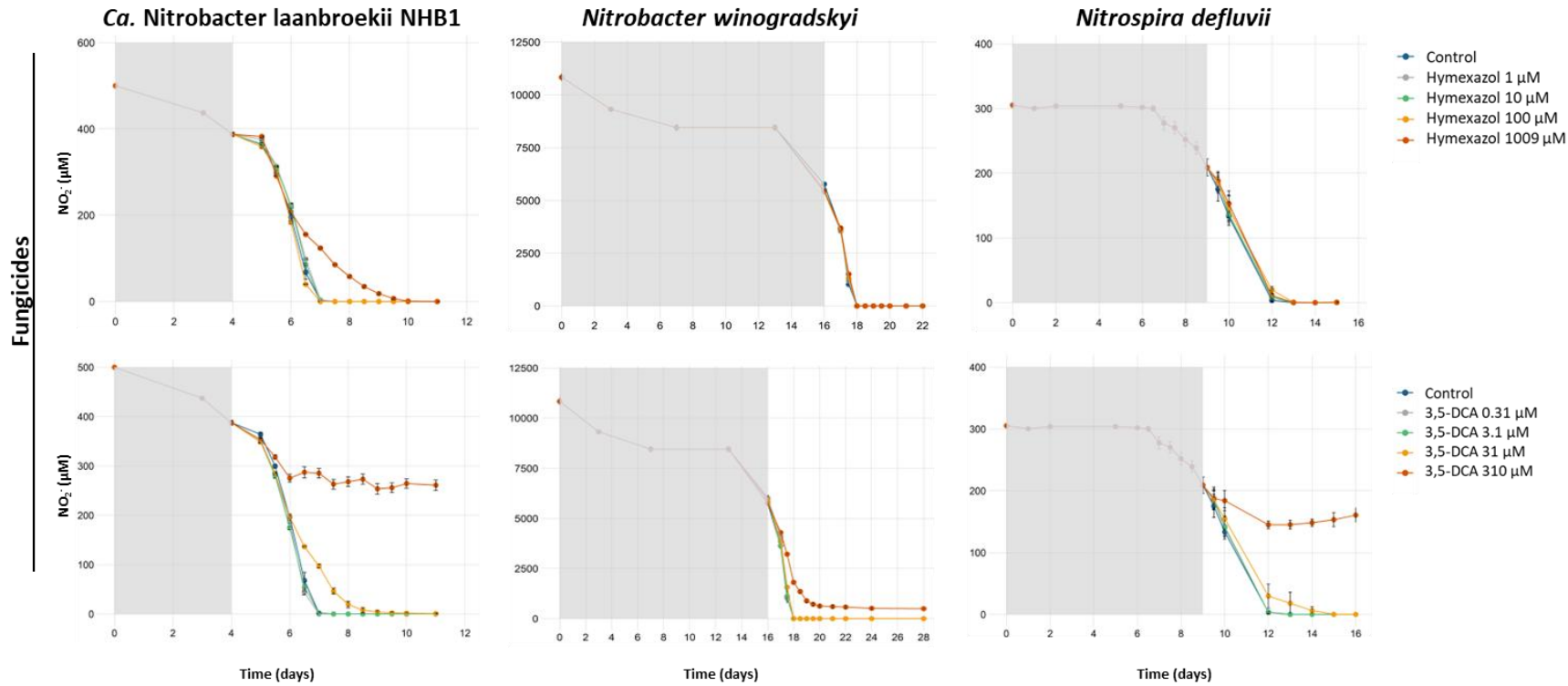
Supplementary Figure S2. The effect of different concentrations of pesticides on nitrite production by ammonia-oxidizing bacteria (AOB) *N. europaea*, *N. communis*, *N. multififormis*, *N. briensis*, *N. ureae* and *N. oligotropha*. Error bars represent the standard error of the mean from triplicate cultures. Grey colour indicate the time when the pesticides were added.



