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Titel | Title

From phospholipids to neutral lipids: Characterising soil microbial communities and their functions

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For Julia and Eduard

Abstract

Soil is a highly dynamic and heterogeneous environment, home to diverse microbial communities that play essential roles in ecosystem functioning. Natural soils frequently experience fluctuating environmental conditions that can have significant effects on these microbial communities. Understanding how soil microbes respond to such perturbations is key to predicting the resilience and overall functioning of soil ecosystems. A critical aspect of this involves analysing marker compounds that can sensitively track the compositional and physiological shifts in microbial communities. This thesis addresses these challenges by exploring the responses of soil microbes to common environmental perturbations, while also evaluating the potential for extending an existing lipid analysis technique to provide deeper insights into microbial community structure and function.

Microbial communities are expected to be physiologically adapted to environmental perturbations which occur commonly in soils, as they have a long history of interactions with them. In this thesis, I investigated the response of soil microbial communities to two perturbations: freeze-thaw cycles and labile substrate (glucose) addition. Using phospholipid and neutral lipid fatty acid (PLFA, NLFA) profiling, I tracked changes in microbial community composition and function over 160 days. Contrary to expectations of high resilience, the perturbations induced long-lasting alterations in microbial community structure as well as nutrient dynamics, indicating persistent shifts in soil microbial functions.

I also evaluated the PLFA extraction method to include glycolipid and neutral lipids, which are potentially sensitive markers for microbial community functions. Specifically, I tested (i) the purity of the lipid fractions with pure lipid standards, (ii) whether the taxonomic specificity of marker fatty acids is retained across lipid fractions in microbial pure cultures, and (iii) if fatty acid profiles differ between the lipid fractions in soil extracts. Satisfactory separation of phospholipids, neutral lipids, and glycolipids was achieved by adding 2% ethanol to chloroform for neutral lipid elution. Taxonomic specificity was consistent across different lipid fractions in pure microbial cultures. In soil extracts, lipid fractions exhibited stronger differences between themselves than between soil types, suggesting that each lipid fraction provides unique insights into microbial community physiology.

Furthermore, I explored the origin of bacterial NLFAs in soils, aiming to clarify whether they derive from carbon storage compounds (triacylglycerols, TAGs) or phospholipid degradation products indicative of necromass. Analysis of the JGI database shows that the key enzyme in bacterial TAG synthesis (WS/DGAT) is abundant in soil bacterial isolate genomes, suggesting the ability to produce TAGs. Additionally, glucose-^{13}C added to soil is rapidly allocated into bacterial NLFAs, indicating storage of excess carbon. These findings support the hypothesis that bacterial NLFAs primarily originate from TAGs, which suggests that they are markers for bacterial carbon storage.

This thesis contributes to a deeper understanding of soil microbial resilience to environmental changes and enhances lipid analysis methodologies. The integration of NLFA and glycolipid fatty acid profiling with PLFA analysis provides a more holistic approach to studying soil microbial communities, with significant implications for analysing microbial community functions. Bacteria-specific NLFAs in particular appear to be an underexplored marker for microbial carbon storage.

Zusammenfassung

Der Boden ist eine hochdynamische und heterogene Umgebung, in der diverse mikrobielle Gemeinschaften leben, die eine zentrale Rolle im Funktionieren von Ökosystemen spielen. Natürliche Böden unterliegen häufig schwankenden Umweltbedingungen, die erhebliche Auswirkungen auf die mikrobiellen Gemeinschaften haben können. Um die Resilienz—und das gesamte Funktionieren—von Bodensystemen zu verstehen, ist es wichtig, zu untersuchen wie Bodenmikroben auf solche Perturbationen reagieren. Ein wesentlicher Aspekt hierbei ist die Analyse von Biomarkern, welche die Zusammensetzung und Physiologie von mikrobiellen Gemeinschaften zuverlässig abbilden. In dieser Arbeit untersuche ich, in welcher Weise Änderungen in der physiko-chemischen Umwelt mikrobielle Gemeinschaften in Böden perturbieren, und erweitere eine gebräuchliche Lipidanalyse, um tiefere Einblicke in die Struktur und Funktion mikrobieller Gemeinschaften zu gewinnen.

