Aerobic methanotrophs and the associated microbial network: resilience and stress response.

Habilitation thesis of
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Preface

Microorganisms are the hidden majority that is often under-appreciated, despite their enormous contribution to many aspects of life on Earth. The study of microorganisms in the environment has gained traction in the past decades with the advent of high throughput sequencing technologies and novel techniques to probe microbial individuality. Still, many aspects of microbial life remain a black box; every discovery is an impetus for further discoveries. This Habilitation thesis investigated the resistance and resilience of the aerobic methanotrophs, a key microbial group responsible for the oxidation of methane (a potent primary greenhouse gas, GHG), to environmental cues and compounded disturbances as anticipated under global climate and land-use change scenarios. Because methanotrophs rarely, if at all, live in seclusion, the methane-driven interaction network comprising of the methanotrophs, as well as the non-methanotrophs was elaborated. A novel strategy to characterize these networks was introduced, coupling stable isotope probing to a co-occurrence network analysis.

This thesis is based on studies conducted partly at the Center for Microbial Ecology and Technology, Ghent University, Belgium (2012 – 2013) and the Department of Microbial Ecology, Netherlands Institute of Ecology (NIOO-KNAW), Wageningen, the Netherlands (2014 – 2017), with the majority of the work performed at the Institute for Microbiology (IFMB), Leibniz University Hannover, Germany (2017 - 2022). Research was funded by the Deutsche Forschungsgemeinschaft (DFG; HO 6234/1-1 and HO 6234/1-2) and the Leibniz University Hannover. This thesis would not have been completed without the ceaseless support of Prof. Marcus Horn. In particular, I am deeply grateful for his endless enthusiasm, guidance, encouragement, and inspiring discussions which frequently lead to exciting experiments, successful publications and grant applications. In short, I am thankful to Prof. Horn for the scientific independence, and for finding time when there is none. Through my scientific adventures in Belgium and the Netherlands, I am thankful to Prof. Nico Boon (Ghent University), and Dr. Paul Bodelier, Prof. Wim v.d. Putten, and Prof. Wietse de Boer (NIOO-KNAW) for giving me the opportunity to work alongside creative and dedicated scientists in their research groups, as well as their patience and guidance in the earlier days of my research career post-doctorate.

Many research ideas would not be realized without the dedication of the PhD candidates, Msc., and BSc. students I worked closely with. For this, I am indebted to Thomas Kaupper, Tanja Heffner, Jiyeon Lim, Giovanni Ganendra, Frederiek Maarten-Kerckhof, Hester van Dijk, Semi Brami, Danica Kynast, Alaa El-Hawwary, Clemens Bothe, Eric van den Brink, Max Reumer, and Rienke Ruijs. I am also thankful to all members of the *Horn* research group for making science fun, and my research stint in Hannover a pleasant and welcoming one; Natalie Röder's and Stefanie Hetz's contributions to the practical courses were invaluable.

No researcher works as an island. I am thankful to my partners-in-science through the years. The collaborations have been fruitful, resulting in publications, if not providing lively scientific discourse. For this, I am grateful to have crossed paths with Svetlana Dedysh, Werner Liesack, Mette Svenning, Kim Heylen, Marc Dumont, Marcela Hernandez, Alexander Tveit, Sascha Krause, Philippe Constant, Zhongjun Jia, Jun Murase, and more recently, Pil Joo Kim, Lucas Mendes, Anja Poehlein, Hyo Jung Lee, Monika Harnisz, Annelies Veraart, Zufarzaana Zulkeflee, Ali Tan Kee Zuan, David Ngugi, Joseph Sawadogo, and Hannu Fritze. I am also thankful to Stefan Zieker, Claudia Lüke, Sukhwan Yoon, Siegfried Vlaeminck, Anahita Rafiei, Anne Daebeler, and Susan Schlimpert for lending an ear, whenever one is needed.

Most importantly, I am grateful to my parents for their love, enduring support and understanding, and giving me the freedom and opportunity to chart my own path.

Adrian Ho Kah Wye, December 2022

Contents

I Summary

Microorganisms are a source, as well as a sink for methane, a potent primary greenhouse gas (GHG). Methane emissions would have been higher if not for the aerobic methane-oxidizers (methanotrophs) consuming the produced methane before being released into the atmosphere. These "low-affinity" methanotrophs thrive in niches where methane and oxygen availability overlap, and are of particular relevance in high methane-emitting environments (e.g., rice paddies, landfill covers, river sediments), whereas the "high-affinity" methanotrophs are responsible for consuming atmospheric methane at trace levels in well-aerated soils. Although shown to be resilient to sporadic disturbances, less is known on how methanotrophs respond to recurring/compounded disturbances, and the role of the accompanying non-methanotrophs in modulating methanotrophic activity remains to be determined. Hence, the central hypothesis was: **Methanotrophs are resilient to environmental disturbances, but recurring or compounded disturbances may have a cumulative effect, compromising methanotrophic activity, which is also modulated by interactions with the biotic environment**. The hypothesis was addressed by microcosm- and mesocosm-based studies, capitalizing on stable isotopes, trace gas analytics, and state-of-the-art molecular analyses of specific genes and gene transcripts.

In contrast to the response and resilience to sporadic or single disturbance events, methane uptake rates were largely impaired after recurring/compounded disturbances as anticipated to occur under a change in land-use (deforestation for agriculture, agriculture land restoration, peat excavation) and climate conditions (recurring and intensified desiccation-rewetting cycles, long-term drought, desertification-induced salinity stress). Emphasis was given to methane mitigation strategies in well-aerated agricultural soils, given the importance of agrosystems to support the global population growth. Soil methane consumption was significantly stimulated in the presence of specific bio-based residues (e.g., compost), unexpectedly facilitated by the apparently "low-affinity", instead of the "high-affinity" methanotrophs. This finding challenges the current paradigm, grouping methanotrophs according to their affinities to methane.

The methane uptake rate was significantly stimulated in the presence of non-methanotrophs, showing emergent properties when non-methanotrophs interact with methanotrophs. The spatial and temporal organization of the methane-driven network, as

well as the relevance of the non-methanotrophs during disturbances were elaborated. A novel methodological strategy was introduced, coupling stable isotope probing with ^{13}C-methane to a co-occurrence network analysis to provide direct ecological linkages between the metabolically active microorganisms involved in the methane-driven trophic interaction, while relating the structure of the interaction networks to activity (methane oxidation). In particular, results showed distinctly co-evolved methane-oxidizing communities in different methane hotpots (rice paddy soil, landfill cover soil, peatlands, and river sediments), suggesting that the methanotrophs exerted a marginal effect on the recruitment of the non-methanotrophs, or more likely, the non-methanotrophs were selected based on their ecological traits, rather than their taxonomic identity. Co-occurrence network analysis revealed the relevance of the non-methanotrophs particularly during recovery from disturbances, where they were identified as the key nodes. Collectively, the non-methanotrophs were not only shown to modulate methane uptake rates, but were also key members of the methane-driven interaction networks in times of disturbances.

Research findings showed the vulnerability of the methanotrophs to intensified disturbances, adversely affecting the methane sink. However, soil methane uptake can be significantly stimulated when implementing regenerative agricultural practices, particularly by applying specific bio-based residues in agrosystems. Interestingly, the non-methanotrophs were shown to be relevant members of the methane-oxidizing community, imposing an interaction-induced effect on community function. Therefore, inclusion of the interaction network, in addition to documenting the community composition, abundances, and abiotic properties, provided a more comprehensive understanding of microbial community responses.

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Chapter 1

General Introduction

1 General Introduction

1.1 Methane and the methanotrophs

Methane is a potent greenhouse gas (GHG), having a 34-fold higher heat retentive capacity in a 100-year time frame than carbon dioxide, and atmosphere methane has increased to approximately 1857 ppm_v in 2018, a 2.6-fold hike from pre-industrial era (IPCC, 2019; Saunois *et al*., 2020). The recent trend in methane growth is a cause for concern, exacerbating the impact of climate-change (Etminan *et al*., 2016; Dean *et al*., 2018), and indicates the imbalance of methane sources and sinks whereby the rate of methane production outpaced consumption (Saunois *et al*., 2020). While the main biogenic methane source, accounting for ~70 % of total methane emission to the atmosphere, is derived from the decomposition of organic matter mediated by the methanogenic archaea, the aerobic and anaerobic methanotrophs form the methane sink (Conrad, 2009; Kirschke *et al*., 2013; Guerrero-Cruz *et al*., 2021).

Discoveries over the past two decades have broadened the diversity of the methanotrophs, particularly the anaerobic ones which were found able to couple anaerobic methane oxidation to a suite of electron acceptors, including iron, sulphate, nitrite, iron, and manganese (Guerrero-Cruz *et al*., 2021). Besides the foreknown aerobic proteobacterial methanotrophs, novel acidophilic and thermophilic/thermotolerant aerobic methanotrophs belonging to Verrucomicrobia were discovered in geothermal springs, but have since been found to be widespread (see review Schmitz *et al*., 2021; Kaupper *et al*., 2021a). Particularly, the aerobic, rather than the anaerobic methanotrophs were often documented to be the active and key methane-oxidizers in many methane-emitting terrestrial environments (Ho *et al*., 2013; Kaupper *et al*., 2022). Compared to the anaerobic methanotrophs, the aerobic ones showed relatively faster growth (doubling time) (Ettwig *et al*., 2010; Blazewicz *et al*., 2012), as well as having distinct ecological traits, and foster interactions with photosynthetic organisms, widening their habitat range to micro-oxic or even anoxic environments (Raghoebarsing *et al*., 2005; Ho & Bodelier, 2015; see review Guerrero-Cruz *et al*., 2021). It follows than high methane-emitting environments (e.g., waste water treatment systems, landfill cover, rice paddies, and peatlands) are also hotspots for the aerobic methanotrophs.

1.2 Aerobic methanotrophs as a relevant biological methane bio-filter and atmospheric methane sink

Aerobic methanotrophs (henceforth, methanotrophs) oxidize methane to methanol using oxygen as the primary electron acceptor with the enzyme methane monooxygenase (MMO). The MMO can be present as a soluble (sMMO) or membrane-bound particulate (pMMO) form, depending on the methanotroph. While the vast majority of methanotrophs harbor the pMMO, the alphaproteobacterial methanotrophs *Methylocapsa* and *Methyloferula* possess only the sMMO (Theisen *et al*., 2005; Vorobev *et al*., 2011). In methanotrophs harboring both the pMMO and sMMO, copper regulates the relative expression of these enzymes, suppressing the sMMO, while stimulating the pMMO (Knapp *et al*., 2007; Trotsenko & Murrell, 2008). The *pmoA* and *mmoX* gene, respectively encoding for the pMMO and sMMO, are frequently targeted in culture-independent studies to characterize the methanotrophs in complex communities (e.g., Liebner & Svenning, 2013; Cai *et al*., 2016; Karwautz *et al.,* 2018). Following methane oxidation, methanol is further oxidized to formaldehyde, where carbon is assimilated *via* the ribulose monophosphate and serine pathway in gamma- and alpha-proteobacterial methanotrophs, respectively. Other characteristics which differentiate the gamma- and alpha-proteobacterial methanotrophs include their distinct phospholipid fatty acid (PLFA) profiles (Ho *et al*., 2019). Depending on the methanotroph, around 50-60% of methane-derived carbon is assimilated into the cell, while the remaining is further oxidized to carbon dioxide *via* formate; in some methanotrophs (e.g., *Methylosinus*), a substantial amount of cell carbon (≥ 60%) may be derived from carbon dioxide (Yang *et al*., 2013; see recent review for biochemical pathways; Dedysh & Knief, 2018). Additionally, some alphaproteobacterial methanotrophs (e.g., *Methylocystis*, *Methylocella*) are facultative, capable of growth on compounds containing carbon-carbon bonds (e.g., acetate, ethanol, succinate), besides methane (Dedysh *et al*., 2005; Chen *et al*., 2010; Im *et al*., 2010; Dedysh & Knief, 2018).

Since the discovery of the first methanotroph in 1906, around 25 genera have been isolated and characterized to date. These include members of the families *Methylococcaceae*, *Methythermaceae*, *Methylocystaceae*, *Beijerinkiaceae*, and *Methylacidiphilales* (Guerrero-Cruz *et al*., 2021). While the majority of cultured methanotrophs are low-affinity methane-oxidizers, typically but not exclusively recovered from high methane-emitting environments (e.g., rice paddies, peatlands, landfill cover, river sediment; Kaupper *et al*., 2022), the high-affinity methanotrophs have, for a long time, only been identified based on their *pmoA* gene diversity and was without any cultured representatives until recently (Cai *et al*., 2016; Pratscher *et al*., 2018; Ho *et al*., 2019; Tveit *et al*., 2019). Among the high-affinity methanotrophs is *Methylocapsa gorgona* isolated from a landfill in the subarctic Norway (Tveit *et al*., 2019). Along with this species, other members of the same genus, *Methylocapsa acidiphila* and *Methylocapsa aurea* have also been shown to grow on atmospheric methane (Tveit *et al*., 2019). Because of their different affinities to methane, the methanotrophs play distinct roles in the environment, with the low-affinity methanotrophs acting as a methane bio-filter at oxic-anoxic interfaces where methane is available at relatively higher concentrations, and the high-affinity ones consuming atmospheric methane at trace levels in well-aerated soils (Figure 1).

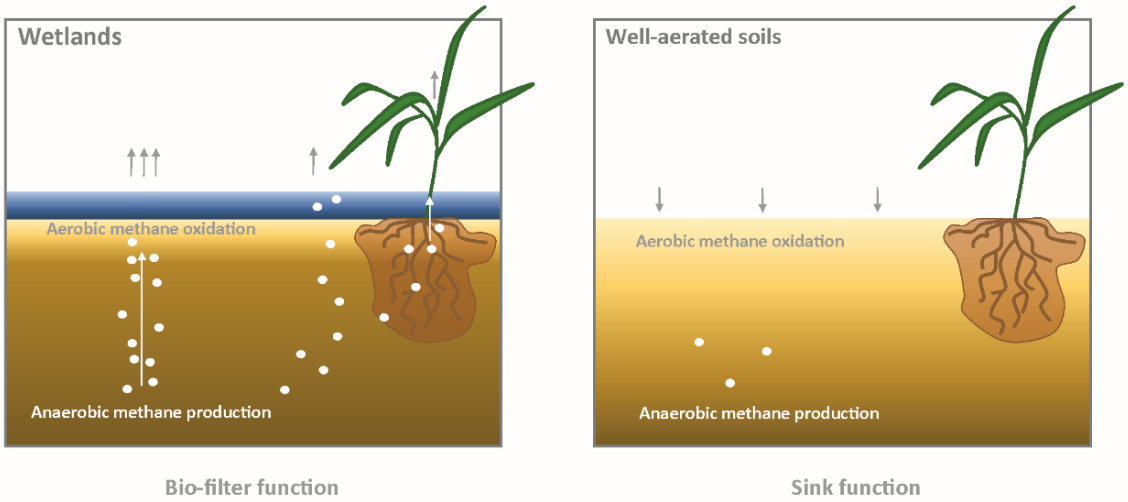

Figure 1: The role of low-affinity methanotrophs as a methane biofilter in high methane-emitting environments (e.g., wetlands; left panel) and atmospheric methane sink in well-aerated soils (right panel). In wetland, a substantial amount of methane (up to 90%) is oxidized at methane-oxygen

counter gradients (e.g., soil-flood water interface, rhizosphere) before being released into the atmosphere. On the other hand, high-affinity methanotrophs consume atmospheric methane, being an effective sink for the GHG.

1.3 Prevalence and life strategies

Versatile metabolic capabilities, combined with the ability to thrive under broad temperature (4 - > 90 ^{O}C) and pH (1-8) ranges, and limited oxygen availability (micro-oxic conditions) contribute to the ubiquity of methanotrophs in widespread environments (see reviews Semrau *et al*., 2010; Knief, 2015; Kwon *et al*., 2019). Accordingly, the methanotrophs have been shown to be remarkably resistant and resilience to disturbances (see Chapter 3). Recovery in methanotrophic activity and community composition were observed after heat and desiccation stress (Ho & Frenzel, 2012; Ho *et al*., 2016a; Kaupper *et al*., 2021b), simulated prolonged drought (Collet *et al*., 2015), mechanical soil aggregate destruction (Kumaresan *et* al., 2011), salt stress (Ho *et al*., 2018), and exposure to pesticides, chemical additives, pharmaceuticals, and high ammonium concentrations (Benner *et al*., 2014; Ho *et al*., 2020; van Dijk *et al*., 2021). Astonishingly, the methanotrophic activity also recovered from recurring disturbances exemplified by a repeated desiccation-rewetting regime, but the trajectory in population dynamics were significantly altered, and activity was severely impaired upon intensification of the desiccation-rewetting cycles (Ho *et al*., 2016b). This suggests that the resilience of the methanotrophic activity was close to a "tipping point", after which, methane uptake rates no longer recovered with further exposure to the disturbance.

The resilience of the methanotrophs to contemporary disturbances may also be attributable to site history (environmental legacy) or prior exposure to the disturbance (Ho *et al*., 2016a; Krause *et al*., 2018; van Kruistum *et al*., 2018; Chapter 3). To this end, historical contingency plays a relevant role by leaving an imprint on the community composition in the form of a microbial seed bank, allowing the emergence and rapid response of a resistant community upon encountering the same disturbance (Pagaling *et al*., 2014; Ho *et al*., 2016a). Interestingly, recovery from a disturbance may also confer resilience to other forms of disturbances (Baumann & Marschner et al., 2013; van Kruistum *et al*., 2018). For instance, a methanotrophic community that was subjected to desiccation recovered significantly faster in the presence of high ammonium stressor than in a community not subjected to desiccation

(van Kruistum *et al*., 2018). In this study, it can be argued that both disturbances may have elicited a similar physiological response, that is, desiccation (causes concentration of solutes) and the addition of ammonium in the form of ammonium chloride increases soil salinity which may have selected for salt-tolerant community members. Although metabolically inactive, the methanotroph seed bank may become relevant, conferring resilience to future disturbances.

The methanotrophs possess distinct functional characteristics, as deduced from their differential responses to disturbances and substrate/nutrient availability; these characteristics, along with their ecological traits have been conceptualized as life strategies placing the methanotrophs at the genus level within the competitor-stress tolerator-ruderal (C-S-R) framework (Figure 2; Ho *et al*., 2013; 2017a). Although initially used for classifying the life strategies of plants (Pierce *et al*., 2016), the C-S-R framework was adopted to accommodate microbial life strategies, allowing the characterization of microbial traits into a three dimensional framework, as opposed to the classification of organisms into copiotrophs and oligotrophs (MacArthur & Wilson, 1967; Fierer *et al*., 2007). The C-S-R framework proposes that the combinations of stressors (i.e., determinants that restrict microbial biomass) and disturbances (i.e., determinants that results in the destruction of microbial biomass) lead to three main life strategies, that is, competitors, stress-tolerators, and ruderals (Pierce *et al*., 2016; Figure 2). As such, the C-S-R framework allows mixed strategies to accommodate the metabolic versatility of microorganisms, and prediction of the distribution and prevalence of the methanotrophs under different environmental conditions.