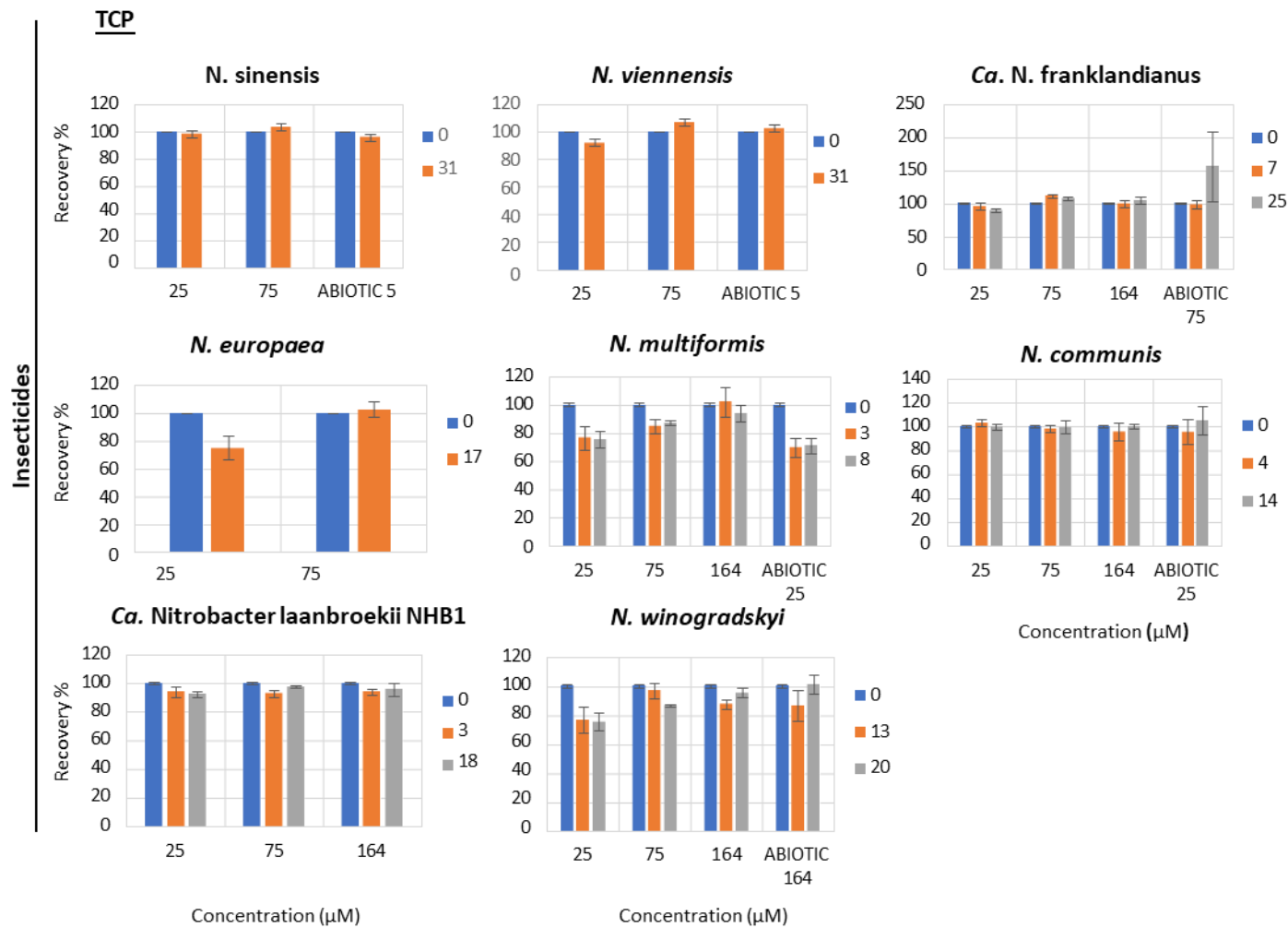
Supplementary Figure S3. The effect of different concentrations of pesticides on nitrite consumption by the nitrite-oxidizing bacteria (NOB) *Ca. Nitrobaacter laanbroekii* NHB1, *N. winogradskyi* and *N. defluvii*. Error bars represent the standard error of the mean from triplicate cultures. Grey colour indicate the time when the pesticides were added.