Gemeinhin ist zu erwarten, dass mikrobielle Gemeinschaften physiologisch an Umweltstörungen angepasst sind, welche häufig in Böden vorkommen, da sie oft unter diesen interagieren. In dieser Arbeit habe ich die Reaktion mikrobieller Gemeinschaften im Boden auf zwei unterschiedliche Perturbationen untersucht: Gefrier-Auftau-Zyklen und die Zugabe eines leicht verfügbaren Substrats (Glukose). Mit der Analyse von Fettsäureprofilen aus Phospholipiden und neutralen Lipiden (PLFA, NLFA) habe ich Veränderungen in der Zusammensetzung und dem Funktionieren der mikrobiellen Gemeinschaften über 160 Tage verfolgt. Obwohl eine hohe Resilienz der mikrobiellen Gemeinschaften zu erwarten war, führten die Perturbationen zu langanhaltenden Veränderungen in der Struktur der mikrobiellen Gemeinschaften, sowie in Kohlenstoff- und Stickstoffdynamiken des Bodens, was auf eine nachhaltige Veränderung der Physiologie der Bodenmikroben hinweist.

Des Weiteren habe ich die PLFA-Extraktionsmethode erweitert, um Glykolipide und neutrale Lipide einzubeziehen. Diese sind potentiell sensitive Marker für physiologische Eigenschaften von Bodenmikroben. Insbesondere habe ich (i) die Ausbeute der Lipidfraktionen mit reinen Lipidstandards getestet, (ii) ob die taxonomische Spezifität von Markerfettsäuren in den unterschiedlichen Lipidfraktionen in mikrobiellen Reinkulturen erhalten bleibt, und (iii) ob sich die Fettsäureprofile zwischen den Lipidfraktionen in Bodenproben unterscheiden. Eine zufriedenstellende Trennung von Phospholipiden, Neutralfetten und Glykolipiden

wurde durch die Zugabe von 2 % Ethanol zu Chloroform für die Elution von neutralen Lipiden erreicht. Die taxonomische Spezifität blieb in verschiedenen Lipidfraktionen in Reinkulturen erhalten. In Bodenproben zeigten die Lipidfraktionen größere Unterschiede zueinander als zwischen den verschiedenen Bodentypen, was darauf hindeutet, dass jede Lipidfraktion spezifische Einblicke in die Physiologie mikrobieller Gemeinschaften bietet.

Darüber hinaus habe ich den Ursprung von bakteriellen NLFAs in Böden untersucht, um zu klären, ob sie aus Kohlenstoffspeicherstoffen wie Triacylglycerolen (TAGs), oder aus Abbauprodukten von Phospholipiden, die auf Nekromasse hindeuten, stammen. Eine Analyse der JGI-Datenbank zeigt, dass das Schlüsselenzym der bakteriellen TAG-Synthese (WS/DGAT) in den Genomen von Isolaten von Bodenbakterien häufig vorkommt, was auf die Fähigkeit zur TAG-Produktion hinweist. Außerdem wird zugeführtes Glukose-^{13}C binnen kurzer Zeit in bakterielle NLFAs eingebaut, was auf eine Speicherung von überschüssigem Kohlenstoff hindeutet. Diese Ergebnisse stützen die Hypothese, dass bakterielle NLFAs hauptsächlich aus TAGs stammen, was darauf hindeutet, dass sie Marker für Kohlenstoffspeicherung in Bakterien sind.

Diese Arbeit trägt zu einem tieferen Verständnis der Resilienz mikrobieller Gemeinschaften im Boden gegenüber Umweltveränderungen bei und verbessert eine Methodik der Lipidanalyse. Die Integration von Fettsäureprofilen aus neutralen Lipiden und Glykolipiden in die PLFA-Methodik bietet einen holistischen Ansatz zur Untersuchung mikrobieller Gemeinschaften in Böden, und hat bedeutende Implikationen für die Analyse mikrobieller Gemeinschaften. Insbesondere NLFAs, die spezifisch für Bakterien sind, scheinen ein noch wenig erforschter Marker für mikrobielle Kohlenstoffspeicherung zu sein.