The ecological traits of specific members of the methanotrophic community were identified (Chapters 3 and 4; see reviews Ho *et al*., 2013; 2017a), and subsequently classified within this scheme (Figure 2). Particularly, the gammaproteobacterial methanotrophs appear to be more responsive to high substrate availability. Although they may not represent the predominant community members, some gammaproteobacterial methanotrophs rapidly proliferated and became the dominant active community members in high methane-emitting environments (e.g., rice paddy, landfill cover, river sediment, Arctic wetlands), as revealed by stable isotope probing (SIP) using ^{13}C-methane and transcript-based analyses (reviews Ho *et al*., 2013; 2017a; Kaupper *et al*., 2022). Accordingly, these methanotrophs (e.g., *Methylomonas*, *Methylobacter*, *Methylomicrobium*, and *Methylosarcina*) were classified as competitiors and competitor-ruderals. In contrast, alphaproteobacterial methanotrophs (e.g., *Methylocystis*, *Methylosinus*) were typically found to become relevant during recovery from

disturbances (e.g., heat shock, desiccation), gradually increasing in their population size over time (Ho *et al*., 2013; 2016a,b). Also, some alphaproteobacterial methanotrophs (e.g., *Methylosinus*) are able to form hardier desiccation- and heat-resistant dormant cells than gammaproteobacterial methanotrophs (Whittenbury *et al*., 1970), indicating their ability to alternate between dormant and vegetative states, depending on the environmental stressors. It is thought that these alphaproteobacterial methanotrophs form a relatively stable community, being present as resting cells which contribute to the methanotroph seedbank in soils (Eller *et al*., 2005; Krause *et al*., 2012; Ho *et al*., 2016a). Besides, alphaproteobacteria methanotrophs exhibit metabolic versatility, having been shown to utilize other substrates (Im *et al*., 2010; Dedysh & Knief, 2018), and may occur under unfavorable conditions (e.g., acidic peatlands; Dedysh, 2011). The alphaproteobacterial methanotrophs were also found to be more competitive under nutrient limitation, suggesting their relevance under oligotrophic conditions (Graham *et al*., 1993). Taken together, accumulating evidence indicated that the alphaproteobacterial methanotrophs were stress tolerators, stress tolerator-ruderals, and stress tolerator-competitors. Because of the lack of environmental data, the competitiveness of other methanotrophic genera remains to be determined. Overall, the C-S-R framework accommodates microbial metabolic versatility, which provides a relatively more comprehensive classification of microbial life strategies.

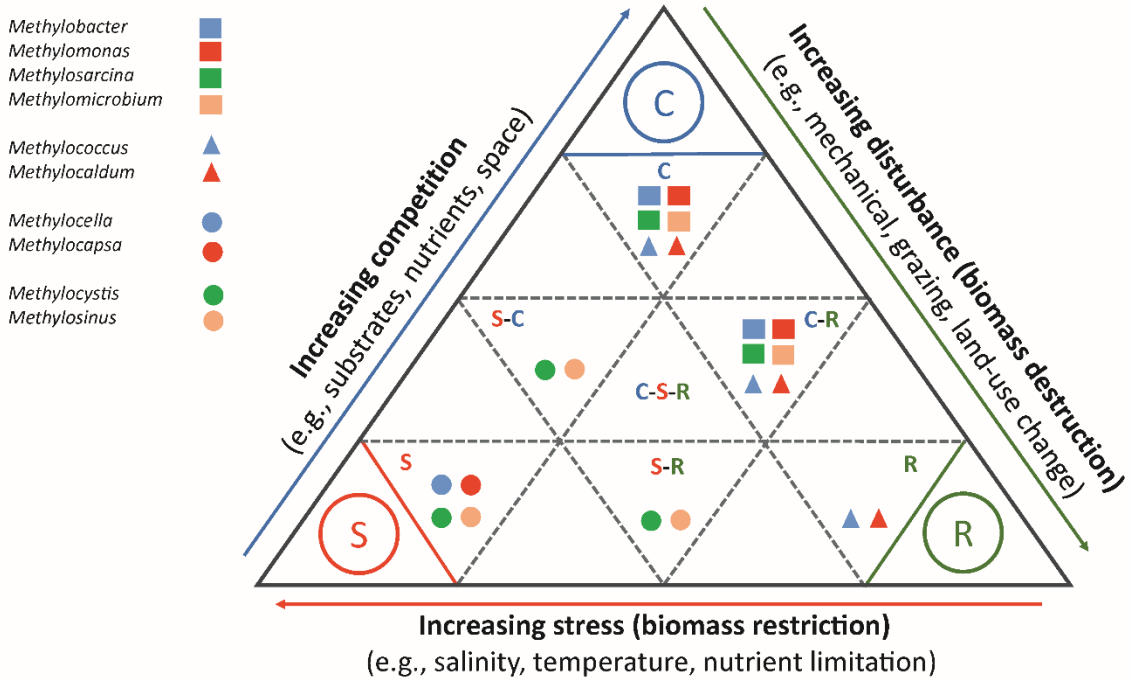

Figure 2: The ecological traits of methanotroph conceptualized as life strategies within the C-S-R framework. Only genera where sufficient environmental knowledge is available are placed within the scheme; placement is based on a literature survey, as given in Ho *et al*. (2013) and Ho *et al*. (2017a).

1.4 The methane-driven interaction network

Although the composition and collective ecological traits of the methanotrophs are relevant for activity during recovery from disturbances and in response to environmental cues, accumulating evidence suggests that methane oxidation is a community function, whereby non-methanotrophs are also important, exerting interaction-induced effects (Chapter 5; Ho *et al*., 2014; 2016).

Co-occurrence network analysis is typically employed to explore microbial interactions in complex communities. Microorganisms that are positively correlated to one another are thought to share the same niche, possessing complementary roles, and are likely to be driven by cross-feeding (Barberan *et al*., 2011; Morris *et al*., 2013; Peura *et al*., 2015; D'Souza *et al*., 2018). Contrastingly, co-occurring microorganisms that are negatively correlated are anticipated to have competing roles in the environmental, occupying distinct niches (niche partitioning), and possibly, involved in a predator-prey relationship (Ghoul & Mitri, 2016; Dann

et al., 2019; Johnson *et al*., 2020). The assessment of the co-occurring interaction networks are based on topological properties, including the number of nodes (representing microbial taxa) and edges (connections between nodes), number of communities, network diameter (longest distance between nodes), average path length (distance between pairs of nodes and length of connections), degree (number of connections per node), clustering coefficient (tendency for nodes to cluster), and modularity (capability to form densely connected communities) (see Chapter 5). Although statistically proven, the correlated microbial taxa, as determined from the network analysis, may not necessarily co-occur in the environment, given the restricted movements of microorganisms. In some instances, sampling was performed at appreciable distances apart (m to km scale), and composited prior to nucleic acid extraction, used to derive the network analysis. Accordingly, the structure (e.g., complexity, connectivity) of the interaction network is likely to be overestimated in DNA-based networks (Kaupper *et al*., 2022), considering that a significant fraction of microorganisms in the environment is metabolically inactive, particularly in soils (Lennon and Jones, 2011).

These limitations were addressed by coupling stable isotope probing (SIP) using ^{13}C-methane to a co-occurrence network analysis to track trophic interactions, thereby providing an ecological linkage between the metabolically active and interacting community members (referred as the methanotroph "interactome") involved in the methane-dependent food web. In effect, a methodological strategy to strengthen and substantiate the biological interaction among community members were introduced to determine the response of the interaction networks to environmental cues and disturbances, as well as to identify the potential mechanisms driving the co-occurrence (see Chapter 5). The inclusion of the SIP-derived interaction networks is thus a step forward, providing a more comprehensive understanding of microbial community responses.

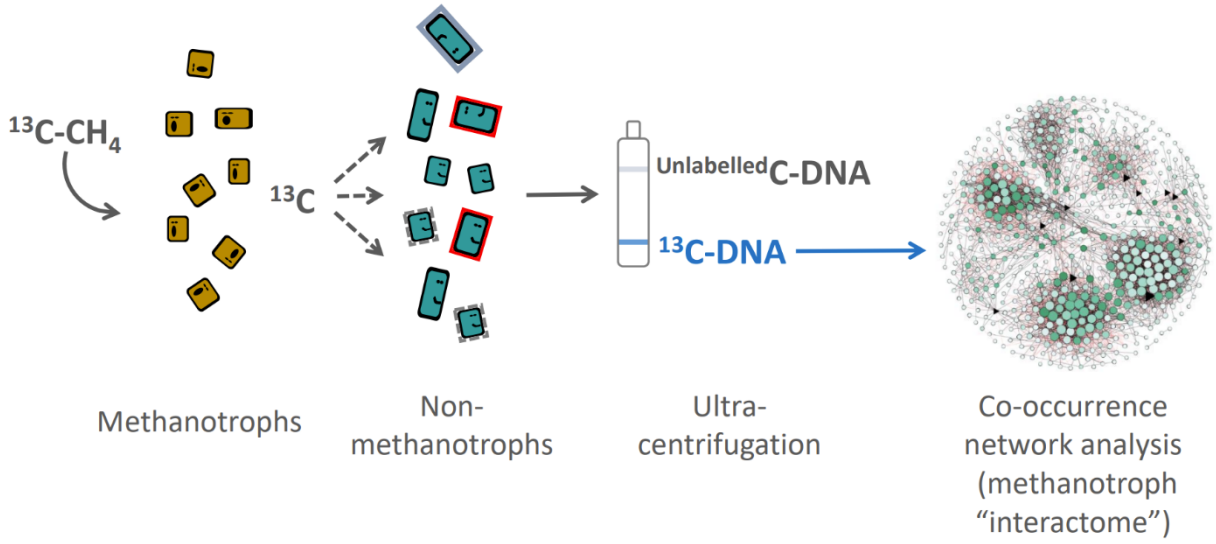

Figure 3: Workflow coupling stable isotope probing (SIP) using ^{13}C-methane to a co-occurrence network analysis to strengthen the interaction network, while reducing spurious connections (Chapter 5). The SIP approach, including identification of the ^{13}C-enriched DNA relative to the ^{unlabelled}C-DNA after ultracentrifugation, was performed as detailed before (Neufeld *et al.,* 2007), whereas the interaction networks were derived from the 16S rRNA gene amplified from the ^{13}C-enriched DNA, representing the metabolically active and replicating members of the community (Kaupper *et al*., 2021a,b; 2022).

1.5 Hypothesis and Objectives

The methanotrophs represent the sole biological methane sink on Earth, playing a crucial role to mitigate methane emissions in widespread environments. Of particular relevance is the role of methanotrophs in agrosystems, given the increasing demand for food and feed to support the ever-growing human population. As such, it is pertinent to elucidate the resistance/resilience and vulnerability of the methanotrophs to environmental disturbances under conditions anticipated with the change in land-use (e.g., deforestation for agriculture, peat excavation), as well as predicated scenarios related to global climate change (e.g., increasing or prolonged drought and temperature, desertification). Equally as important are the associated non-methanotrophs, which can exert an effect on the activity of the methanotrophs. These interactions modulate diverse aspects of microbial life, affecting not only the activity, but also shape the diversity, composition, abundance, stability, and assembly of microbial communities. Little is known of how interactions within the methane-driven network may affect methane oxidation. The central hypothesis was:

Methanotrophs are resilient to environmental disturbances, but recurring or compounded disturbances may have a cumulative effect, compromising methanotrophic activity, which is also modulated by interactions with the biotic environment.

The main objectives, corresponding to the three chapters are:

- To determine the resilience of the methanotrophs to compounded and intensified disturbances, as anticipated under land-use and global climate change scenarios (Chapter 3).
- To determine the resilience of the methanotrophs and the methane sink function to agriculture-induced effects in well-aerated upland soils (Chapter 4).
- To determine the role of the non-methanotrophs in modulating methanotrophic activity in methane-emitting environments and in response to disturbances (Chapter 5).

Chapter 2

List of Publications in the "Habilitation" Thesis

2 List of Publications in the "Habilitation" Thesis

(Own contributions are given in italics; corresponding author)*

Chapter 1

General Introduction

Review

1. **Ho A**, Kerckhof F-M, Lüke C, Reim A, Krause S, Boon N, Bodelier PLE (2013) Conceptualizing functional traits and ecological characteristics of methane-oxidizing bacteria as life strategies. Environmental Microbiology Reports 5: 335-345.
   (Concept with PLE Bodelier and writing).

2. **Ho A***, Di Lonardo DP, Bodelier PLE (2017) Revisiting life strategy concepts in environmental microbial ecology. FEMS Microbiology Ecology 93: fix006.
   (*Concept and writing; corresponding author).*

3. Guerrero-Cruz S, Vaksmaa A, Horn MA, Niemann H, Pijuan M, **Ho A*** (2021) Methanotrophs: discoveries, environmental relevance, and a perspective on current and future applications. Frontiers in Microbiology 12: 678057.
   (*Concept and part of manuscript writing; corresponding author).*

Chapter 3

The resilience of the aerobic methanotrophs to disturbances

Primary research literature

4. Collet S, Reim A, **Ho A**, Frenzel P (2015) Recovery of paddy soil methanotrophs from long term drought. *Soil Biology and Biochemistry* 88: 69-72.
   (*Concept, co-supervision, and part of manuscript writing).*

5. **Ho A***, Lueke C, Reim A, Frenzel P (2016) Resilience of (seed bank) aerobic methanotrophs and methanotrophic activity to desiccation and heat stress. Soil Biology and Biochemistry 101: 130-138.
   (*Concept, performed study, and writing; corresponding author).*

6. **Ho A***, van den Brink E, Reim A, Krause S, Bodelier PLE. (2016) Recurrence and frequency of disturbance have cumulative effect on methanotrophic activity, abundance, and community structure. Frontiers in Microbiology 6: e1493.
   (*Concept, performed study with E van den Brink, and writing; corresponding author).*

7. Reumer M, Harnisz M, Lee HJ, Reim A, Grunert O, Putkinen A, Fritze H, Bodelier PLE, **Ho A*** (2018) Impact of peat mining, and restoration on methane turnover potentials and methane-cycling microorganisms in a northern bog. Applied and Environmental Microbiology 84: e02218-17.
   (*Concept, supervision, and writing; corresponding author).*

8. **Ho A***, Mo Y, Lee HY, Sauheitl L, Jia Z, Horn MA (2018) Effect of salt stress on aerobic methane oxidation and associated methanotrophs; a microcosm study of a natural community from a non-saline environment. Soil Biology and Biochemistry 125: 210-214.
   (*Concept with MA Horn, performed study, and writing; corresponding author).*

9. Kaupper T, Luehrs J, Lee HJ, Mo Y, Jia Z, Horn MA, **Ho A*** (2020) Disentangling abiotic and biotic controls of aerobic methane oxidation during re-colonization. Soil Biology and Biochemistry 142: 107729.
   (*Concept with MA Horn, supervision, and writing; corresponding author).*

10. Mo Y, Jin F, Zheng Y, Baoyin T, **Ho A***, Jia Z. (2020) Succession of bacterial community and methanotrophy during lake shrinkage. Journal of Soils and Sediments 20: 1545-1557.
    (*Concept with Z Jia and part of writing; corresponding author).*

11. **Ho A***, Zuan ATK, Mendes LW, Lee HY, Zulkeflee Z, van Dijk H, Kim PJ, Horn MA (2021) Aerobic methanotrophy and co-occurrence networks of a tropical rainforest and oil palm plantations in Malaysia. Microbial Ecology; https://doi.org/10.1007/s00248-021-01908-3.
(*Concept, performed study, and writing; corresponding author).*

Review

12. Kaupper T, Hetz S, Kolb S, Yoon S, Horn MA, **Ho A*** (2020) Deforestation for oil palm: impact on microbially mediated methane and nitrous oxide emissions, and soil bacterial communities. Biology and Fertility of Soils 56: 287-298.
(Concept with MA Horn and S Kolb, supervision, and writing; corresponding author).

Chapter 4

The role of aerobic methanotrophs in climate-smart agriculture

Primary research literature

13. Ho A*, Reim A, Kim SY, Meima-Franke M, Termorshuizen A, de Boer W, van der Putten WH, Bodelier PLE (2015) Unexpected stimulation of soil methane uptake as emergent property of agricultural soils following bio-based residue application. Global Change Biology 21: 3864-3879.
(Concept with PLE Bodelier, WH van der Putten, and W de Boer, performed study, and writing; corresponding author).

14. **Ho A***, El-Hawwary A, Kim SY, Meima-Franke M, Bodelier PLE (2015) Manure-associated stimulation of soil-borne methanogenic activity in agricultural soils. Biology and Fertility of Soils 51: 511-516.
(Concept, supervision, and writing; corresponding author).