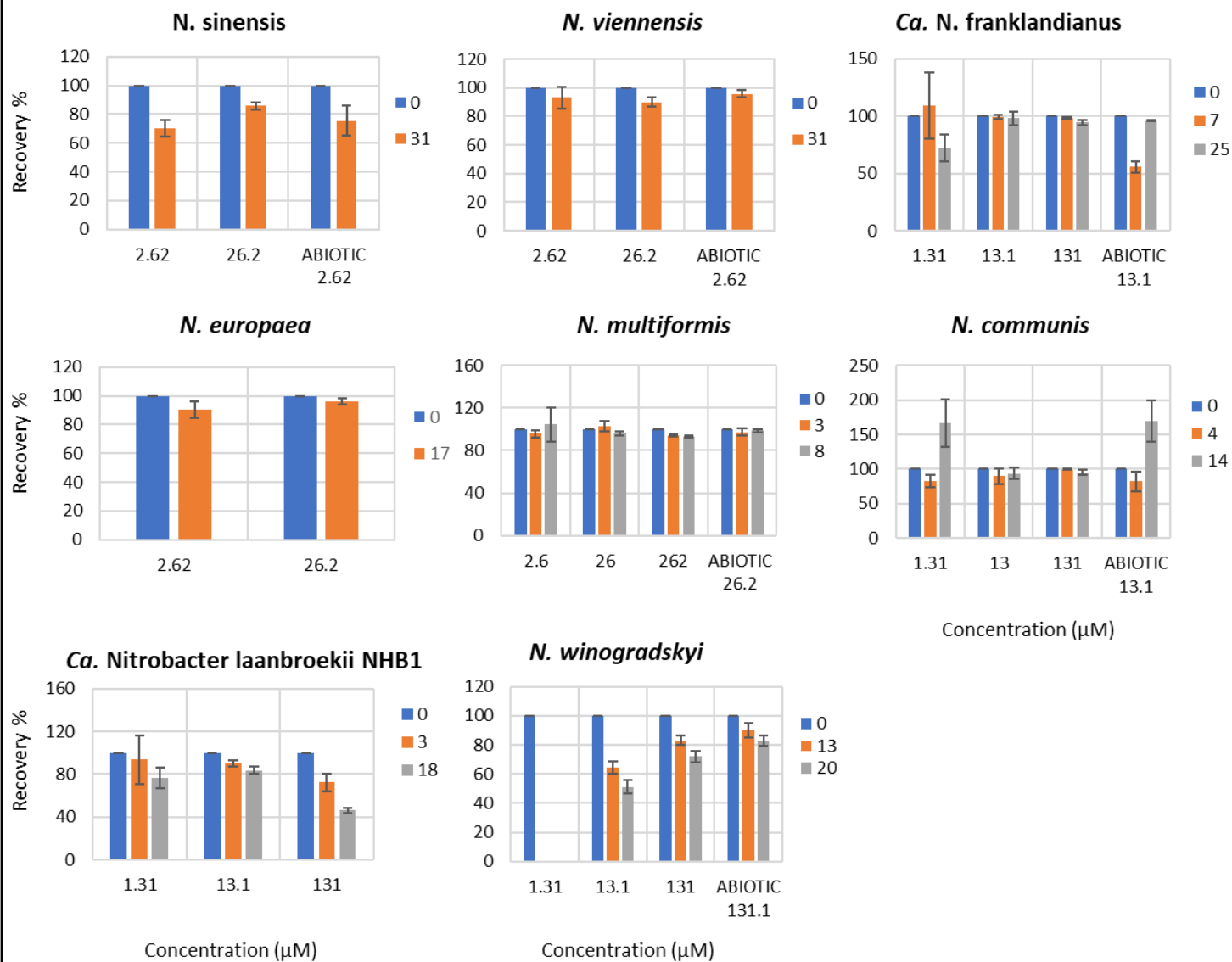
Supplementary Figure S3. The effect of different concentrations of pesticides on nitrite consumption by the nitrite-oxidizing bacteria (NOB) *Ca. Nitrobacter laanbroekii* NHB1, *N. winogradskyi* and *N. defluvii*. Error bars represent the standard error of the mean from triplicate cultures. Grey colour indicate the time when the pesticides were added.



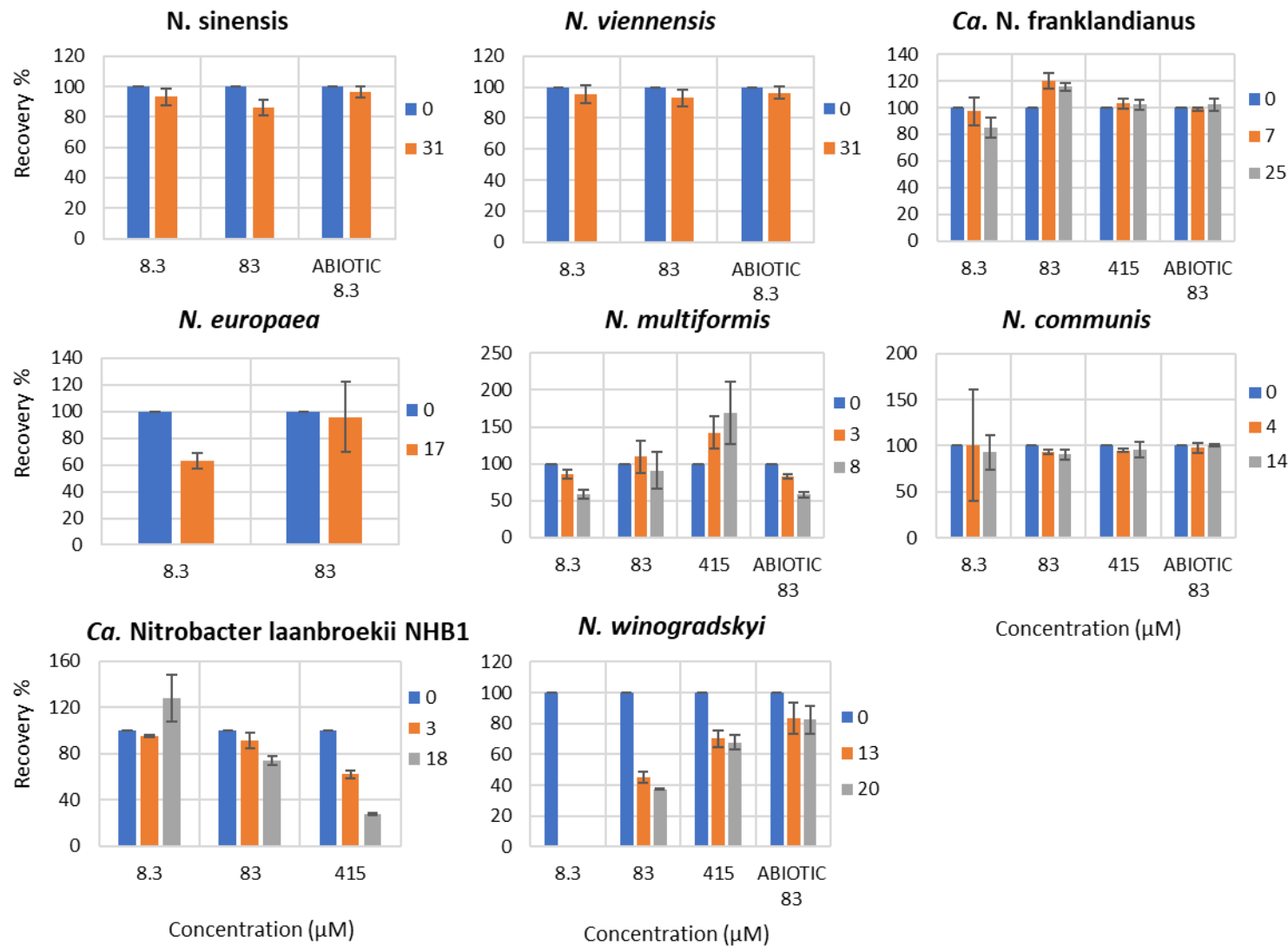
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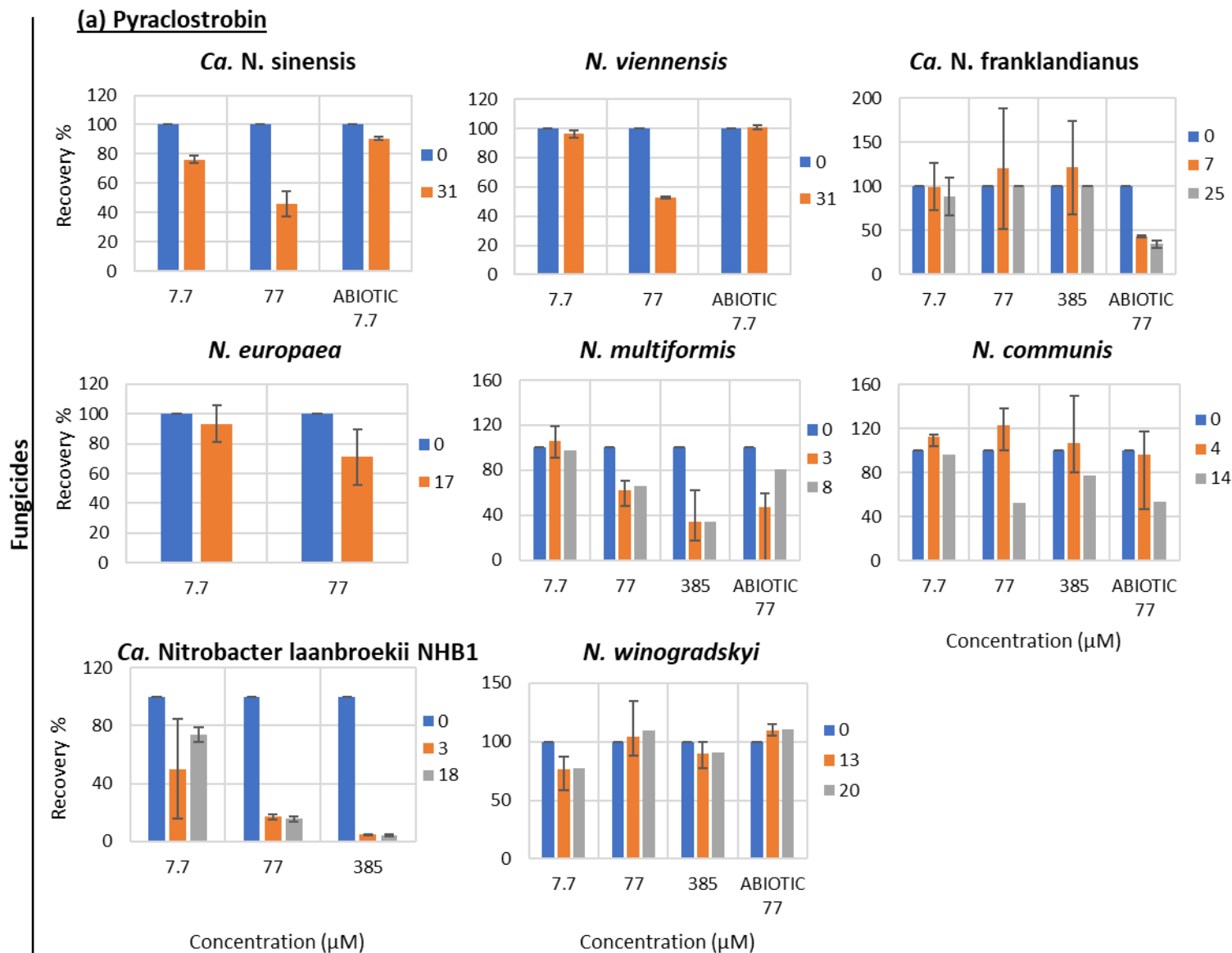
Supplementary Figure S4. The degradation of insecticide TCP applied over a range of concentrations in the liquid cultures of the AOA *N. sinensis*, *N. viennensis* and *Ca. N. franklandianus*, the AOB *N. europaea*, *N. multiformis* and *N. communis*, and the NOB *Ca. Nitrobacter laanbroekii* NHB1 and *N. winogradskyi* and in abiotic non-inoculated controls (at one selected concentration), determined at least at two time points. Error bars represent the standard error of the mean of triplicate cultures.