15. **Ho A***, Ijaz UZ, Janssens TKS, Ruijs R, Kim SY, De Boer W, Termorshuizen A, van der Putten WH, Bodelier PLE (2017) Effects of bio-based residue amendments on

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Chapter 5

The methanotroph interactome: greater than the sum of its parts

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Review

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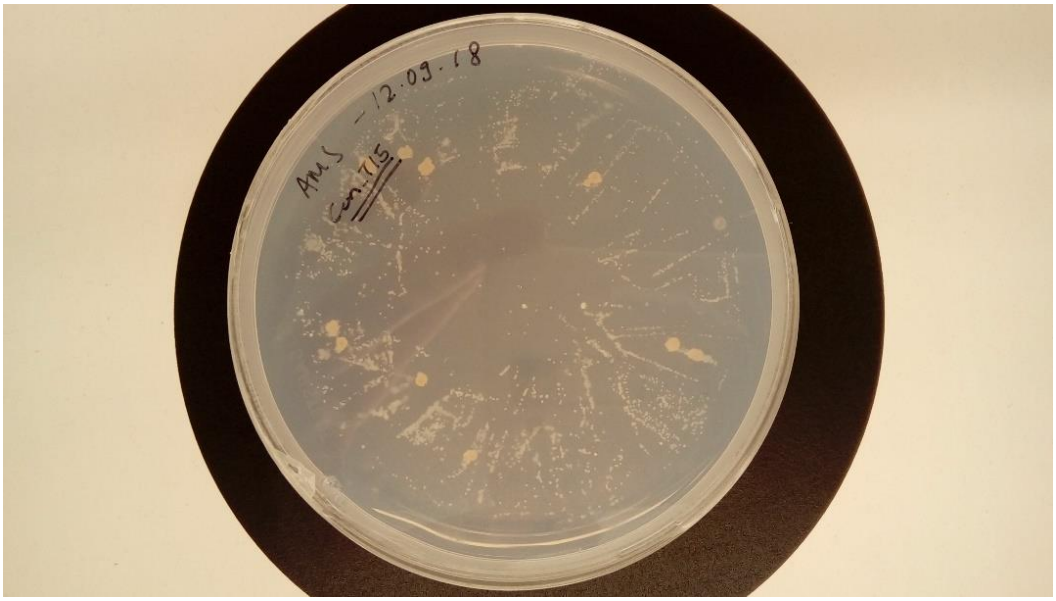
Chapter 3

The Resilience of the Aerobic Methanotrophs to recurring and compounded disturbances



Highly enriched desiccation- and heat-resistant methane-oxidizing community grown in Ammonium Mineral Salts (AMS) medium by Adrian Ho

3 The resilience of the aerobic methanotrophs to recurring and compounded disturbances

Aerobic methanotrophs are remarkably resilient to single or one-off disturbances, recovering from heat shock (Ho & Frenzel, 2012), physical disruption to soil structure (Kumaresan *et al*., 2011), increasing salinity (Bissett *et al*., 2012), and disturbance-induced mortality (Ho *et al*., 2011; Pan *et al*., 2014), among other environmental perturbations (e.g., contamination of heavy metal, and pollutants such as pharmaceuticals, pesticides, and chemical additives; Semrau *et al*., 2010; Benner *et al*., 2015), given sufficient time (within days to weeks) and substrate (methane and oxygen) availability. At times, the methanotrophs even over-compensated for disturbance-induced activity and diversity loss during recovery (e.g., Ho *et al*., 2011; Ho & Frenzel, 2012). However, a shift in the methanotrophic community composition is often detected, indicating the differential response of the methanotroph sub-groups to the disturbance resulting in the altered trajectory in community succession. Particularly, the alphaproteobacterial methanotrophs (*Methylosinus*, *Methylocystis*) which showed habitat preference for relatively oligotrophic environments (e.g., ombrotrophic peatlands, upland soils), appeared to be favored by or were generally more resistant to disturbances (Dedysh, 2011; Ho *et al*., 2013; 2017a; Knief *et al*., 2015). This suggested advantageous ecological traits inherent to some methanotrophs, likely reflecting on their life strategies, and enabled their persistence following disturbances (Ho *et al*., 2013).

The unexpected resilience of the methanotrophs may be partly explained by previous exposure to the same disturbance, prompting rapid recovery of a community which had survived the event (Krause *et al*., 2017a; van Kruistum *et al*., 2018; section 3.1). This begs the question whether the methanotrophs are also resilient to disturbances without prior exposure (section 3.1), and whether the methanotrophs remain resilient in the face of (intensified) recurring (section 3.2) and prolonged (section 3.3) disturbances. Following disturbances, nutrients and space (as a result of cell die-off), two relevant factors restricting microbial growth in the environment may become available. During re-colonization after disturbances, the modified abiotic (edaphic properties), and/or biotic (methanotrophic community composition) parameters may determine the success of the early colonizers, benefiting the fast-growing methanotrophs under these favorable conditions (section 3.4). Although methanotrophs may re-colonize open niches, and their activity, as well as community

composition/abundances may recover, the methanotrophs may nonetheless be challenged by compounded disturbances or environmental changes, as anticipated under land-use and global climate change scenarios, that is, peat mining and restoration (section 3.5), deforestation for palm oil production (section 3.6), and desertification-induced salt stress (section 3.7). The objectives were:

(i) To determine the role of site history (i.e., prior disturbance exposure) in conferring resilience during contemporary disturbances (desiccation-rewetting and heat stress).

(ii) To determine the resilience of the methanotrophs to (intensified) recurring desiccation-rewetting.

(iii) To determine the resilience of the methanotrophs to prolonged drought spanning over 18 years.

(iv) To determine the relative importance of the abiotic (edaphic properties) and/or biotic (methanotrophic community composition) parameters during re-colonization after a simulated disturbance-induced die-off.

(v) To determine the resilience of the methanotrophs to land-use change; a case study on peat excavation and restoration.

(vi) To determine the resilience of the methanotrophs to land-use change; a case study on deforestation for palm oil production.

(vii) To determine the resistance of the methanotrophs to global climate change; a case study on desertification-induced salt stress after lake shrinkage.

3.1 The resilience of the methanotrophic activity and community composition to desiccation-rewetting and heat stress, disturbances without prior exposure [Objective 3 (i); based on Ho *et al*., 2016a]

Repeated exposure to disturbances is anticipated to leave an imprint on the microbial community composition (Ge *et al*., 2008; Allison *et al*., 2013; Meisner *et al*., 2013) in the form of a microbial seed bank (Lennon & Jones, 2011), which may confer resilience to the contemporary community upon exposure to future recurring disturbances. Therefore, depending on the site history, the methanotrophic (seed bank) community indigenous to different environments may show varying degrees of resistance and/or resilience to desiccation-rewetting and heat stress; methanotrophs inhabiting an environment with prior exposure to drought may thus recover more effectively from desiccation-rewetting and heat stress, compared to a drought-free community. The methanotrophic activity and community composition from a rice paddy soil experiencing recurring desiccation-rewetting and heat stress was compared to two lake sediments with sporadic (Lake Neusiedl, Austria) or no (Lake Constance, Germany) previous exposure to these disturbances. Drought was induced combining desiccation and heat treatment at ambient (25^{o}C, designated mild stress), and elevated (75^{o}C, designated severe stress) temperatures to induce the formation of resting cell (Whittenbury *et al*., 1970). Fresh samples served as reference.

Unexpectedly, the methane uptake rates recovered after mild and severe stress in the rice paddy soil and both lake sediments, when compared to the reference after 40 days incubation, albeit activity was lower < 3 days after severe stress. More pronounced after severe stress, the methanotroph population size was appreciably reduced by up to three orders of magnitude, but abundances rapidly recovered reaching values exhibited by the reference incubations already at < 26 days upon rewetting the soil/sediments. Generally, a shift towards the predominance of gammaproteobacterial methanotroph was detected over time after stress, based on group-specific qPCR analyses. The overwhelming numerical abundance of gammaproteobacterial methanotrophs masked the exponential increase of the alphaproteobacterial methanotrophs (*Methylosinus*, *Methylocystis*), particularly after heat stress at 75^{o}C. This suggests that desiccation and heat stress induced the emergence of some dormant alphaproteobacterial methanotrophs from the seed bank community.

The recovered methanotrophic community was distinct after mild and severe stress, compared to the reference, as revealed by a canonical correspondence analysis derived from the *pmoA*-based diagnostic microarray. Hence, the induced stress shifted the trajectory of population dynamics, but the community members were sufficiently redundant to sustain overall methane uptake. Among the gammaproteobacteria, some methanotrophs were more stress tolerant (e.g., *Methylocaldum*- and *Methylosarcina*-related) than others (e.g., *Methylomonas*–related and the yet-uncultured freshwater cluster 1), indicating niche differentiation within this subgroup.

Overall, the methanotrophic activity was remarkable resilient to the induced stress, regardless of site history. This was particularly evident when comparing the most contrasting sites whereby methane uptake rates in the permanently inundated lake sediment (Lake Constance) recovered well after mild and severe stress relative to the rice paddy soil experiencing higher temperatures and alternating desiccation-rewetting cycles. Nevertheless, stress induced a shift in the composition of the recovered community, suggesting that repeated exposure to adverse conditions will change the composition of the (seed bank) methanotroph. Overall, while site history may not directly reflect the response of methanotrophic activity to desiccation-rewetting and heat stress, the methanotroph seed bank can still be a repository for future contingencies.

3.2 Impaired methanotrophic activity, and shift in the community composition with (intensified) recurring desiccation-rewetting [Objective 3 (ii); based on Ho *et al*., 2016b]

Although the methanotrophs showed remarkable recovery to a suite of disturbances induced as single events, their resilience may reach a "tipping point" with recurring (intensified) disturbances where methanotrophic activity and abundances may no longer recover and community composition profoundly altered. To this end, alternate drought and heavy rainfall is anticipated to intensify with global warming, modifying the soil quality (e.g., organic matter content, soil aggregate size and distribution) and nutrient contents (e.g., total C and N, as a result of mineralization) in turn, drives microbially-mediated processes in soils, including methane oxidation (Denef *et al*., 2001; Mikha *et al*., 2005). Three cycles of desiccation-rewetting (termed, moderate disturbance) were induced representing a recurring disturbance, and the frequency of the disturbance was further increased twofold, that is, from

every 14 days to every 7 days (termed, severe disturbance), allowing less recovery time between cycles for a total of 6 cycles (disturbance intensification; Figure 4) in a soil microcosm study.

Methane uptake rate was significantly impaired immediately after each desiccation-rewetting cycle in the moderately disturbed microcosms when compared to the un-disturbed reference (Figure 4). Although methane uptake was more varied (increased, decreased, or no significant changes) after desiccation-rewetting in the severely disturbed microcosms, the adverse effect was more pronounced after four consecutive desiccation-rewetting cycles (Figure 4). This indicates that the methanotrophic activity was generally not resistant to desiccation-rewetting. However, given sufficient recovery time (14 days in the moderately disturbed microcosms), the methanotrophic activity was resilient to recurring disturbance, but activity diminished with intensified recurring disturbance in the severely disturbed microcosms. By the last desiccation-rewetting cycle, methanotrophic activity was neither resistant nor resilient to severe disturbance, relative to the reference. Accordingly, the resilience index, RL (Orwin & Wardle, 2004) reflects the trend in the activity measurements, showing a decreasing RL value on the third and from the fourth cycle onwards under moderate and severe disturbances, respectively. This indicates that the activity was becoming less resilient with consecutive cycles, and approaching a “tipping point”.

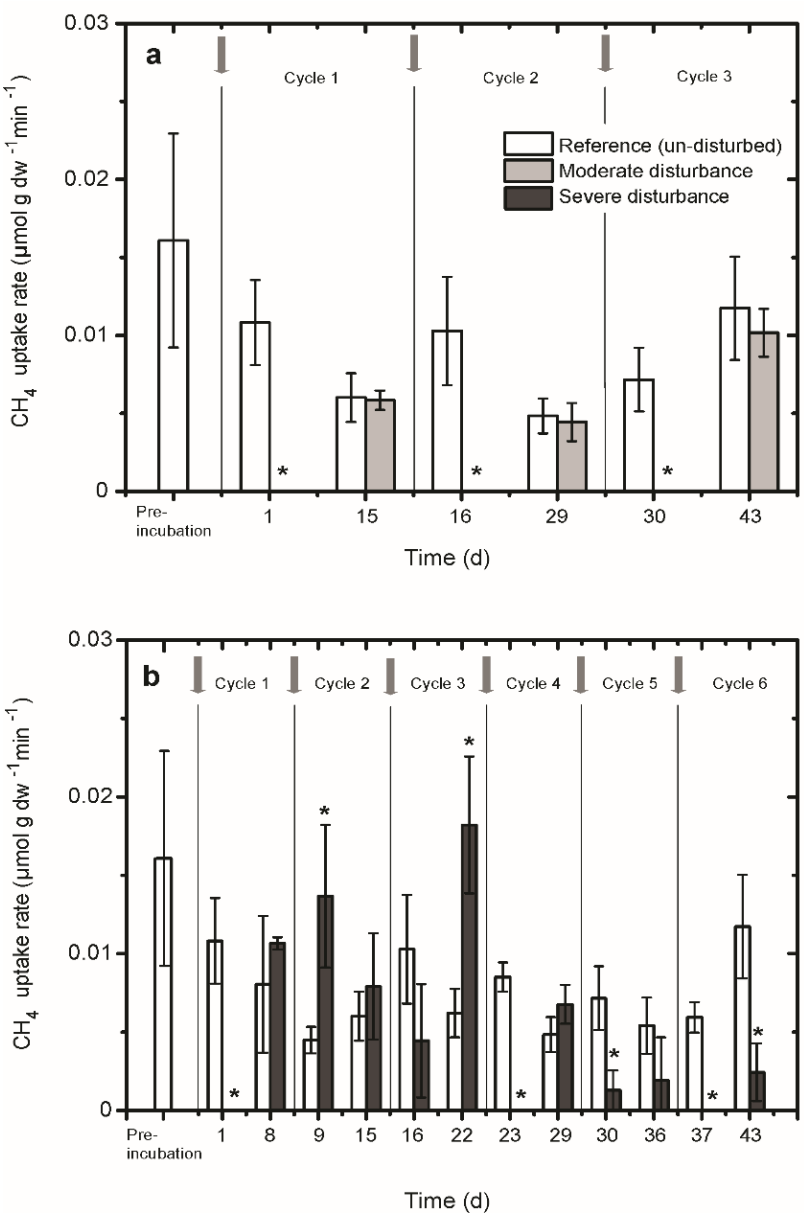


Figure 4: Methane uptake rates determined immediately after desiccation re-wetting (resistance) for each cycle, and after 14 days (3 desiccation-rewetting cycles, moderate disturbance; a) or 7 days (6 desiccation-rewetting cycles, severe disturbance; b) during recovery (resilience). Arrows indicate when desiccation-rewetting was induced. The soil was pre-incubation for 14 days prior to the first desiccation-rewetting cycle. The asterisk indicates the level of significance at $p<0.01$, comparing the methane uptake rates in the un-disturbed (reference) and disturbed microcosms.

On the basis of the quantitative (qPCR) and qualitative (terminal restriction fragment length polymorphism, t-RFLP) analyses, the recovering methanotrophic community was predominantly comprised of *Methylobacter*, whereas other gammaproteobacterial (*Methylocaldum*, *Methylococcus*), as well as alphaproteobacterial (*Methylocystis* and

Methylosinus) methanotrophs were immediately adversely affected after desiccation-rewetting. The rapid recovery of *Methylobacter* was unexpected, given that members of this genus are thought to form relatively less desiccation resistant resting cells that those of *Methylocystis*. Nevertheless, the carbon and nitrogen flush induced by desiccation and subsequent rewetting (i.e., mobilization of nutrients and/or increased nutrient availability from lysed cells; Mikha *et al*., 2005) may have spurred proliferation of the surviving *Methylobacter*, known to be rapid responders to abrupt nutrient availability (Ho *et al*., 2013). Although the alphaproteobacterial methanotrophs were initially affected by the disturbance, population gradually recovered with consecutive desiccation-rewetting cycles, indicating their resilience to the disturbance over time. The differential response of the methanotrophs to recurring disturbances revealed yet unrecognized ecological traits among community members.

While the methanotrophs may recover after sporadic disturbances, their resilience eventually reached a "tipping point" with intensified recurring disturbance, even in the presence of methane availability. It appears that increasing the frequency of the disturbance before recovery from the preceding disturbance has a cumulative effect, compromising the methanotrophic activity and altering the temporal trajectory of population dynamics.

3.3 The resilience of the methanotrophic activity and transcriptionally active community to prolonged drought [Objective 3 (iii); based on Collet *et al*., 2015]

The resistance and resilience of the methanotrophs to simulated prolonged drought were investigated by rewetting air-dried paddy soils that were stored under ambient temperature since 2010 (1 year since sampling), 2006 (5 years), 1998 (13 years), and 1993 (18 years) in microcosm incubations. Astonishingly, methane uptake, as well as the *pmoA* gene transcripts were detected already 2 days after rewetting. Methane uptake rates were comparable in all soils, but values diverged after 3 weeks incubation, whereby the more recently stored soils (since 2006 and 2010) exhibited significantly higher methane uptake than the older soils (since 1993 and 1998), likely caused by population growth under methane availability. Hence, the recovery of the methane uptake rate was affected upon rewetting, which may be related to the change in the surviving methanotrophic community after long term drought.

Noteworthy, the methanotroph diversity (Shanon-Weaver) was lower in the older soils stored since 1998 and 1993, than in the recently stored soils. Among the methanotrophs, alphaproteobacterial ones (*Methylocystis*) were detected at higher relative abundances at < 5 days during the incubation using a diagnostic microarray analysis targeting the *pmoA* gene. This indicates that members of this subgroup were able to form desiccation-resistant spores or resting stages to survive drought, supporting earlier pure culture work (Whittenbury *et al*., 1970; Higgins *et al*., 1981). However, gammaproteobacterial methanotrophs (mainly, *Methylobacter* species) appeared to be early rapid colonizers > 5 days incubation, as indicated by the *pmoA* gene transcript-targeted diagnostic microarray analysis. Because the alphaproteobacterial, rather than the gammaproteobacterial methanotrophs were largely shown to form desiccation-resistant resting stages, gammaproteobacterial methanotrophs were anticipated to deplete over time from the methanotroph seed bank with prolonged drought. In contrast, results indicate that some gammaprotepbacterial methanotrophs belonging to *Methylobacter* were desiccation-resistant. Summarized, prolonged drought (> 13 years) adversely affected the methanotrophic activity, and selected for specific methanotrophs (members of *Methylocystis* and *Methylobacter*) in the seed bank community.

3.4 Edaphic properties more strongly affected the methanotrophic activity than the initial community composition during re-colonization after a simulated disturbance-induced die-off [Objective 3 (iv); based on Kaupper *et al*., 2020a]

Methanotrophic activity is modulated by abiotic (e.g., soil physico-chemical parameters) and biotic (e.g., community composition, diversity) parameters during re-colonization after disturbances (Ho *et al*., 2011; Pan *et al*., 2014; Kaupper *et al*., 2020). Among the abiotic parameters, nutrient availability and space upon disturbance-induced cell lysis and death significantly affected the methanotrophic activity and growth (Ho *et al*., 2011; Pan *et al*., 2014). However, the relative contribution of the abiotic and biotic parameters is often confounded in the soil environment. To disentangle the relative effects of abiotic (collective edaphic properties) and biotic (initial community composition) determinants governing methanotroph re-colonization, and hence growth and activity, a reciprocal inoculation experiment was performed using two different soils with distinct characteristics (paddy and upland agricultural soils) and the gamma-irradiated (sterilized) fraction of these soils (Figure

5). If abiotic determinants more strongly affect methanotrophic activity, the same gamma-irradiated soil (i.e., abiotic environment) will consistently support higher methane uptake regardless of the initial methanotrophic community composition. However, if biotic determinants more strongly affect methanotrophic activity, the same soil (i.e., initial methanotrophic community composition) will consistently support higher methane uptake, regardless of the edaphic properties. Moreover, in the event of no consistent effects, stochastic factors (e.g., legacy or site history, priority effect) may play a role, overriding the effects of abiotic and biotic determinants.