(a) Metsulfuron-methyl

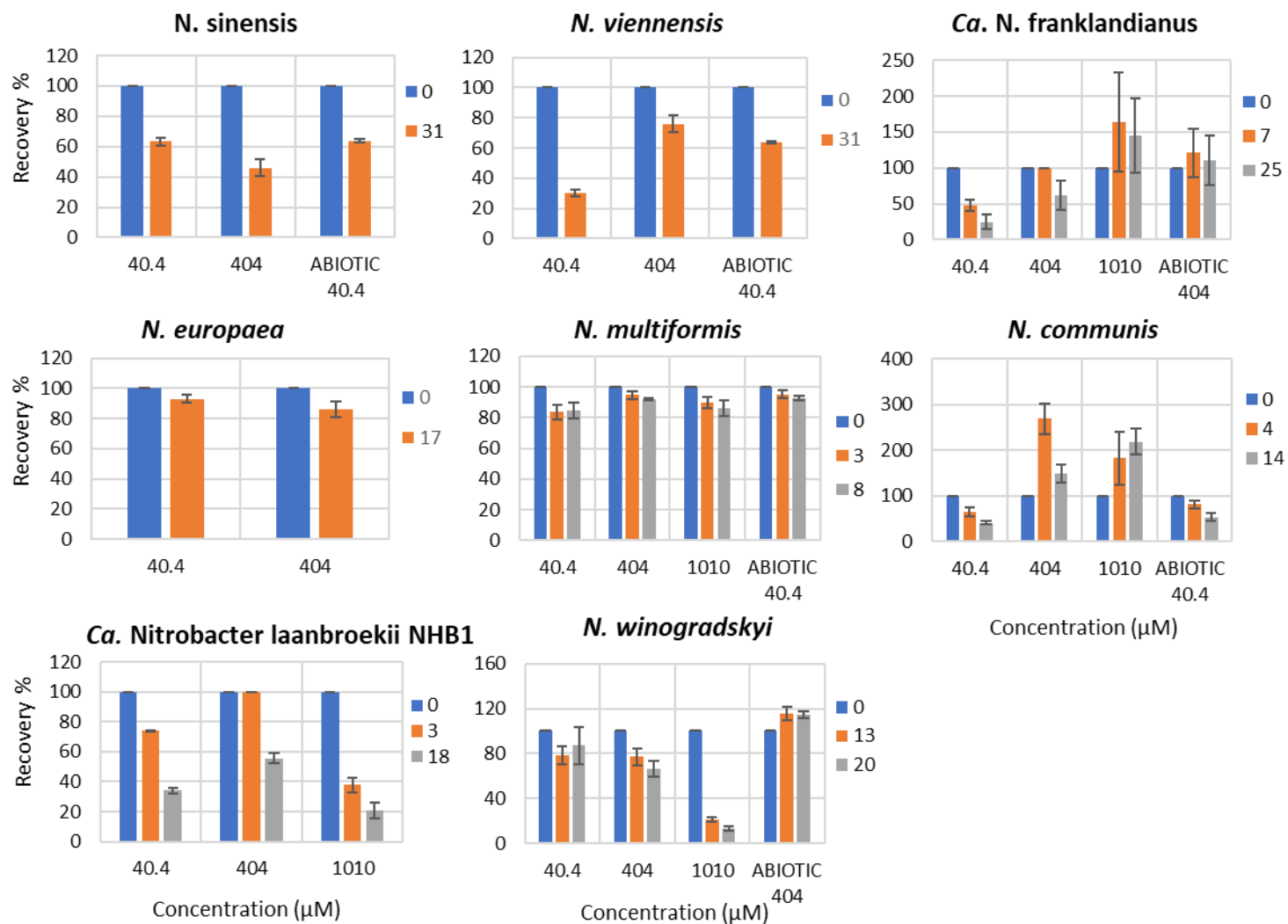
Supplementary Figure S5. The degradation of herbicides (a) metsulfuron-methyl and (b) clethodim applied over a range of concentrations in the liquid cultures of the AOA *N. sinensis*, *N. viennensis* and *Ca. N. franklandianus*, the AOB *N. europaea*, *N. multiformis* and *N. communis*, and the NOB *Ca. Nitrobacter laanbroekii* NHB1 and *N. winogradskyi* and in abiotic non-inoculated controls (at one selected concentration), determined at least at two time points. Error bars represent the standard error of the mean of triplicate cultures.

(b) Clethodim

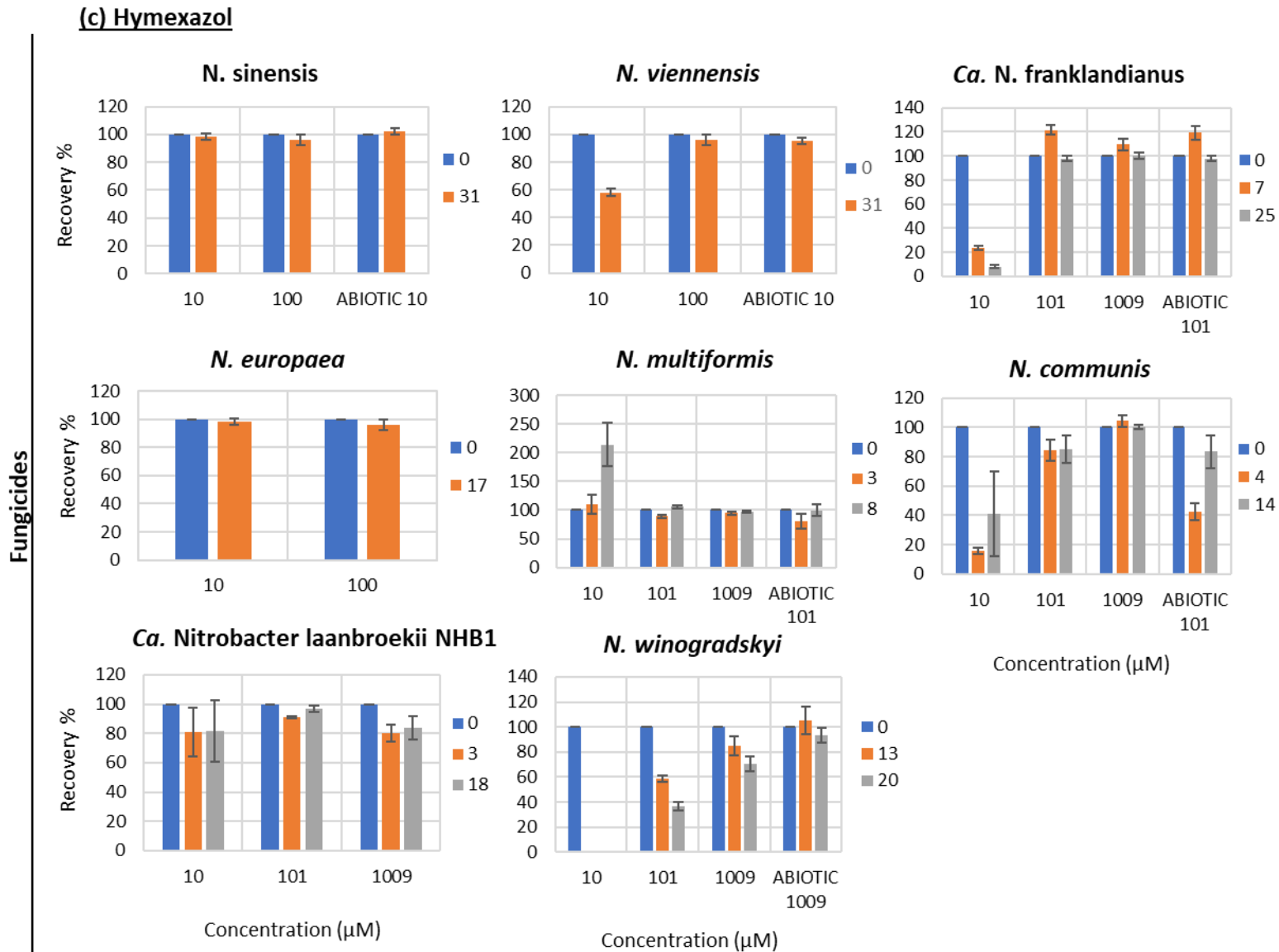
Supplementary Figure S5. The degradation of herbicides (a) metsulfuron-methyl and (b) clethodim applied over a range of concentrations in the liquid cultures of the AOA *N. sinensis*, *N. viennensis* and *Ca. N. franklandianus*, the AOB *N. europaea*, *N. multiformis* and *N. communis*, and the NOB *Ca. Nitrobacter laanbroekii* NHB1 and *N. winogradskyi* and in abiotic non-inoculated controls (at one selected concentration), determined at least at two time points. Error bars represent the standard error of the mean of triplicate cultures.