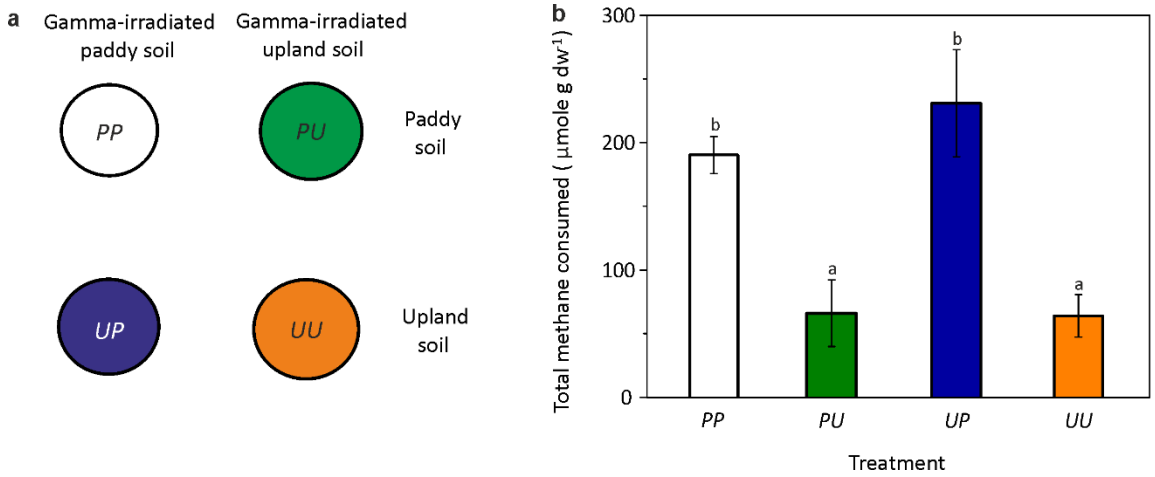


Figure 5: Experimental design showing the reciprocal inoculation of soils in gamma-irradiated fractions of the soils (a), and the total methane consumed during the 35-day incubation of the different treatments (mean ± s.d.; *n* = 3) (b). In (a), the treatments consisted of paddy soil + gamma-irradiated paddy soil (designated as *PP*), paddy soil + gamma-irradiated upland soil (*PU*), upland soil + gamma-irradiated paddy soil (*UP*), and upland soil + gamma-irradiated upland soil (*UU*). In (b), the total methane consumed was determined by integrating the area below the curve of methane uptake rates.

Despite the differences in the initial methanotrophic community composition, methane uptake rate was consistently and appreciably higher in the incubations containing the gamma-irradiated paddy soil compared to the upland agricultural soil (Figure 5). This suggests that the abiotic parameters in the paddy soil more strongly regulated the methanotrophic activity than the initial methanotrophic community composition. The significantly higher methane consumed was not caused by an initial higher methanotroph abundance, as confirmed by the qPCR analysis. However, the initial methanotrophic community composition influences the trajectory of community succession over time, but not

at the expense of the methane uptake rates. Hence, findings support the supposition that methane uptake was primarily regulated by the edaphic properties, while the initial community composition exerted a less pronounced effect on the activity. In light of accumulating evidence indicating the importance of biotic determinants in modulating methane oxidation (see Chapter 5; Stock *et al*., 2013; Change *et al*., 2018; Veraart *et al*., 2018), it is likely that while soil edaphic properties may strongly affect activity at the pioneering stages of re-colonization, biotic determinants may become relevant in established microbial communities.

3.5 Recovery of the methanotrophic activity and community composition after peat excavation and restoration. [Objective 3 (v); based on Reumer *et al*., 2018]

Northern ombrotrophic peatlands are a net carbon sink, but are also a source of methane. Together with other wetlands, peatlands contribute approximately 23% of the total methane budget of 500-600 Tg per annum (Conrad, 2009). Total methane emitted would have been significantly higher if not for the methanotrophs that oxidize methane at the oxic-anoxic interfaces, mitigating the release of the greenhouse gas into the atmosphere (Basiliko *et al*., 2007; Liebner *et al*., 2011). Mining modifies the peat characteristics (e.g., increase pH, remove inorganic compounds such as P, K, and Na; Andersen *et al*., 2006; Basiliko *et al*., 2007) in turn, may adversely affect the methanotrophic activity, causing the loss or reducing the efficiency of the methane bio-filter function. Therefore, harvested peatlands are dammed, allowing peat regrowth as a restoration strategy. With the return of *Sphagnum* during restoration, the potential for methane oxidation and the methanotrophic community composition are anticipated to resemble those in a pristine peatland.

Although peat mining adversely affected the potential for methane oxidation, activity fully recovered, comparing the activity of an actively mined, abandoned, and restored peatlands after 15 years to a pristine site (as reference) in a microcosm study. The trend in the methanotrophic activity was largely reflected in the methanogenic activity, which was significantly higher in the restored site than in the actively mined and abandoned sites, suggesting that methane oxidation was fueled by substrate availability (Andersen *et al*., 2006; Juottonen *et al*., 2015). Correspondingly, the methanotrophic abundance recovered, and the community composition became more similar in the pristine and restored peatlands, showing

the predominance of alphaproteobacterial methanotrophs (*Methylocystis*, *Methylosinus*) thought to be the active members of the methanotrophic community in widespread acidic peatlands (Kip *et al*., 2010; Dedysh, 2011). Although community composition tended to converge, a longer duration (> 15 years) is presumably needed to fully reverse the effects of peat mining on the methanotrophic community.

3.6 Recovery of the methanotrophic activity and community composition after conversion of a tropical rainforest to oil palm plantation in Malaysia [Objective 3 (vi); based on Kaupper *et al*., 2020b; Ho *et al*., 2021]

Malaysia, together with Indonesia are the major palm oil producers globally, contributing approximately 85% of the total palm oil production (Carlson *et al*., 2013; Yan, 2017). The conversion of rainforest mineral soils to oil palm plantations has been associated to a weaker methane sink, and increased nitrous oxide emission after high nitrogen fertilization (i.e., > 200 kg N ha^{-1} $year^{-1}$; Hassler *et al*., 2017; Hewitt *et al*., 2009; Figure 6). Among the abiotic parameters, total C and N is anticipated to decrease with less litter deposition in the converted sites, and acidic soil pH will be neutralized after liming, a common agricultural practice to promote crop growth (Figure 6). A shift in the microbial community composition has been detected after deforestation for oil palm plantation, with an overall decrease in bacterial abundance, in contrast to the documented rise in fungal abundance (Figure 6). Oil palm agriculture-induced shift to the microbial community composition and abundance may also affect the microorganisms involved in methane emission. Given that the oil palm plantations were converted from well-aerated mineral soils with marginal or negligible methane production, methane uptake rates under initial low (< 30 ppmv; "high-affinity" methane oxidation), as well as high (~ 1.3%$_{v/v}$; "low-affinity" methane oxidation) methane concentrations were determined comparing a tropical rainforest to converted oil palm plantations since 2012, 2006, and 1993 (Ho *et al*., 2021).

The potential for "high-affinity" methane oxidation was significantly impaired in the oil palm agricultural soils compared to the tropical rainforest, but rates gradually recovered; the methane uptake rate in the oil palm agricultural soil since 1993 was significantly higher than in the recently converted soil since 2012 (Figure 6). Likewise, the potential for "low-affinity" methane oxidation was adversely affected only in the recently converted oil palm plantations;

rates recovered already after 13 years following conversion (Figure 6). Hence, both "high-affinity" and "low-affinity" methane oxidation exhibited resilience in the face of land-use change.

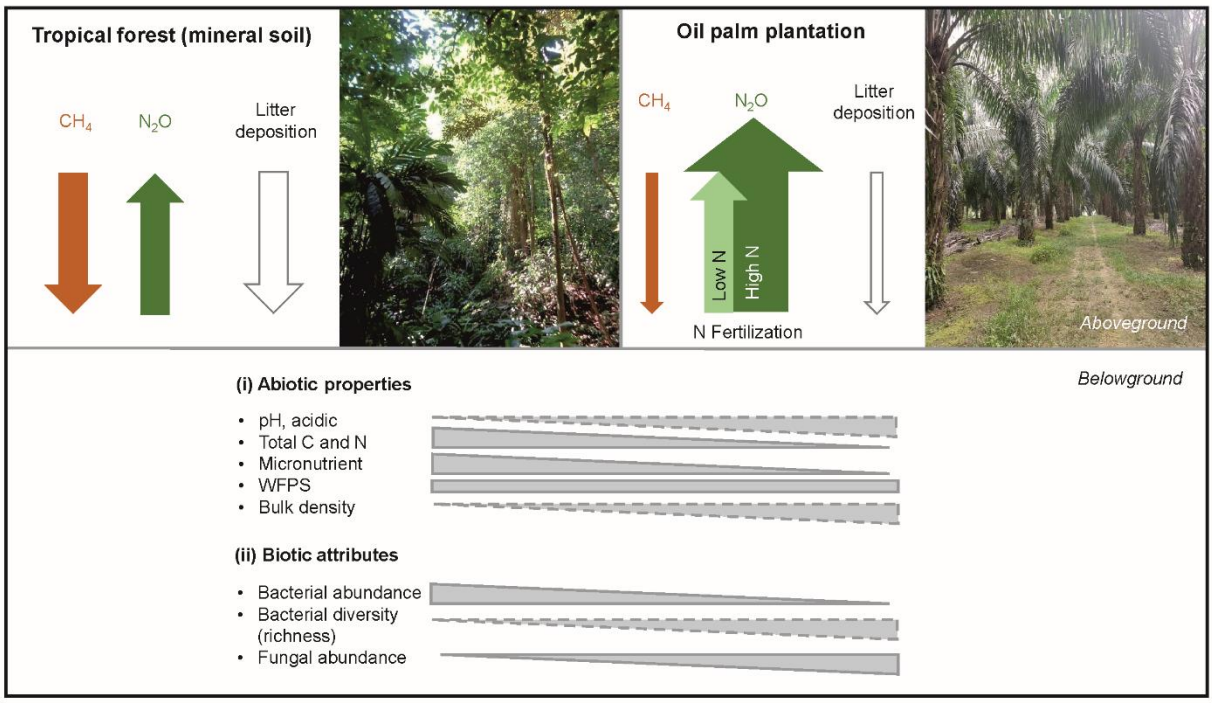


Figure 6: Scheme summarizing the effects of the conversion of a tropical rainforest (mineral soil) to oil palm plantation on the aboveground GHG fluxes and belowground (a)biotic parameters, based on a literature review (Kaupper *et al*., 2020). Orange, green, and white arrows, respectively indicate the magnitude change in the methane and nitrous oxide fluxes, and litter deposition after the conversion of a tropical rainforest to oil palm agriculture. Note that nitrous oxide emission does not appear to be significantly affected at a low nitrogen fertilization rate (< 88 kg N ha^{-1} $year^{-1}$), comparing the fluxes in a tropical rainforest and oil palm plantation. Shifts in the belowground (a)biotic parameters after land conversion are indicated by triangles. Triangles tapered towards the end indicate a decrease (e.g., total C, N, and micronutrients), whereas triangles with dashed lines indicate an increase/decrease or remained unchanged (e.g., pH increased or remained unchanged). Abbreviations: N, nitrogen; WFPS, water-filled pore space.

Considering the methane uptake rates in incubations under low methane concentration (i.e., "high-affinity" methane oxidation, anticipated to be predominant in well-aerated soils) and the methanotroph abundance based on qPCR analysis (assuming one methanotroph harbours two *pmoA* gene copies; Semrau *et al*., 1995), the apparent cell-specific activity was on average higher in the tropical rainforest (5.0 x 10^{-17} mol CH_4 h^{-1} $cell^{-1}$) than in the oil palm plantation (ranged from 1.9 x 10^{-17} to 1.9 x 10^{-18} mol CH_4 h^{-1} $cell^{-1}$) soils. Therefore, although methane uptake rates may recover over time, the amount of methane oxidized per cell was negatively affected with long-term oil palm agriculture.

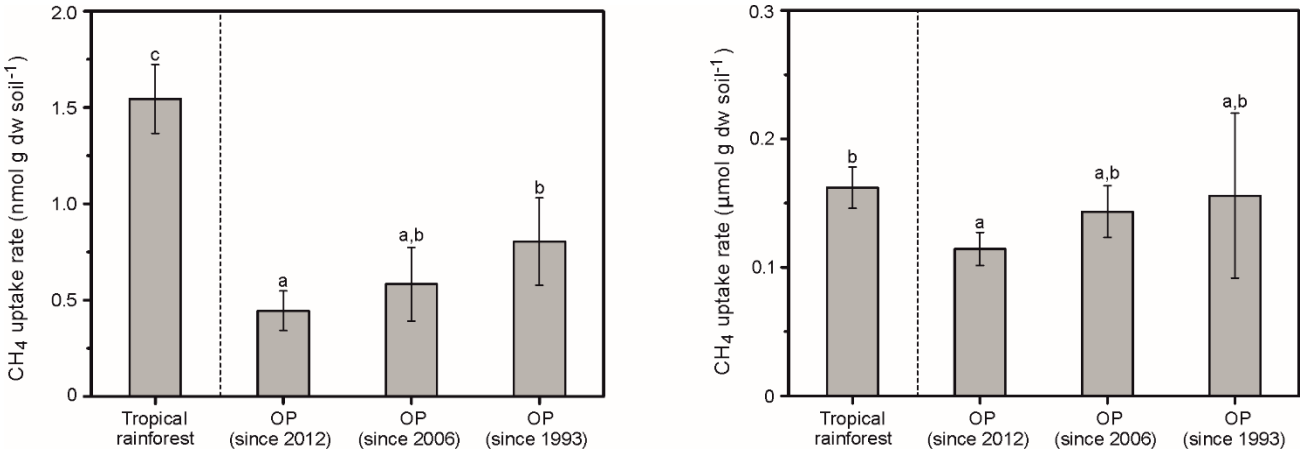


Figure 7: Potential "high-affinity" (left panel) and "low-affinity" (right panel) methane oxidation in a tropical rainforest and oil palm plantations since 2012, 2006, and 1993. Rates were determined from sampling in 2019. Lowercase letters indicate the level of significance at $p<0.05$. Note the different values in the y-axis.

Accordingly, the methanotrophic community composition shifted from unclassified methanotrophs to canonical alphaproteobacterial methanotrophs (*Methylocystis*) and yet-uncultured "high-affinity" methanotrophs (e.g., tropical upland soil cluster, upland soil cluster-alpha) with on-going oil palm agriculture. The methanotrophic community composition also appeared to be relatively stable within each site (with the exception of the oil palm plantation since 1993), exhibiting little temporal variation between the sampling times (January, 2019 and 2020). Although the trend in methane uptake indicates potential recovery with long-term oil palm agriculture, the methanotrophic community composition was soon altered (< 7 years) after the change in land-use, but remained relatively stable thereafter.

3.7 Changes in the methanotrophic activity and community composition after desertification-induced lake shrinkage, and resistance to salt stress [Objective 3 (vii); based on Ho *et al*., 2018; Mo *et al*., 2020]

Exacerbated lake shrinkage in (semi-)arid regions could be associated to desertification, attributable to the changing climate (e.g., reduced precipitation, increasing temperatures). Because of increased evapotranspiration, concentrated solutes in the soil can potentially induce salt stress, affecting the methanotrophs. Changes in the potential for methane oxidation and the composition of the methanotrophs were characterized along a transect of a shrinking lake induced by desertification (Lake Dali, China, ~81% of the total area since 1972), covering the saline lake sediment, saline riparian soil (transition zone), and a grassland soil (Mo *et al*., 2020), before explicitly determining the resistance of the methanotrophs to short-term salt stress (Ho *et al*., 2018).

Methane uptake rates were significantly different in all sites, with the values documented in the following order, saline riparian soil > saline lake sediment > grassland soil. Notably, methane uptake rate in the saline lake sediment (~50 μg g sediment^{-1} d^{-1}) was appreciably lower than in other freshwater lake sediments (section 3.1; ~155 μg g sediment^{-1} d^{-1} in Lake Constance, Germany and ~ 290 μg g sediment^{-1} d^{-1} in Lake Neusiedl, Austria), suggesting a lower potential for methane oxidation with higher salinity. Based on SIP using ^{13}C-methane, the predominantly (> 50% of the total community) active methanotrophs were distinct in all sites, albeit belonging to the same subgroup (gammaproteobacteria). While the predominant active methanotrophs in the saline lake sediment were affiliated to *Methylomonas*, the saline riparian and grassland soil harbored a *Methylomicrobium*- and *Methylobacter*-dominated community, respectively. Alphaproteobacterial methanotrophs (mainly, *Methylocystis*) represented only a minor overall fraction (< 5%) of the active community in all sites. The detection of distinct active community members along the transect indicates niche differentiation among the gammaproteobacterial methanotrophs, as community diverges over time. Presumably, these methanotrops were favored by the saline condition, consistent with previous findings (e.g., mangroves, alkaline lakes, estuaries; Deng *et al*., 2017; Osudar *et al*., 2017; Shiau *et al*., 2018), and a salt-tolerant community may emerge with gradually increasingly salinity in the long-term.

Furthermore, to determine the resistance of the methanotrophs to salt stress in the short-term (i.e., a salt-tolerant community would unlikely to develop within days), microcosm incubations using a rice paddy soil in increasing NaCl concentrations (i.e., 0 M as reference, and from 0.005 M to 0.6 M, seawater salinity) were performed. Methane uptake rate was not significantly affected at < 0.3 M NaCl, but activity was completely inhibited at 0.6 M NaCl. The trend in methane uptake was largely reflected in the abundances of the gamma-and alpha-proteobacterial methanotrophs, in that, *pmoA* gene abundances were significantly lower after incubation at 0.6 M NaCl. Noteworthy, the gammaproteobacterial methanotrophs were more responsive to salt stress, significantly increasing (type Ia-specific qPCR assay) or decreasing (type Ib-specific qPCR assay) in abundances at increasing NaCl concentrations up to 0.3 M, while alphaproteobacterial methanotroph abundance remained relatively unchanged at < 0.3 M NaCl. Sequence analysis revealed that *Methylobacter* and other yet-uncultured type Ia-related gammaproteobacterial methanotrophs were favored under salinity at < 0.3 M NaCl.

Although methanotrophic activity was sustained in the saline sediment and riparian soil, likely resulting from the selection and/or adaption of a salt-tolerant community in the long-term (Mo *et al*., 2020), activity can be adversely affected when exposed to high salinity levels in the short-term (Ho *et al*., 2018). Still, the methanotrophs were resistant to moderate salinity levels up to 0.3 M NaCl. In both studies, it is clear that specific gammaproteobacterial methanotrophs (e.g., *Methylobacter*, *Methylomonas*, *Methylomicrobium*) were favored under saline conditions.

3.8 Conclusion

The recovery of microbially-mediated processes and associated microorganisms from disturbances can be attributable to prior exposure to the disturbance, but specifically for the methanotrophs, results indicate the marginal role of site history in conferring resilience to contemporary disturbances (section 3.1). Nevertheless, prior disturbances likely selected for a reservoir of (seed bank) community members that were resistant, or were even favored by the disturbance. Accordingly, the recovery of the methanotrophic activity was deterministically governed by edaphic factors, rather than the composition of the methanotrophic community (section 3.4). Although the methanotrophs were resilient, recovering from sporadic disturbances given sufficient time and substrate availability, results

showed that their resilience was challenged in the face of intensified recurring and prolonged disturbances; following these disturbances, methanotrophic activity did not fully recovered, and the disturbances induced a compositional shift in the recovered community (sections 3.2 & 3.3; Figure 8). Likewise, the methanotrophic activity was significantly impaired by compounded disturbances as anticipated during the change in land-use (Figure 8). The methane uptake rates recovered after 15 years of restoration following peat mining, but the community composition had not fully recovered (section 3.5). Although the potential for "high-affinity" methane oxidation gradually increased with on-going oil palm agriculture, rates were still significantly lower than in the reference site (tropical rainforest) after almost three decades of land conversion (section 3.6). Salt stress can adversely affect the potential for methane oxidation in the short-term, but a salt stress-tolerant methanotrophic community is likely selected over time during gradual desertification-induced increase in lake salinity, sustaining methane uptake (section 3.7). The differential response of specific methanotrophic genera to the induced disturbances (sections 3.1 – 3.7) suggests niche differentiation among community members, with the gammaproteobacterial methanotrophs that survived the disturbance can be regarded as rapid colonizers (see section 1.3). Summarized, the resilience of the methanotrophic activity may reach a "tipping point" in the event of multiple or compounded disturbances, and when disturbance intensified and persisted. As in sections 3.2 and 3.3, the shift in the methanotrophic community composition immediately post-disturbance (sections 3.5 & 3.7) may alter the collective traits of the community, with consequences for community functioning under fluctuating environmental conditions.

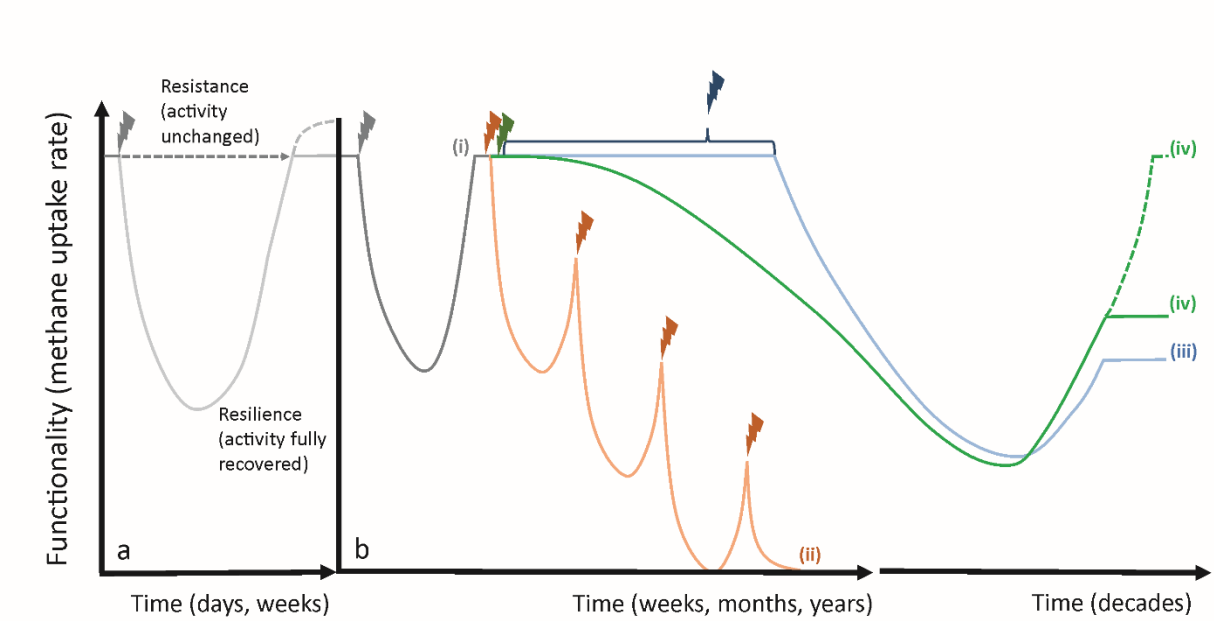


Figure 8: The effects of sporadic (a) and recurring (i, grey line; ii, orange line), prolonged (iii, blue line), and compounded (iv, green line; b) disturbances on the methanotrophic activity. These projections are based on research findings in sections 3.1 through 3.6. Note that in many cases, the recovery in methane uptake rates is not reflected in the recovery of the methanotrophic community composition, indicating redundancy among the community members. Given sufficient recovery time under substrate (methane and oxygen) availability, methanotrophic activity will recover (light grey line), or even over-compensate for initial activity loss (dashed light grey line) likely attributable to higher nutrient and space availability (derived from disturbance-induced cell lysis and death) after sporadic disturbances (a). In (b), prior exposure to a disturbance may select for a seed bank community resistant to the disturbance for future contingencies. Hence, upon exposure to the same disturbance, activity will fully recover, and may even be less adversely affected (i, grey line; b). Without allowing a full recovery from prior disturbances, the methanotrophic activity eventually reached a "tipping point", and thereafter, activity no longer recover, with intensified recurring disturbance (ii, orange line). Following prolonged disturbances (iii, blue line), methanotrophic activity was profoundly altered, and did not recover to pre-disturbance levels. Likewise, compounded disturbances (iv, green line) as expected under land-use change scenarios (i.e., peat mining, deforestation for oil palm agriculture) significantly impaired the methanotrophic activity (particularly, "high-affinity" methane oxidation), but activity may return requiring extended recovery time spanning over decades (iv, dashed green line).

Chapter 4

The Role of Aerobic Methanotrophs in Climate-Smart Agriculture

Vredepeel agricultural field, Wageningen University and Research, the Netherlands, and flux chambers (inset) by Adrian Ho

4 The role of aerobic methanotrophs in climate-smart agriculture

4.1 Aerobic methanotrophs in upland agricultural soils

The increasing demand for food and feed to support the growing human population necessitates a shift towards sustainable circular economy, which entails in the re-investment of bio-based residues to agricultural lands. However, the incorporation of bio-based residues in agricultural practices may have undesirable side effects, including the production of primary GHG by stimulating the soil indigenous microorganisms and/or the addition of residue-derived microorganisms into the soil (section 4.1). Consequently, the impact of bio-based residue input in agricultural soils and the effects on the soil chemistry influencing crop growth, have led to the need to devise a comprehensive organic amendment climate-smart strategy that can attenuate agriculture-related GHG emissions, while maintaining soil fertility and crop yield (Paustian *et al*., 2016; sections 4.2 – 4.4). *Vice verse*, the restoration of former agricultural lands to semi-natural state may reduce GHG emissions (Nazaries *et al*., 2013; McDaniel *et al*., 2019; El-Hawwary *et al*., 2022; section 4.5). GHG turnover processes which lead to emissions in agricultural soils are largely catalyzed by soil microorganisms (Liesack *et al*., 2000; Yvon-Durocher *et al*., 2014; Yoon *et al*., 2019; Kaupper *et al*., 2020).

Particularly, methane is a potent greenhouse gas, and accounts for up to 17% of total global warming (IPCC, 2019). Understanding the sources and sinks of atmospheric methane is thus crucial to devise methane mitigation strategies when applying bio-based residues in agricultural soils. Indeed, agricultural practices (e.g., organic and inorganic fertilization regime, agriculture intensification) modifies the soil physico-chemical properties and nutrient turnover, adversely affecting the methane sink function, and the methanotrophs (Levine *et al*., 2011; Tate *et al*., 2015; Meyer *et al*., 2017). In contrast to being methane biofilters at oxic-anoxic interfaces in high methane-emitting environments (i.e., “low-affinity” methane oxidation), the “high-affinity” methanotrophs are methane sinks in well-aerated upland agricultural soils, consuming methane at (circum-)atmospheric concentrations (< 40 ppm_v; Singh *et al*., 2010; section 4.4). Whether an upland agricultural soil becomes a methane source or sink is more dependent on the activity of the methanotrophs, rather than the methanogenic archaea (Meyer *et al*., 2017). Hence, the methanotrophs are the focus of this chapter. The objectives were:

(i) To determine whether manure-induced methane production in agricultural soils is caused by a stimulation of soil-borne methanogens and/or manure-derived methanogens.

(ii) To resolve the effects of ammonium as anticipated under increasing fertilization regime, on the methanotrophic activity and community composition.

(iii) To determine the impact of bio-based residue addition on the GHG emissions, microbial communities, and crop yield, with emphasis on methanotrophy.

(iv) To determine the metabolically active "high-affinity" methanotrophs after bio-based residue addition in agricultural soils, as revealed by phospholipid fatty acid (PLFA)-based SIP.

(v) To determine GHG emissions after agriculture abandonment.

4.2 Manure-induced stimulation of soil-borne methanogens and methane production in agricultural soils [Objective 4 (i); based on Ho *et al*., 2015b]

Manure is an N-rich by-product of livestock farming, and has been attributed to higher methane production after application in agricultural soils (Steed & Hashimoto, 1994; Kim *et al*, 2016). However, it remains unclear whether increased methane production was caused by stimulated soil-borne methanogens upon substrate (i.e., manure) availability and/or increased manure-derived methanogens. Here, the relative contribution of soil-borne and manure-derived methanogens to methane production was determined using an upland and a wetland agricultural soil in a series of microcosm incubations consisting of (i) manure + soil, (ii) sterilized manure + soil, and (iii) manure + sterilized soil at 10%, 20%, and 40% manure application rates, as well as microcosms containing solely (iv) manure and (v) soil as reference incubations. During the incubation, the methane production rate was determined, and the methanogens were enumerated by targeting the *mcrA* gene (encoding for the methyl coenzyme M reductase) as proxy for the methanogenic abundance.

Methane production rate was significantly higher in microcosms containing manure + soil and sterilized manure + soil relative to the microcosms containing manure + sterilized soil, whereas methane production rate was significantly lower in the reference microcosm containing only soil (Figure 9). Considering that the presence of manure (both sterilized and un-sterilized) in the soil induced significantly higher methane production rates than in the

microcosm containing manure + sterilized soil, the methanogens indigenous to the soil was thus significantly stimulated by manure addition. Accordingly, the methane production rate was significantly correlated ($p<0.005$, linear regression) to the *mcrA* gene abundance, indicating growth of the methanogenic population. However, the stimulatory effect did not correspond to the application rate of the manure; further manure input (from 10% to 40%) did not proportionally increase methane production rate (Figure 9). Possibly, methane production was restricted by the population size of the methanogens in the soil.

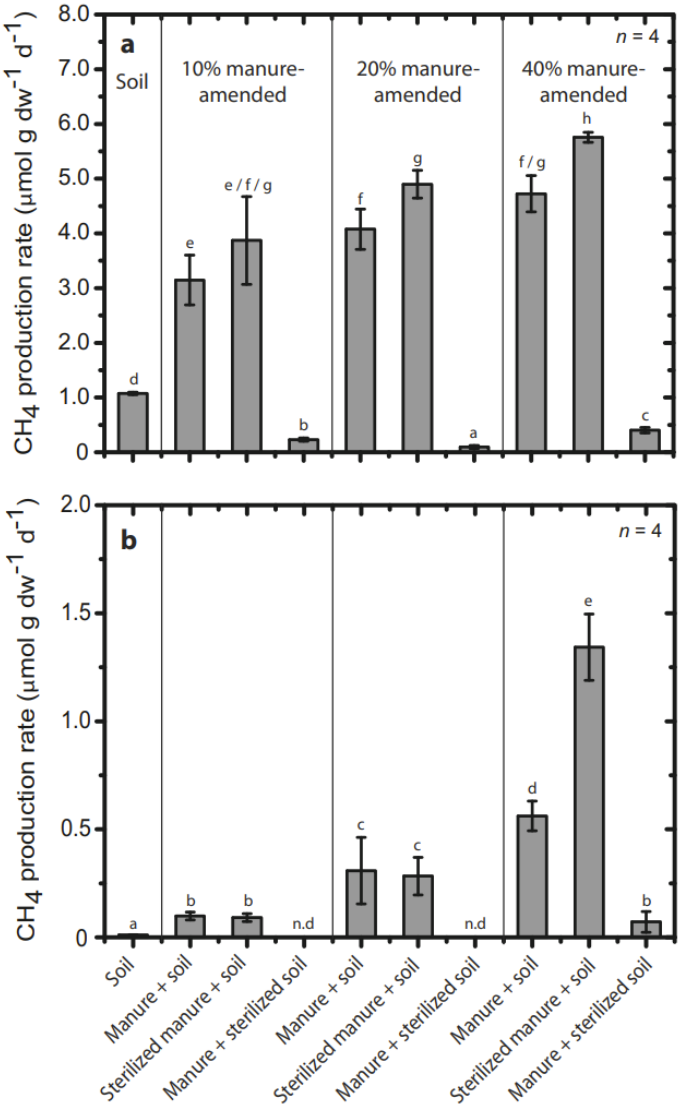


Figure 9: Methane production rate in microcosms containing soil (reference), manure + soil, sterilized manure + soil, and manure + sterilized soil at 10%, 20%, and 40% manure application rates in wetland (a) and well-aerated upland (b) agricultural soils (n=3). Microcosms containing only manure and

autoclaved manure (sterility check) are given in Ho et al. (2015b). The letters indicate the level of significance (ANOVA) at $p < 0.05$ between treatments. Abbreviation; n.d., not detected.

Manure promoted the soil-borne methanogens, resulting in significantly higher methane production. Although previous work inferred that higher methane emissions may be attributable to the addition of methanogens *via* manure input in agricultural soils (Radl *et al*., 2007; Gattinger *et al*., 2007), this study showed that the methanogens seeded from the manure contributed only marginally to methane production, at least in the short-term. The manure-derived methanogens showed no incipient activity in the soil. Generally, this study points to the relevance of the physico-chemical properties of a bio-based residue when considering methane mitigation strategies in agrosystems.

4.3 Soluble ammonium, rather than the total (exchangeable) ammonium concentrations, determines ammonium-induced effects on the methanotrophic activity and community composition [Objective 4 (ii); based on van Dijk *et al*., 2021]

Ammonium-based fertilizers are commonly applied in agricultural systems. However, nitrogen use efficiency is low, with nitrogen recovery in crops at < 50% worldwide (Fageria & Bligar, 2005). Ammonium may be loss or rendered inaccessible *via* leaching, volatilization at higher pH, adsorption to soil and biological consumption by soil microorganisms (King & Schnell, 1998; Fageria & Bligar, 2005; Ho *et al*., 2018). Particularly, the effects of ammonium on soil methane uptake and the methanotrophs remain contentious.

Ammonium has been documented to both stimulate and inhibit methane uptake in agricultural soils, or have no apparent effects (Bodelier & Laanbroek, 2004; Noll *et al*., 2008; Alam & Jia, 2012; Krause *et al*., 2012). The seemingly contrasting response of methane uptake to ammonium has been attributed to the application rate and mineral forms of ammonium, as well as the methanotrophic community composition (references within van Dijk *et al*., 2021), but may also be explained by the difference in the total (exchangeable) and soluble (i.e., bioavailable) ammonium fractions. Ammonium (cation) is readily adsorbed to negatively charged soil particles and organic matter (King &Schnell, 1998; Ho *et al*., 2018), and becomes inaccessible to the soil microorganisms. Hence, considering the total (exchangeable)

ammonium concentration may not reflect on the ammonium-induced effects on microbially-mediated soil processes.

The effects of ammonium on the methanotrophic activity and community composition were resolved in a microcosm study, comparing soil incubations with a 1000-fold (i.e., relatively less ammonium adsorption sites) and 10-fold (i.e., relatively more ammonium adsorption sites) dilutions, after pre-incubation to allow comparable starting methanotroph abundances. Ammonium was supplemented as NH_4Cl at 0.5 – 4.75 g L^{-1} (Treatment, T1 to T6; Figure 10). The soluble ammonium fraction was considered to be accessible to the soil microorganisms (i.e, bioavailable), while the exchangeable ammonium as determine in 2 M KCl, was regarded as the total ammonium (i.e., adsorbed and soluble fractions; for details, see van Dijk *et al*., 2021).

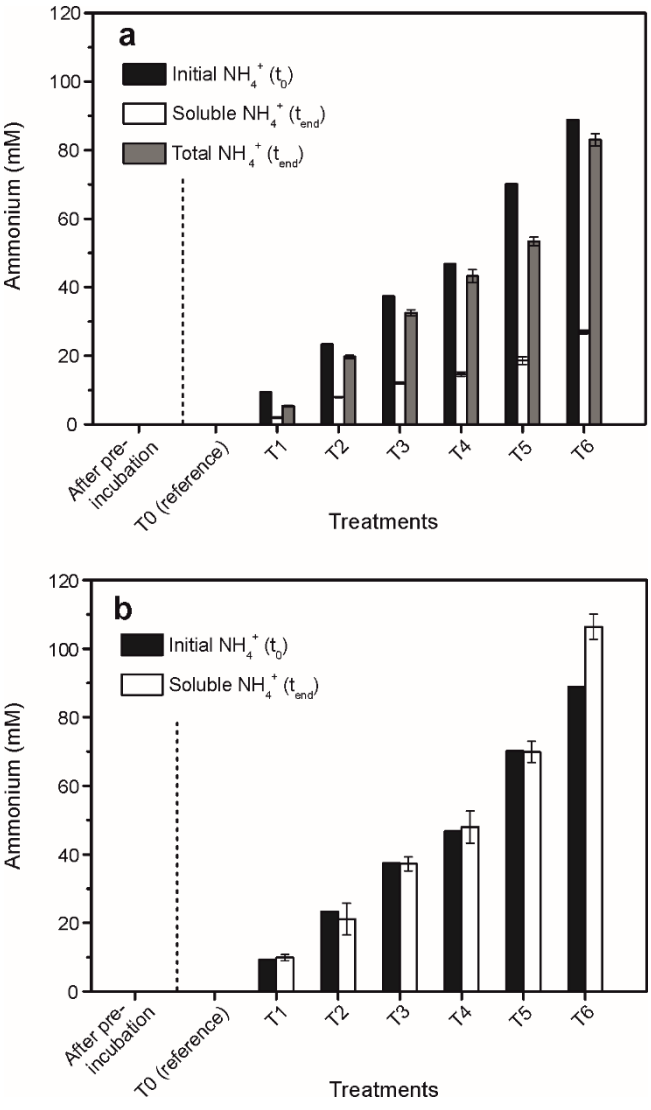


Figure 10: Total (exchangeable) and soluble ammonium concentrations in the 10-fold (a) and 1000-fold (b) diluted soil suspension incubations (n=3). The initial ammonium concentration (t_0) was calculated based on the NH_4Cl supplemented. Ammonium was measured after the incubation (t_{end}) for all treatments (T0, un-amended reference; T1, 0.50 g L^{-1}; T2, 1.25 g L^{-1}; T3, 2.00 g L^{-1}; T4, 2.50 g L^{-1}; T5, 3.25 g L^{-1}; T6, 4.75 g L^{-1}). Ammonium was below the detection limit of the colorimetric assay (< 0.02 mM). In (a), the total ammonium concentration was significantly higher ($p<0.05$) than the soluble fraction in all treatments after incubation. In (b), only soluble ammonium was determined because the soil was no longer visible after a 1000-fold dilution.