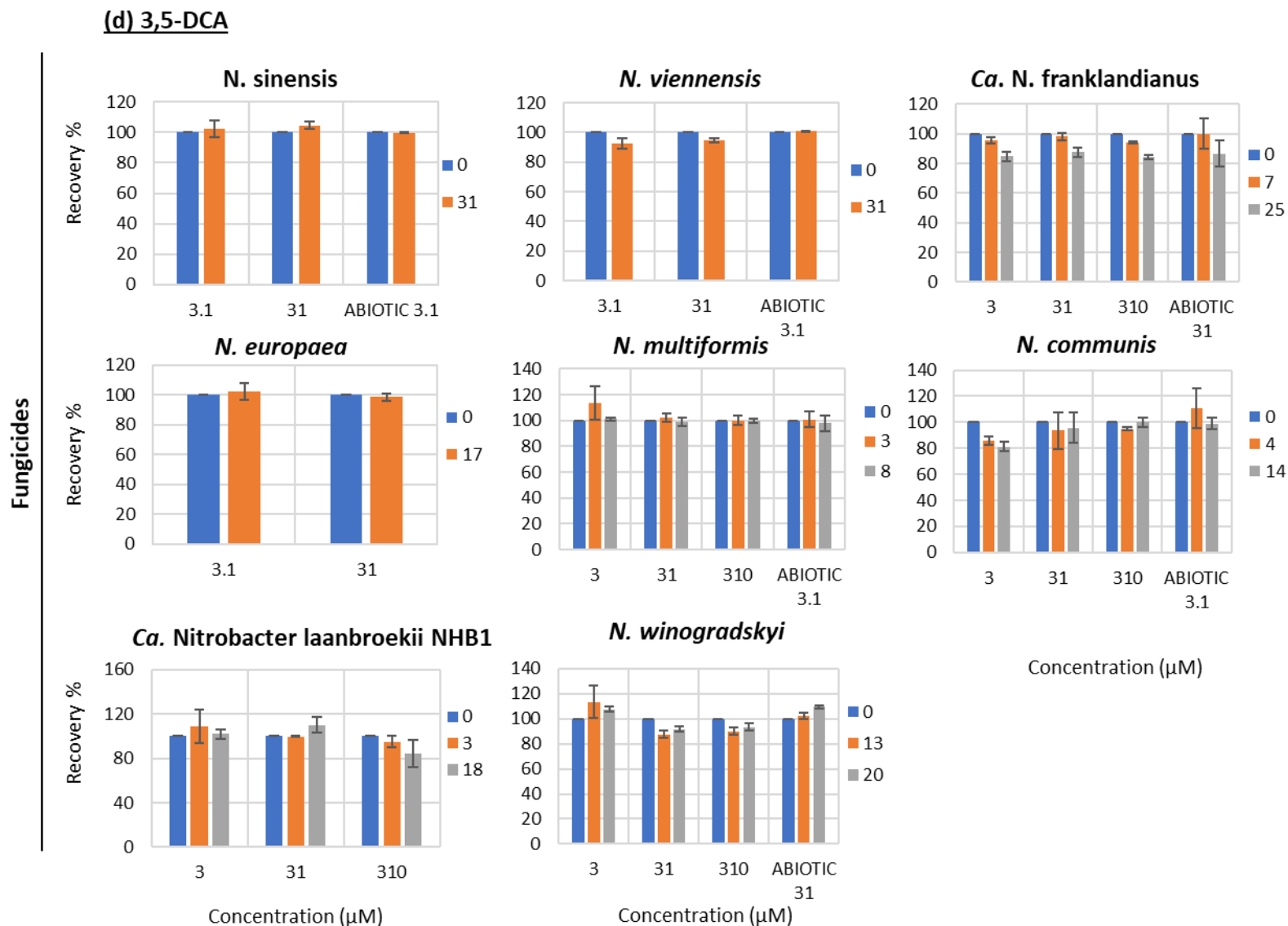
Supplementary Figure S6. The degradation of fungicides (a) pyraclostrobin, (b) etridiazole, (c) hymexazol and (d) 3,5- DCA applied over a range of concentrations in the liquid cultures of the AOA *N. sinensis*, *N. viennensis* and *Ca. N. franklandianus*, the AOB *N. europaea*, *N. multiformis* and *N. communis*, and the NOB *Ca. Nitrobacter laanbroekii* NHB1 and *N. winogradskyi* and in abiotic non-inoculated controls (at one selected concentration), determined at least at two time points. Error bars represent the standard error of the mean of triplicate cultures.

(b) Etridiazole

Supplementary Figure S6. The degradation of fungicides (a) pyraclostrobin, (b) etridiazole, (c) hymexazol and (d) 3,5- DCA applied over a range of concentrations in the liquid cultures of the AOA *N. sinensis*, *N. viennensis* and *Ca. N. franklandianus*, the AOB *N. europaea*, *N. multiformis* and *N. communis*, and the NOB *Ca. Nitrobaeter laanbroekii* NHB1 and *N. winogradskyi* and in abiotic non-inoculated controls (at one selected concentration), determined at least at two time points. Error bars represent the standard error of the mean of triplicate cultures.



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AUTORISATION DE SOUTENANCE

Vu les dispositions de l'arrêté du 25 mai 2016 modifié par l'arrêté du 26 août 2022,

Vu la demande du directeur de thèse

Monsieur G. NICOL

et les rapports de

M. G. PATON
Professor - University of Aberdeen

et de

Mme K. PAPADOPOULOU
Professor - University of Thessaly

Madame BACHTSEVANI Eleftheria

est autorisée à soutenir une thèse pour l'obtention du grade de **DOCTEUR**

Ecole doctorale ELECTRONIQUE, ELECTROTECHNIQUE, AUTOMATIQUE

Fait à Ecully, le 6 décembre 2024

Pour le directeur de l'École Centrale de Lyon
Le directeur de la recherche



Christophe CORRE