Ammonium significantly stimulated the methanotrophic activity at all supplemented concentrations in the incubations with 10-fold diluted soil suspension, whereas methane uptake exhibited a dose-dependent effect in the incubation with 1000-fold dilution, where methane uptake was significantly stimulated at < 2.5 g L^{-1}. Notably, the trend in methane uptake in both incubations could be explained by the soluble ammonium concentration, which was proportionate to the supplemented ammonium in the 1000-fold diluted soil suspension, but was appreciably lower in the incubation with 10-fold dilution (Figure 10). Here, soluble ammonium was < 1.6 g L^{-1}, regardless of the supplemented amount of ammonium. Approximately 36-63% of the supplemented ammonium was determined to be adsorbed to the soil, comparing the initial ammonium concentration to the total and soluble fractions after the incubation (Figure 10a). Therefore, the ammonium-induced effect was influenced by the ion exchange capacity (adsorption/desorption) of the ammonium in the soil.

Methylosarcina, a gammaproteobacterial methanotroph, predominated the community in both incubations supplemented with > 2.50 g NH_4Cl L^{-1}, indicating the relevance of this methanotroph at higher ammonium levels. This is in agreement with previous work showing that the gammaproteobacterial methanotrophs (e.g., *Methylobacter*, *Methylomicrobium*), including *Methylosarcina* were generally more responsive to abrupt ammonium availability than the alphaproteobacterial methanotrophs (Noll *et al*., 2008; Qiu *et al*., 2008; Ho *et al*., 2020). Collectively, this chapter emphasizes the importance of considering the soluble or bioavailable fraction, rather than the total ammonium concentrations (and by extension, other cations) when determining ammonium-induced effects on the methanotrophs.

4.4 Bio-based residue amendments significantly stimulated the methane sink function in upland agricultural soils, based on mesocosm studies [Objective 4 (iii); based on Ho *et al*., 2015c; 2017b]

In contrast to rice paddies (wetland agricultural soil), the methanotrophic potential in well-aerated upland agricultural soils had received little attention, probably due to the low or even negligible methane uptake capacity when compared to relatively un-disturbed upland soils (Table 1; Ho *et al*., 2015c). Here, the response of methane emissions, as well as the other primary GHG (carbon dioxide and nitrous oxide) to the application of locally-sourced bio-

based residues with a wide C:N range (5.5 – 28.0), including sewage sludge, aquatic plant material, compost, wood material, compressed beet leaves, and paper pulp (listed in the order of increasing C:N ratio) were determined using two upland agricultural soils (sandy loam and clay) to screen for suitable organic residues that exert a low global warming potential (GWP) and promote crop yield.

Table 1: Methane flux and methane uptake rate of well-aerated un-disturbed and agricultural soils from diverse environments, as summarized in Ho *et al*. (2015c).

Soils	**Methane flux ($\mu mol\ m^{-2}\ h^{-1}$)#**	**Methane uptake rates ($nmol\ g\ dw^{-1}\ h^{-1}$)***	**Reference**
Un-disturbed soil.	-2 to -10	0.1 to 0.7	Literature review in Ho *et al*. (2015c)
Agricultural soils.	-0.1 to -4.5	0.002 to 0.02	Literature review in Ho *et al*. (2015c)
Agricultural soils (un-amended).	-10 to + 7.2	0.03 to 0.07	Ho *et al*. (2015c)
Bio-based residue-amended agricultural soils.	< -24	0.04 to 0.1	Ho *et al*. (2015c)

#Flux range (min – max) determined from a literature review (Ho *et al*., 2015c).

*Initial methane concentration to determine soil methane uptake; ambient air or circum-atmospheric methane levels (< 40 ppm_v).

Among the bio-based residues, the addition of N-rich materials such as sewage sludge and aquatic plant material yielded significantly higher crop biomass compared to un-amended soils, but also imposed appreciably higher GWP, mainly as a result of heightened nitrous oxide emission. On the other hand, compost imposed the least GWP comparable or even lower than the un-amended soil, with little modification to the composition of the soil bacterial community and fungal abundance (Figure 11; Ho *et al*., 2017b). However, the addition of compost had no apparent effect on crop production, but may benefit other aspects of soil

functioning (e.g., longer carbon sequestration in soil; Ryals *et al*., 2015). Also, compost addition unexpectedly suppressed methane emission, albeit in the short-term (< 2 months incubation). As methane emission is a balance of methane production and oxidation, a further methane oxidation assay was performed showing that the increased methane sink function was the result of significantly higher methane uptake rates rather than decreased methane production, following compost addition. Therefore, compost transiently stimulated the methane uptake rates in agricultural soils, likely caused by induced cell-specific activity given that the *pmoA* gene copy numbers (proxy for methanotroph abundance) remained relatively constant during the incubation. Although transient, the stimulated methane uptake offset up to 16% of the total carbon dioxide emitted. The agricultural soils may thus benefit from repeated compost amendments to further reduce the GWP, and compensate for the loss of the methane sink function after land conversion. Evidently, higher crop yield was trade-off for lower GWP, but the carbon dioxide offset by increased methane uptake suggests that crop productivity can be improved considering compost addition complemented with other N-rich soil additives at optimal combinations to minimize overall GHG emissions.

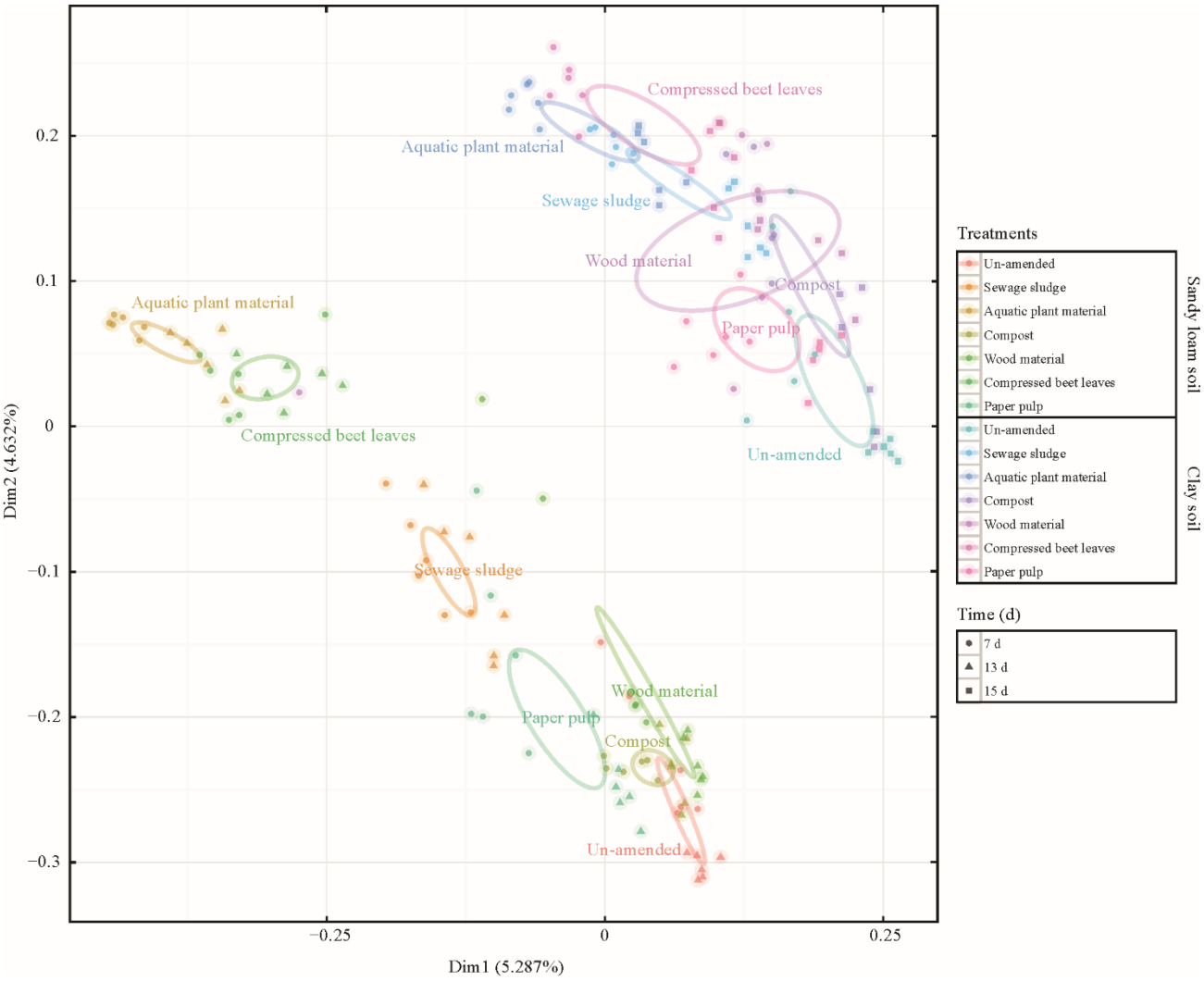


Figure 11: Principal coordinate analysis (PCoA) showing the effects of bio-based residue addition on the bacterial community composition in a sandy loam and clay agricultural soil, based on the 16S rRNA gene sequencing analysis (IonTorrent™ sequencing; primer pair, 515F/806R). The bacterial community composition was distinct in both soils, with the community in the un-amended and compost-amended soils consistently clustered closely together. The ellipse shows 95% confidence interval of the standard errors. The symbols depict the incubation days (circle, day 7; triangle, day 13; square, day 15).

With distinct bacterial communities in the sandy loam and clay soils even after residue addition (Figure 11), soil properties appear to be key drivers shaping the soil microbial community, which take precedence over residue-induced effects. Noteworthy, residue type rather than the C:N ratio more strongly affected the decomposition of the residues, as shown in a litter bag assay determining decomposition by weight loss and microbial respiration (carbon dioxide flux). This is in contrast to previous assumption that the C:N ratio is indicative

of decomposability (Cayuela *et al*., 2010). More comprehensive approaches (Brenzinger *et al*., 2021), considering GHG emissions, carbon retention, nutritional status, crop yield, and plant disease suppression, among other desirable attributes, are relevant when assessing climate-smart agriculture management practices in future.

4.5 Canonical "low-affinity" methanotrophs are responsible for the methane sink function in well-aerated upland agricultural soils [Objective 4 (iv); based on Ho *et al*., 2019]

Further confirmation for the stimulatory effect of compost on soil methane uptake rate was documented in an independent study using the same sandy loam and clay soils (as in Section 4.3) sampled 1-2 years apart. In this study, ^{13}C-methane was used in a phospholipid fatty acid (PLFA)-based SIP to detect the metabolically active "high-affinity" methanotrophs. Because well-aerated upland agricultural soils are anticipated to be methane sinks (Table 1), incubations were performed at circum-atmospheric concentrations (< 40 ppmv ^{13}C-methane; Singh *et al*., 2010), and the ^{13}C-enriched PLFA was subsequently retrieved. A more rapid incorporation of the ^{13}C into the PLFA than in nucleic acids (DNA or RNA) makes the PLFA-based SIP a suitable technique to detect the "high-affinity" methanotrophs.

Soil methane uptake was sustained throughout the incubation (130 days), albeit the stimulatory effect of compost addition was detected at < 50 days. Methane uptake was concomitant to the accumulation of headspace ^{13}C-carbon dioxide, confirming the oxidation of ^{13}C-methane. Interestingly, the ^{13}C-enriched PLFA profile was distinct in both soils and could be unambiguously assigned to conventional "low-affinity" alphaproteobacterial methanotrophs affiliated to *Methyloferulla*, *Methylocystis*, and *Methylosinus*. Sequencing of the 16S rRNA and *pmoA* genes excluded the presence of the as-yet-uncultured "high-affinity" methanotrophs in these soils.

Although canonical "low-affinity", and "high-affinity" methanotrophs have frequently been co-detected, the role of the canonical methanotrophs as a methane sink in upland soils remains enigmatic until recently. This study, along with others (Cai *et al*., 2016; Pratscher *et al*., 2018; Tveit *et al*., 2019), revealed that the supposedly "low-affinity" methanotrophs are capable of "high-affinity" methane oxidation under specific conditions and thus, represent an overlooked reservoir of canonical methanotrophs mediating (circum-)atmospheric methane uptake in agricultural soils.

4.6 Reversion of GHG emission trends after the abandonment of agriculture [Objective 4 (v); based on El-Hawwary *et al*., 2022]

While the conversion of pristine to agricultural lands and the introduction of agricultural management practices induce GHG emissions, the abandonment of agriculture and reversion to a semi-natural state may reduce the GHG footprint (Nazaries *et al*., 2013; McDaniel *et al*., 2019). GHG emissions were monitored along a chronosequence of abandoned agricultural soils spanning from 9 to 32 years to be compared to an on-going agricultural soil. Soil incubations without (reference) and with supplemented manure were performed in a mesocosm-scale study. The recovery of GHG emissions after agriculture abandonment was determined in the reference incubations, whereas the legacy of agriculture (i.e., capacity to emit GHG that is still present in the soils after abandonment) was assessed by determining the response of GHG emissions after contemporary manure addition.

The agricultural soil is a methane source, in contrast to the abandoned sites which are methane sinks (Figure 12). Following manure input, methane emission appreciably increased (9-10 folds) in the agricultural soil, and the methane sink function in the abandoned sites were impaired, with no apparent effect of time since abandonment (Figure 12). On the other hand, carbon dioxide emission significantly increased, more pronounced < 24 years after agriculture cessation compared to the sites abandoned for a longer time (> 29 years), suggesting a shift in soil respiration. Expectedly, carbon dioxide emissions appreciably increased in all sites after manure input, generally following the trend in the soils without manure whereby the recently abandoned soils emitted significantly higher carbon dioxide that the foremost abandoned soils. This, together with the trend shown in a litter bag assay (i.e., manure weight loss in the abandoned sites) indicate a relatively higher C mineralization up to 24 years after agriculture abandonment.

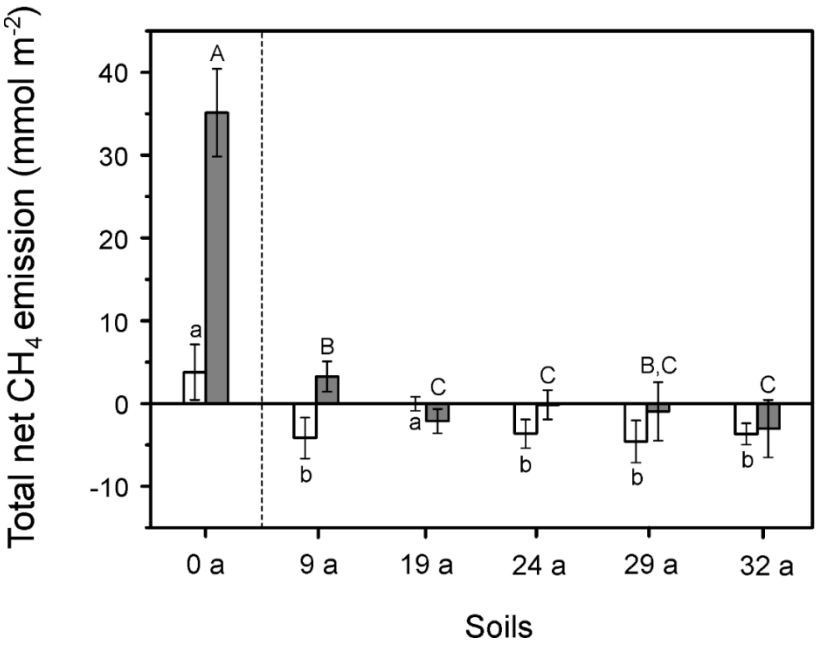


Figure 12: Total methane emissions over 56 days incubation in the un-amended (blank bars) and manure-amended (grey bars) soils with on-going agriculture (0 a), and 9, 19, 24, 29, and 32 years after agriculture abandonment. Lower and upper case letters indicate the level of significance (ANOVA; $p<0.05$) among the soils without and with manure input, respectively.

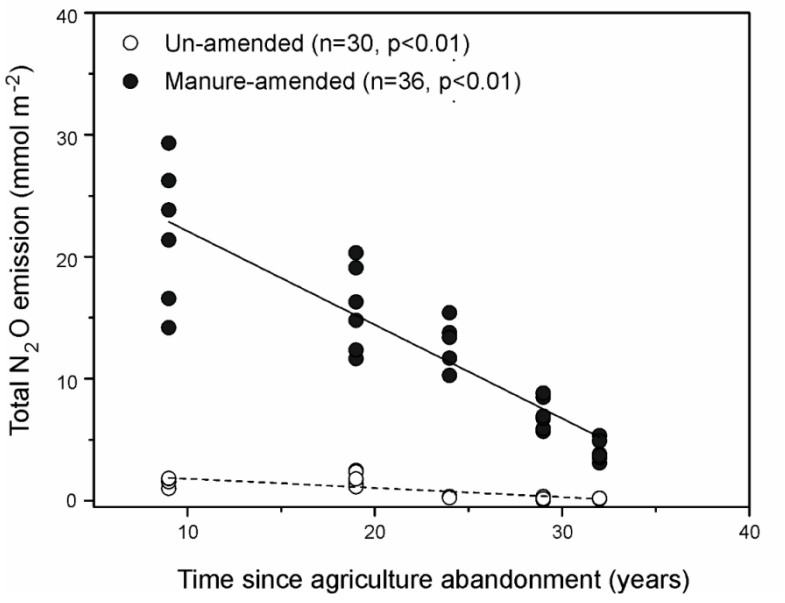


Figure 13: The relationship between total nitrous oxide emission (56 days incubation) and years after agriculture abandonment in the soils without (open circles) and with (black circles) manure addition. The linear regression showed significant correlation ($p<0.05$) between the two variables, considering all replicate.

Astonishingly, total nitrous oxide emission exhibited a monotonic decrease over time since abandonment in both the soils with and without supplemented manure, reflecting on a modified capacity for (de)nitrification (Figure 13). In particular, the loss of the ammonium-oxidizing archaea, and a weakened response in the denitrifier abundances (i.e., based on the *nirS*, *nirK*, and *nosZ* genes) to manure addition were documented over time since agriculture abandonment. Therefore, the capacity for GHG emissions significantly diminishes over time, with an overall lower GWP after agriculture abandonment.

4.7 Conclusion

Summarized, this chapter demonstrated that N-fertilization in the form of manure stimulated the soil-borne methanogens, significantly increasing methane production in agricultural soils (section 4.1). Generally, ammonium-induced effects on microbially-mediated processes, including methane uptake in agricultural soils may be more accurately captured, determining the effects of the soluble, rather than the total (exchangeable) ammonium fraction (section 4.2). Although manure input increased methane production, the application of other bio-based residues such as compost may exert an opposing effect by stimulating the methane uptake rate to levels even exceeding undisturbed or pristine environments (section 4.3; Table 1; Figure 14). This is surprising given that upland agricultural soils were expected to contribute marginally to atmospheric methane consumption because of the vulnerability of the “high-affinity” methanotrophs to perturbation. Interestingly, the active methanotrophs shown to oxidize methane at (circum-)atmospheric levels in agricultural soils were canonical “low-affinity” alphaproteobacterial methanotrophs (section 4.4). These findings suggest that the choice of bio-based residues is pertinent when introducing climate-smart agriculture management practices, and the need to re-visit the role of the “low-affinity” methanotrophs as a methane sink in agricultural soils. However, agriculture-associated GHG emissions can be reversed following land restoration after agriculture abandonment (section 4.5).

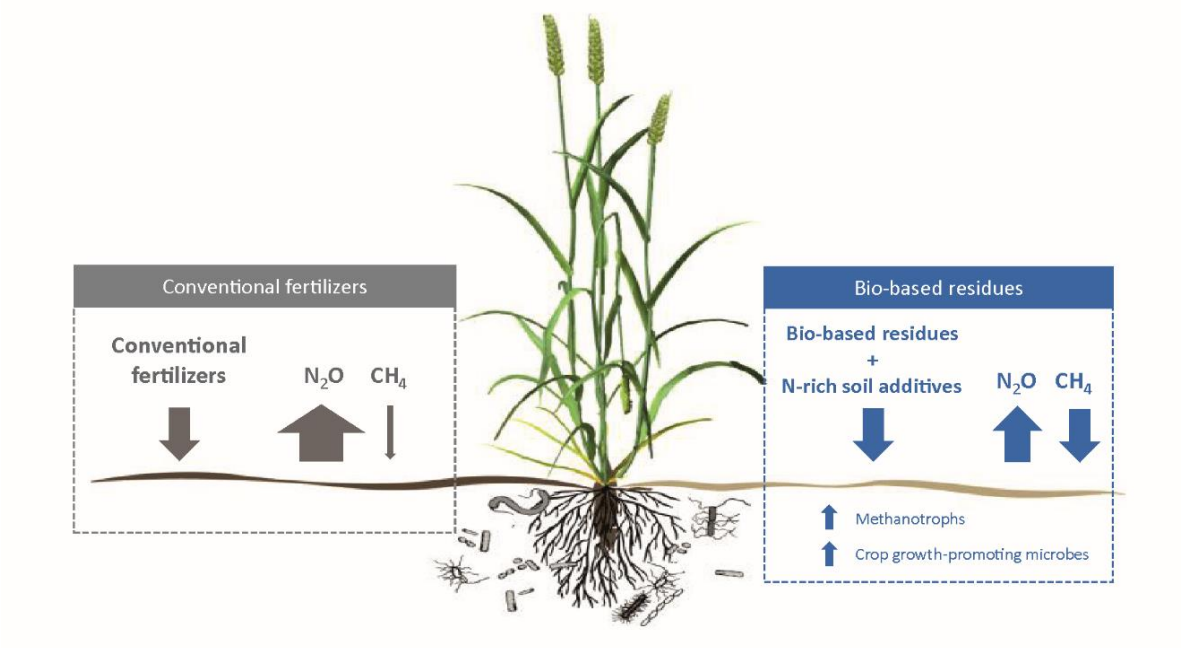
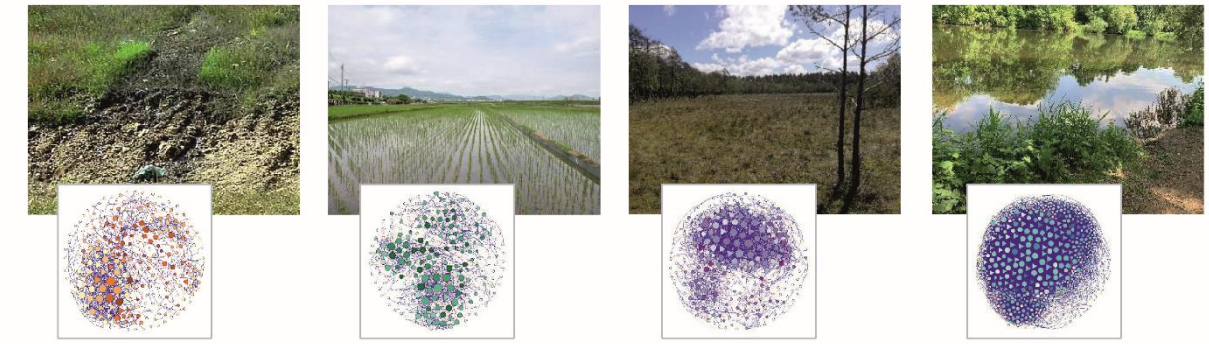


Figure 14: GHG emissions in upland agricultural soils after conventional chemical fertilization and specific bio-based residue addition. A low nitrogen use efficiency in crops will result in the flow of nitrogen to (de)nitrification, leading to nitrous oxide emission. The introduction of agricultural management practices may hinder the methane sink in agricultural soils. However, the incorporation of specific bio-based residues stimulated the soil methane uptake rate, strengthening the methane sink function. Although the stimulatory effect was transient, repeated residue addition may prolong the positive effect. Because the heightened methane uptake offset up to 16% of carbon dioxide produced, the use of bio-based residues can be combined with other nitrogen-rich soil additives to promote crop growth. Accordingly, supplementing agricultural soils with bio-based residues also favored crop growth-promoting fungus and bacteria (Brenzinger *et al*., 2021).

Chapter 5

The Methanotroph Interactome: Greater than the Sum of its Parts



Methane-emitting environments and corresponding interaction networks. Left – right: landfill cover (Germany), rice paddy (Italy), peatland (Poland), and river sediment (Germany) by Adrian Ho

5 The methanotroph interactome: Greater than the sum of its parts

5.1 The methane-driven interaction networks

Biological interactions modulate diverse aspects of microbial life, shaping the activity, diversity, composition, abundance, stability, and assembly of microbial communities (Kaupper *et al*., 2021a,b; Peura *et al*., 2015; Ratzke *et al*., 2020; Dal Co *et al*., 2022). Specifically for the microbial community mediating aerobic methane oxidation, the non-methanotrophs have been shown to be highly relevant members of the interaction network, despite their lack of the enzymatic repertoire to oxidize methane. The non-methanotrophs may exert an interaction-induced effect leading to emergent properties arising from the interacting community (section 5.1). To this end, the non-methanotrophs significantly stimulated the methanotrophic activity and growth, as well as increased methanotroph-mediated micropollutant degradation (Ho *et al*., 2014; Benner *et al*., 2015; Krause *et al*., 2017b; Veraart *et al*., 2018). Therefore, elucidating the interaction of microbial communities, and the organization and assembly of the interacting community (section 5.2) are crucial to provide a comprehensive understanding of microbial community responses to environmental cues and disturbances (sections 5.3 and 5.4).

While the response of aerobic methanotrophy to environmental parameters have been widely documented (see reviews Semrau *et al*., 2010; Ho *et al*., 2013; Guerrero-Cruz *et al*., 2021), little is known of how interacting methanotrophs and non-methanotrophs may exert an impact on aerobic methane oxidation. Microbial interactions in the environment have often been explored using a co-occurrence network analysis (Barberán *et al*., 2012; Mo *et al*., 2020). Here, we defined the co-occurring methanotrophs and non-methanotrophs as the methanotroph "interactome", which can be tracked *via* the flow of ^{13}C-methane from the methanotrophs to the other (micro)organisms in the soil food web. Stable isotope probing (SIP) using ^{13}C-methane effectively differentiates the metabolically active community members from the largely dormant fraction (e.g., Dumont *et al*., 2011; Altshuler *et al*., 2022), and also enables identification of the non-methanotrophs that assimilate methane-derived substrates. Therefore, coupling SIP to a co-occurrence network analysis provides an ecological link between the actively interacting members of the methanotroph interactome, while directly relating the methanotrophic activity to the structure of the interaction networks. The objectives were:

(i) To determine whether the methanotrophic activity is affected by the presence of the non-methanotrophs in an artificially assembled community.

(ii) To determine, in a proof-of-principle study, the feasibility of coupling SIP to a co-occurrence network analysis, and to apply this approach to elaborate the spatial and temporal organization of the methanotroph interactome.

(iii) To determine how the methanotroph interactome is affected by disturbances, exemplified by desiccation-rewetting and peat excavation, representing a single and compounded disturbance event, respectively.

(iv) To determine the response of the methanotroph interactome to disturbance intensification.

5.2 Methanotrophic activity under increasing non-methanotroph richness [Objective 5 (i); based on Ho *et al*., 2014]

The methanotrophs are in close association with other microorganisms in the environment, and have often been co-cultured alongside specific non-methanotrophs (e.g., non-methanotrophic methylotrophs, *Rhizobium*; see review Ho *et al*., 2016c), suggesting a mutually beneficial relationship. Therefore, not only is the richness of methanotrophs essential for aerobic methane oxidation (Levine *et al*., 2011; Schnyder *et al*., 2018), the diversity and variation of the co-occurring non-methanotrophs are also highly relevant for community functioning. To determine the importance of the diversity of the non-methanotrophs in supporting the methanotrophic activity (aerobic methane oxidation), a community comprising of randomly selected one to ten non-methanotrophs were co-cultured together with a methanotroph (*Methylomonas methanica* NCIMB 11130^{T}), while holding the starting cell numbers equal in all co-cultures. The methanotroph was also grown alone, serving as a reference.

The methane oxidation rates were significantly higher, and were proportionate to the richness of the non-methanotrophs (Figure 15). However, the stimulatory effect was not detected when the methanotroph was cultured in the spent medium of the non-methanotrophs (i.e., filter-sterilized medium after culturing the non-methanotrophs). This indicates that a close physical association of the methanotrophs and non-methanotrophs was

required to stimulate the methanotrophic activity and/or the stimulatory effect was unlikely caused by compounds exuded by the non-methanotrophs. The heightened methane oxidation rates may thus be attributable to a relief of inhibition when the non-methanotrophs consume compounds (e.g., products of methane oxidation, methanol and formate) that adversely affect the methanotrophs when accumulated at high concentrations.

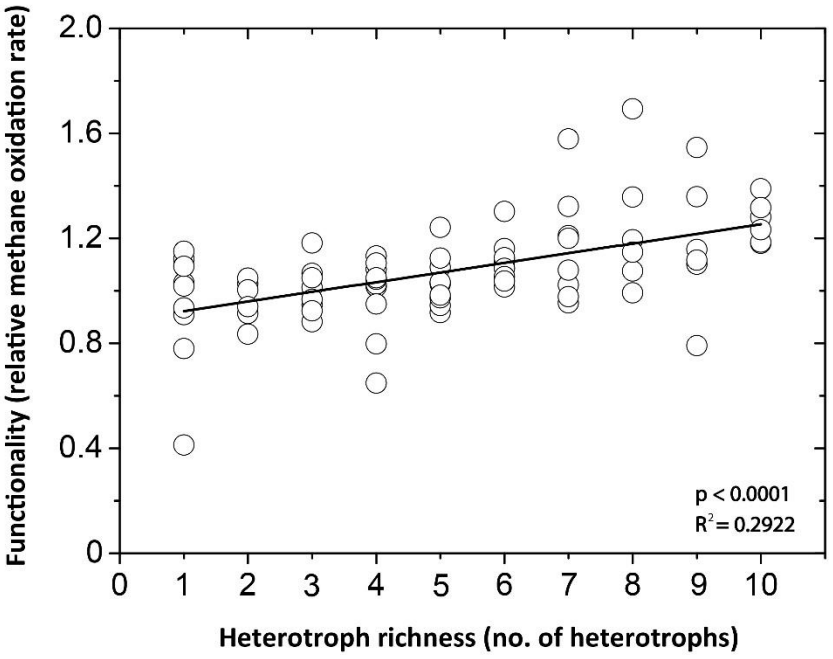


Figure 15: Proportional increase in functionality (methane oxidation rate) and non-methanotroph richness. The methane oxidation rates in the co-cultures were normalized to the reference incubation containing only the methanotroph i.e., ratio of the methane oxidation rates in the co-culture and reference incubations (*n*=86). Functionality was significantly correlated to the non-methanotroph richness ($p<0.01$); the significant positive correlation was consistently shown in two independent batch incubations.

Conversely, almost all non-methanotrophs showed significant or average higher growth in the methanotroph spent medium, indicating that other microorganisms benefited from the methanotroph exudates. Overall, a complex interaction underlies the co-existence of the methanotrophs and other microorganisms, with the richness of the non-methanotrophs favoring methanotrophic activity.

5.3 Coupling SIP to a co-occurrence network analysis to elaborate the spatial and temporal organization of the methanotroph interactome [Objective 5 (ii); based on Ho *et al*., 2016c and Kaupper *at al*., 2022]

Methane-based SIP has typically been employed to detect the primary consumers of the labelled substrate, while the co-detection of cross-feeding microorganisms (non-methanotrophs) has often been a caveat against the approach (Dumont & Murrell, 2005; Altshuler *et al*., 2022). Yet, cross-feeding is a ubiquitous feature of naturally-occurring microbial communities (Morris *et al*., 2013; D'Souza *et al*., 2018); the cross-feeding non-methanotrophs are potentially key members of the methane-driven food web, given their close association to the methanotrophs. In a meta-analysis, previous studies employing SIP using ^{13}C-methane combined with 16S rRNA gene amplicon sequencing of the ^{13}C-enriched DNA were reviewed. In total, five datasets derived from diverse methane-emitting environments met the criteria (see Ho *et al*., 2016c) to be further considered in a proof-of-principle study to determine the feasibility of coupling SIP to a co-occurrence network analysis to track the flow of ^{13}C through the methanotroph interactome and to characterize the interaction network. Although the meta-analysis provided a strong basis for the application of the methodological approach (i.e., SIP-network analysis) to elaborate methane-driven trophic interactions, the inconsistencies between datasets of multiple independent studies limits the interpretation of the meta-analysis.

After demonstrating the feasibility of SIP-network analysis, the approach was applied to widespread methane hotspots (i.e., methane-emitting pristine and restored peatlands, and rice paddy, riparian, and landfill cover soils) and over time to elaborate the spatial and temporal organization of the methanotroph interactome. Interestingly, the network analysis revealed unique co-occurring taxa from the different environments (i.e., no shared co-occurring taxa between all environments), despite the close clustering of the active bacterial, including the methanotrophic community composition particularly in the rice paddy, riparian, and landfill cover soils. Contrary to expectations, this indicates a distinctly co-evolved methanotroph interactome that was more strongly affected by other parameters besides high substrate availability, and/or that the methanotrophs exerted a marginal effect on the recruitment of the non-methanotrophs. Nevertheless, more shared co-occurring taxa from the acidic peatlands (pristine and restored sites), and circum-neutral freshwater ecosystems

(rice paddy and riparian soil) were detected, indicating some commonalities in the environmental selection of these co-occurring microorganisms. Temporal changes in the network topology are anticipated as the soil is a dynamic environment. Nevertheless, monitoring the networks over time (19 days), results indicate a relatively stable network structure, at least in the short-term; the range of the network topology was narrower over time than between sites. Considering the co-occurrence patterns between sites and over time suggest that members of *Chthoniobacter* are yet-unrecognized interacting partners, favoring the gammaproteobacterial methanotrophs. Hence, SIP-network analysis identified potentially important members of the methanotroph interactome.

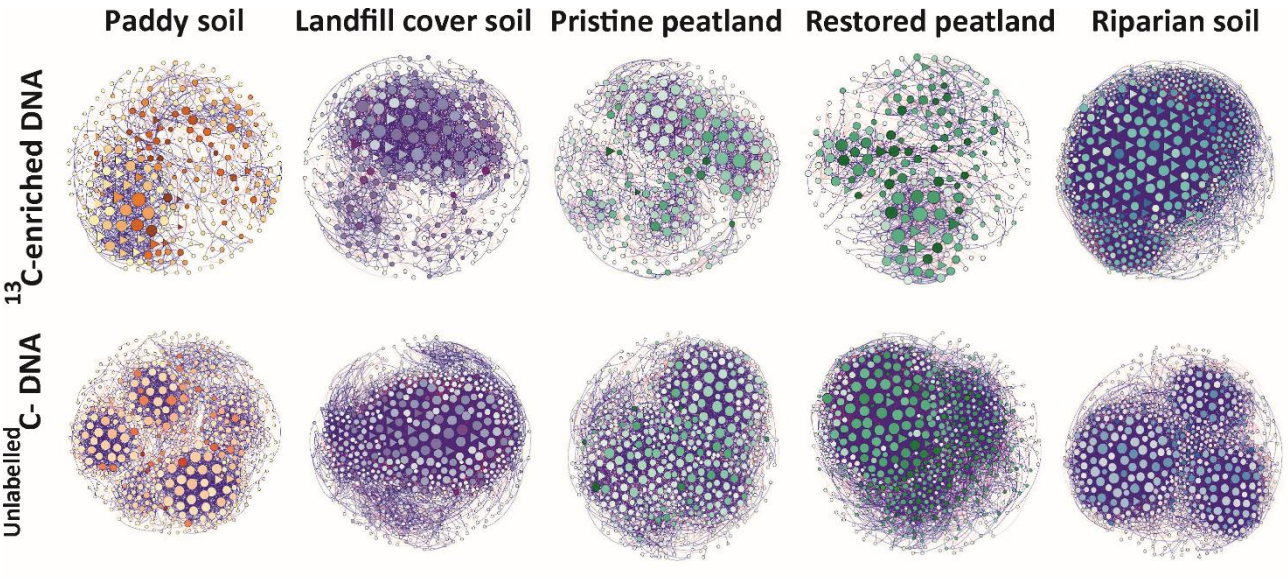


Figure 16. Co-occurrence network analysis derived from the ^{13}C-enriched and ^{Unlabelled}C-DNA in methane hotspots. True SparCC correlations with a magnitude of > 0.8 (positive correlations) or < -0.8 (negative correlations), and statistical significance of $p<0.01$ were considered for the network construction (sequence analysis and network construction are detailed in Kaupper *et al*., 2022). The network topology properties are given in Table 3, comparing the networks derived from the ^{13}C-enriched and ^{Unlabelled}C-DNA.

Because SIP-co-occurrence network analysis would minimize spurious and weak connections, and exclude the largely dormant community members, the networks derived from the ^{13}C-enriched DNA generally showed a less complex and connected topology compared to the ^{unlabelled}C-DNA-based (i.e., DNA isolated directly from the environmental sample) networks, as indicated by the lower number of nodes, edges, and degree (average

number of connections per node) in the peatlands, rice paddy, and landfill cover soils (Figure 16 & Table 3). Therefore, applying the network analysis on DNA directly isolated (i.e., without SIP) from soils may overestimate the network complexity.

Table 2: Top five key nodes (OTUs) with more betweenness centrality in the un-disturbed and disturbed incubations after desiccation-rewetting (Kaupper *et al*., 2021b), peat mining (Kaupper et al., 2021a), and increasing ammonium stress (disturbance intensification; Ho *et al*., 2020). Comparison of betweenness centrality was performed per dataset (experiment). Methanotrophs are emboldened.

Time after disturbance	Un-disturbed (reference)		Disturbed	
	Affiliations (OTU)[a]	Betweenness centrality	Affiliations (OTU)[a]	Betweenness centrality
Desiccation-rewetting				
1-7 days	Gemmatimonadaceae	1244.8	*Sphingomonas*	1006.8
	Methylomonas	1169.8	Blastocatellia Sub 4	816.0
	Noviherbaspirillum	1077.1	*Ideonella*	785.4
	Beijerinckiaceae	967.1	Methylophilaceae	666.6
	Acidobacteria Sub 6	946.2	*Chthoniobacter*	662.9
27-71 days	*Paenibacillus*	1151.5	Proteobacteria	884.9
	Acidobacteria Sub 7	1067.5	Unclassified Bacteria	869.5
	Sulfuritalea	936.8	Chitinophagaceae	669.6
	Opitutaceae	830.1	Microscillaceae	583.6
	Unclassified Bacteria	829.5	*Crenothrix*	574.9
Peat mining				
	Rhodospirillales	10656.9	***Methylomonas***	6872.3
19 years	Unclassified Bacteria	9894.4	*Bdellovibrio*	6312.0
	Burkholderiaceae	9755.8	Magnetospirillaceae	5617.5
	Methylomonas	8653.7	***Methylomonas***	5426.7
	Candidatus Solibacter	8011.1	Acidimicrobiia	5061.8
Increasing ammonium stress				
1.25 – 1.75	Burkholderiaceae	3276.7	Burkholderiaceae	1034.0
g NH_4^+ ml^{-1}	Burkholderiaceae	2277.6	*Aeromicrobium*	898.9
	Fimbriiminadaceae	2063.3	***Methylobacter***	888.5
	Burkholderiaceae	2028.68	Rhizobiaceae	834.3
	Reyranella	2017.7	*Bosea*	828.8
2.5 – 3.25	*Hyphomicrobium*	672.4	*Flavisolibacter*	567.6
g NH_4^+ ml^{-1}	Chitinophagaceae	589.8	Fimbriimonadaceae	527.1
	Hyphomicrobium	466.3	*Parvibaculum*	489.1
	Sphingobacteriales	316.8	*Ramlibacter*	477.4
	Chitinophagaceae	269.9	Unclassified Bacteria	469.3
3.75 – 4.75	*Caulobacter*	244.2	*Parvibaculum*	714.8
g NH_4^+ ml^{-1}	Chitinophagaceae	211.2	Chitinophagaceae	571.1
	Variovorax	209.2	*Lacunisphaera*	413.5
	Bdellovibrio	204.2	*Parvibaculum*	411.2
	Methylocystis	160.0	*Ramlibacter*	364.5

[a]OTUs are classified to the finest taxonomic affiliation, whenever possible.

Table 3: Topological properties of the co-occurrence network analysis derived from the ^{13}C-enriched and $^{Unlabelled}C$-DNA in methane hotspots.

Network properties	Paddy Soil		Landfill cover soil		Pristine peatland		Restored Peatland		Riparian soil	
	^{13}C	$^{Unlabelled}C$	^{13}C	$^{Unlabelled}C$	^{13}C	$^{Unlabelled}C$	^{13}C	$^{Unlabelled}C$	^{13}C	$^{Unlabelled}C$
Number of nodes[a]	299	536	329	655	344	622	258	681	737	667
Number of edges[b]	980	2839	2078	8899	1684	4219	846	6918	10003	6261
Modularity[c]	0.817	1.166	1.557	2.067	2.316	1.734	1.812	1.894	0.692	0.992
Number of communities[d]	55	91	32	30	39	67	38	40	19	74
Network diameter[e]	16	14	10	10	11	10	11	11	12	10
Average path length[f]	5.913	5.803	3.969	3.607	4.402	4.372	5.01	4.228	4.201	4.433
Average degree[g]	6.55	10.59	12.63	27.173	9.79	13.566	6.55	20.317	27.14	18.774
Av. clustering coefficient[h]	0.365	0.416	0.409	0.418	0.413	0.397	0.397	0.413	0.319	0.345

[a]Microbial taxon (at genus level) with at least one significant ($p < 0.01$) and strong (SparCC > 0.8 or < -0.8) correlation;

[b]Number of connections/correlations obtained by SparCC analysis;

[c]The capability of the nodes to form highly connected communities, that is, a structure with high density of between nodes connections (inferred by Gephi);

[d]A community is defined as a group of nodes densely connected internally (Gephi);

[e]The longest distance between nodes in the network, measured in number of edges (Gephi);

[f]Average network distance between all pair of nodes or the average length off all edges in the network (Gephi);

[g]The average number of connections per node in the network, that is, the node connectivity (Gephi);

[h]How nodes are embedded in their neighborhood and the degree to which they tend to cluster together (Gephi).

5.4 The response of the methanotroph interactome as derived from SIP-network analysis to disturbances [Objective 5 (iii); based on Kaupper *et al*., 2021a,b]

Disturbance-induced effects on the methanotrophs may also impact other members of the interactome, given their close-association and the reliance on the methane-derived carbon for the entire community. Therefore, the interaction network may be re-structured, reflecting on the response of the methanotroph interactome to resist and/or recover (resilience) from disturbances. In this chapter, the response of the methanotroph interactome to two model disturbances exemplified by desiccation-rewetting (single disturbance event), and peat excavation representing a compounded form of disturbance were determined.

Both disturbances significantly re-structured the methanotroph interactome. The methanotrophic activity was significantly adversely affected by desiccation-rewetting in the short-term (< 5 days), but methane uptake rates soon recovered thereafter, and was comparable to the values exhibited in the un-disturbed community (Kaupper *et al*., 2022b). Likewise, the methanotrophic community composition and abundances fully recovered. However, the interaction network was profoundly altered, becoming more complex, but less modular after the disturbance. Hence, desiccation-rewetting fostered closer association among the members of the methanotroph interactome. With the elimination of microorganisms that were sensitive to desiccation-rewetting, the community members which survived the disturbance were thus forced to interact among themselves, likely increasing metabolic exchange driving their co-occurrence over time. Also, desiccation-rewetting increases nutrient availability (e.g., inorganic N) resulting from the lysed cells, fuelling the metabolic exchange (Ho *et al*., 2016a; Ho & Frenzel, 2012). On the other hand, modularity, that is, the ability to form independently connected groups or compartments within the network (Zhou *et al*., 2010), decreased following desiccation-rewetting. An interaction network with a high modularity is anticipated to localize or restrict the effects of disturbances to compartments within the network (Zhou *et al*., 2010), potentially shielding the entire community to the effects of the disturbance. The loss of modularity would thus indicate that the recovered community may be more vulnerable to future or recurring disturbances.

Likewise, the methane-driven interaction network was significantly altered in the restored peatlands after mining, when compared to the pristine peatlands (Kaupper *et al*.,

2021a). These sites have been well-characterized, and showed largely comparable methanotrophic activity, community composition, and abundances, which indicates that the aerobic methanotrophs have generally recovered 19 years post-mining (Reumer *et al*., 2018). However, re-structuring of the recovered interaction network resulted in the loss of complexity, as well as modularity, which may have consequences in the face of future disturbances and environmental changes.

Cross-feeding non-methanotrophs are anticipated to form close knit networks centered around the methanotrophs, given that the methanotrophs are the sole "primary" consumer and the source of methane-derived carbon. Interestingly, many nodes with high betweenness centrality comprised of non-methanotrophs. The nodes with high betweenness centrality are regarded as the "key nodes" with a higher frequency acting as a bridge between two other nodes along the shortest path (Table 2; Borgatti, 2005; Poudel *et al*., 2016). The key nodes thus represent microbial taxa that likely play a significant role within the methanotroph interactome; systematic removal of these nodes may lead to the collapse of the interaction networks. The detection of non-methanotrophs as the predominant key nodes thus implicate these microorganisms in the modulation of the interaction networks during disturbances.

The mode of disturbances and shift in the nutritional status after disturbances may affect metabolic exchange in the recovered community, in turn, influence the complexity of the networks. Also, it is becoming evident that the networks consistently became less modular after both model disturbances (Figure 17). Overall, disturbances re-structured the methane-driven interaction networks, despite the recovery in the methanotrophic activity, community composition, and abundances, which may have consequences for community functioning (section 5.4).

5.5 The response of the methanotroph interactome to disturbance intensification [Objective 5 (iv); based on Ho *et al*., 2020]

Environmental disturbances are not necessarily sporadic events, and may re-occur and/or intensified over time, exerting an effect on microbially-mediated processes and the microbial communities (e.g., frequent freeze-thaw cycles, increasing drought and herbivory). This chapter investigated the response of the methanotrophs, including the methane-driven

interaction network, to disturbance intensification as induced by a step-wise increase in ammonium concentration (0.5 – 4.75 g NH_4Cl L^{-1}, at 0.25 g NH_4Cl L^{-1} increments). Although the effects of ammonium on methane oxidation are conflicting in environmental studies, ammonium inhibition on methanotrophic activity has consistently been shown to be dose-dependent in pure cultures (see references therein Ho *et al*., 2020). In addition to ammonium, by-products of ammonium oxidation (e.g., hydroxylamine, nitrite) may also inhibit the methanotrophs (Nyerges & Stein, 2009; Poret-Peterson *et al*., 2008). Here, ammonium was inhibitive at concentrations exceeding 2.25 g NH_4Cl L^{-1}, significantly reducing the methane uptake rates and delayed the onset of methane uptake, when compared to the reference incubation containing 0.5 g NH_4Cl L^{-1} in the growth (i.e., ammonium mineral salts, AMS) medium.

The adverse effect of ammonium on the methane uptake rates is reflected in the methane-driven interaction network, showing a less complex community structure, accompanied by reduced modularity with step-wise increase in ammonium concentrations (Figure 17). Like in the previous studies (section 5.3; Kaupper et al., 2021a,b), the key nodes were overwhelming comprised of non-methanotrophs, albeit represented by different taxa (Table 2). This was not entirely unexpected, as the co-occurring taxa were found to be distinct from different environments, but may possess similar ecological traits (Kaupper *et al*., 2022). Overall, the complexity of the interaction network was not sustained, eventually reaching a "tipping point", and unraveled under disturbance intensification; the gradually reduced network complexity and modularity could be linked to the significantly impaired methanotrophic activity, indicating the relevance of a complex and modular network for community functioning and to resist disturbances.

5.6 Conclusions

The non-methanotrophs are crucial members of the methanotroph interactome, influencing community function *via* interaction-induced effects (section 5.1). The co-occurring non-methanotrophs were unique to different high methane-emitting environments (section 5.2), suggesting that other factors besides methane availability drive their co-occurrence. Alternatively, the non-methanotrophs may have been co-enriched alongside the methanotrophs based on their functional traits (rather than taxonomic identity), which favor the methanotrophs (Krause *et al*., 2017b). Importantly, the non-methanotrophs have been implicated in the resilience of the methane-driven interaction network during recovery from disturbances, given their role as key node species (sections 5.3 and 5.4). Despite the recovery in the methane uptake rates, bacterial community composition, and abundances following induced disturbances, the interaction networks were significantly modified, with differing levels of complexity, and consistently becoming less modular over time (sections 5.3; Figure 17), which may have consequences for community function with recurring disturbances (section 5.4). It thus appears that the legacy of the disturbance persisted in the interaction network. Moving beyond associating activity (e.g., specific process rates) to microbial community composition, abundances, and abiotic parameters, determining the interaction network is crucial to provide a comprehensive understanding on the dynamics of community function.

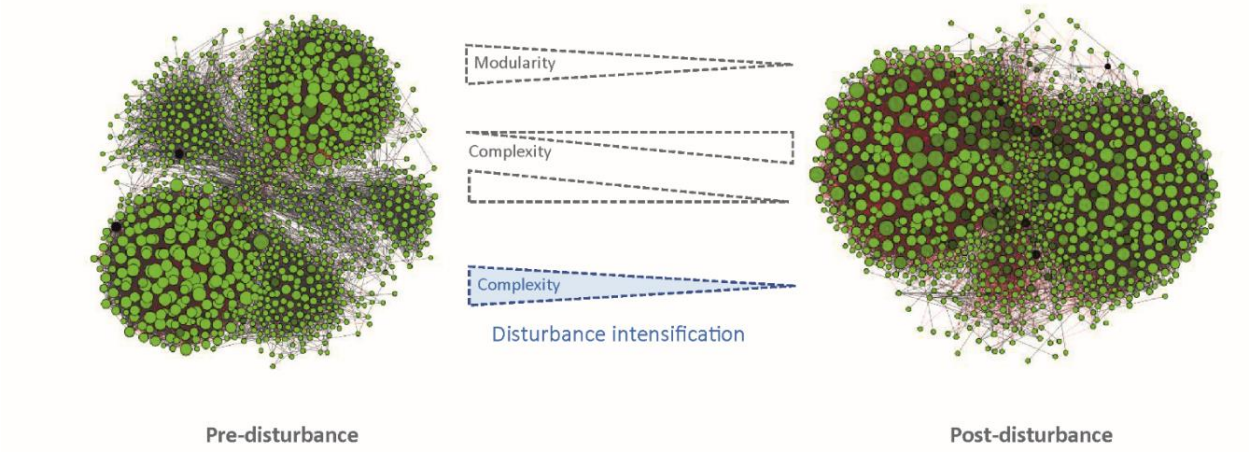


Figure 17. The impact of disturbances on the methane-driven interaction network. The network complexity is modified (increase or decrease), depending on the mode of disturbance and accessible nutrients post-disturbance, but modularity consistently decreased post-disturbance (Section 5.3). However, both complexity and modularity are negatively affected with recurring disturbance at increasing intensity, concomitant to significantly impaired methanotrophic activity (section 5.4).

6 General Conclusions and Perspectives

Owing to their vast taxonomic and functional diversity, soil microorganisms were thought to be sufficiently redundant to compensate for diversity loss. However, some microorganisms catalyze rather specific processes, and these specialists are relatively less diverse and can constitute only a minor fraction of the soil microbiome. The methanotrophs have been regarded as members of the rare biosphere in soils. Despite being present at a relatively low abundance and diversity, the methanotrophs were previously documented to be remarkably resilience to single disturbance events. However, further studies in this thesis showed their vulnerability to recurring and compounded disturbances associated to change in land-use and climate, which significantly altered the composition and interaction network of the methane-driven interactome. These changes were accompanied by impaired methanotrophic activity, adversely affecting the methane sink function in soils. Although the agriculture-associated change in land-use may negatively affect soil methane uptake, these effects can be attenuated by implementing specific agricultural management practices (e.g., choice of soil additives). In this thesis, research results derived from mesocosm-scale studies provide a basis for pragmatic approaches in the field, devising strategies to mitigate methane emissions in agrosystems, while improving crop yield. Hence, the vulnerability, and (a)biotic factors driving the methanotrophic activity, as well as shaping the methanotroph interactome were determined. Research findings addressed the objectives and support the hypothesis of this thesis:

Methanotrophs are resilient to environmental disturbances, but recurring or compounded disturbances may have a cumulative effect, compromising methanotrophic activity, which is also modulated by interactions with the biotic environment.

Considering that the soil harbors diverse organisms besides bacteria and archaea, the interaction of microorganisms with other members of the soil microbiome (viruses, fungi, protists, nematodes, macrofauna), and the impact of these interactions on community function remain largely unresolved, deserving future attention.

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Appendices

A 1 Curriculum Vitae

A 2 Complete list of publications

A1 Curriculum Vitae

Dr. rer. nat. Adrian Ho Kah Wye
(05.02.1981, Georgetown, Malaysia)

Education

2007 – 2010 PhD, Microbiology, Max-Planck-Institute for Terrestrial Microbiology and Philipps University Marburg, Germany.

2005 – 2007 MSc., Food- and Bio-Technology, Lund University, Sweden.

2002 – 2005 BSc., Biotechnology, Murdoch University, Western Australia.

Career

2017 – *wissenschaftlicher Assistant*, Leibniz University Hannover, Germany.

2013 – 2017 Post-doctoral researcher, Netherlands Institute of Ecology (NIOO-KNAW), Wageningen, the Netherlands.

2012-2013 Post-doctoral researcher, Ghent University, Belgium.

2010 – 2011 Post-doctoral researcher, Max-Planck-Institute for Terrestrial Microbiology, Germany.

2004 – 2005 Research intern, Commonwealth Scientific and Industrial Research Organization (CSIRO), Western Australia.

Professional Membership and Esteem Indicators

2020 - Member, German Association for General and Applied Microbiology (VAAM).

2021 - Editorial board member, FEMS Microbiology Ecology journal.

2015 - Reviews editor/Guest editor, Frontiers in Microbiology journal.

2011 - Ad-hoc reviewer for > 25 international peer-reviewed journals (e.g., Nature Geoscience, Microbiome, ISME Journal, Soil Biology & Biochemistry).

Reviews for Funding Agencies

- Deutsche Forschungsgemeinschaft (DFG).
- Fonds voor Wetenschappelijk Onderzoek (FWO-Vlaanderen, Belgium).
- The Research Council of Norway.
- National Science Centre Poland.

A2 Complete List of Publications

Peer-reviewed Research Articles (*Corresponding author)

1. Rohrbach S, Gkoutselis G, Hink L, Weig AR, Obst M, Diekmann A, **Ho A**, Rambold G, Horn MA. Microplastic polymer properties as deterministic factors driving terrestrial plastisphere microbiome assembly and succession in the field. Environmental Microbiology; Accepted.

2. El-Hawwary A, Brenzinger K, Lee HJ, Veraart AJ, Morriën E, Schloter M, van der Putten WH, Bodelier PLE, **Ho A*** (2022) Greenhouse gas (CO_2, CH_4, and N_2O) emissions after abandonment of agriculture. Biology and Fertility of Soils 58: 579-591.

3. Kaupper T, Mendes LW, Poehlein A, Frohloff D, Rohrbach S, Horn MA, **Ho A*** (2022) The methane-driven interaction network in terrestrial methane hotspots. Environmental Microbiome 17: 15.

4. Sultana N, Zhao J, Cai Y, Rahman GKMM, Alam MS, Faheem M, **Ho A**, Jia Z (2022) Methanotrophy-driven accumulation of soil organic carbon in four paddy soils of Bangladesh. *Pedosphere* 32; 348-358.

5. **Ho A***, Zuan ATK, Mendes LW, Lee HY, Zulkeflee Z, van Dijk H, Kim PJ, Horn MA (2021) Aerobic methanotrophy and co-occurrence networks of a tropical rainforest and oil palm plantations in Malaysia. Microbial Ecology; https://doi.org/10.1007/s00248-021-01908-3

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