Overview of manuscripts

Chapter 2

Manuscript title: One-time freeze-thawing or carbon input events have long-term legacies in soil microbial communities

Authors: Stefan Gorka, Christian Ranits, Shasha Zhang, Bruna Imai, Ksenia Guseva, and Christina Kaiser

Reference: Gorka, S., Ranits, C., Zhang, S., Imai, B., Guseva, K., Kaiser, C., 2023. One-time freeze-thawing or carbon input events have long-term legacies in soil microbial communities. Geoderma 432, 116399. https://doi.org/10.1016/j.geoderma.2023.116399

Author contributions: CK conceived the initial idea for the study. SG, CR, SZ, and BI performed the lab experiment. SG took care of the incubations, carried out the chromatographic measurements and data analysis, and drafted the manuscript. CR, KS, and CK contributed to the revision of the manuscript.

Chapter 3

Manuscript title: Beyond PLFA: Concurrent extraction of neutral and glycolipid fatty acids provides new insights into soil microbial communities

Authors: Stefan Gorka, Sean Darcy, Julia Horak, Bruna Imai, Moritz Mohrlok, Erika Salas, Andreas Richter, Hannes Schmidt, Wolfgang Wanek, Christina Kaiser, and Alberto Canarini

Reference: Gorka, S., Darcy, S., Horak, J., Imai, B., Mohrlok, M., Salas, E., Richter, A., Schmidt, H., Wanek, W., Kaiser, C., Canarini, A., 2023. Beyond PLFA: Concurrent extraction of neutral and glycolipid fatty acids provides new insights into soil microbial communities. Soil Biology and Biochemistry 187, 109205. https://doi.org/10.1016/j.soilbio.2023.109205

Author contributions: AC conceived the initial idea for the study, and refined it with CK and SG. JH, BI, MM, ES, AR, HS, and WW grew and provided the microbial strains. SG, SD, AC, and BI extracted lipid-derived FAMEs. SG, BI, and AC performed chromatographic measurements and data analysis of the pure lipid tests. SG, SD and AC performed chromatographic measurements and data analysis of microbial pure cultures. SG performed the soil FAME extractions and ergosterol tests, and refined the chromatographic method under supervision of AC and CK. All co-authors contributed to the revision of the manuscript.

Chapter 4

Manuscript title: Soil bacterial neutral lipid fatty acids: Markers for energy storage or necromass?

Authors: Stefan Gorka, Alberto Canarini, Hannes Schmidt, and Christina Kaiser

Reference: Draft manuscript

Author contributions: CK conceived the initial idea for the study. SG conducted the complete NLFA profile literature search. SG and AC performed the chromatographic measurements and data analysis of microbial pure culture FAMEs. SG performed the chromatographic measurements and data analysis of soil FAMEs. SG conducted the JGI library search of soil bacterial isolate genomes under supervision of HS. All co-authors contributed to the revision of the manuscript.

Chapter 1

General introduction

and thesis outline

1. Soil as a habitat for microbial communities

Soil hosts an astonishing abundance and diversity of microbial life. Some authors estimate that a single gram of soil contains up to 10^{10} bacterial cells, several metres of fungal mycelium, and between 10^3 and 10^6 microbial species (Torsvik and Øvreås, 2002; Balser et al., 2005; Gans et al., 2005; Mummey and Rillig, 2008; Raynaud and Nunan, 2014). These microbes fulfil important ecosystem functions, as they closely interact with plants and stimulate their growth and stress tolerance, mediate transformations of carbon inputs by decomposing plant material, and contribute to the formation of stable soil organic matter (Hayat et al., 2010; van der Heijden et al., 2015; Liang et al., 2017; Begum et al., 2019; Kästner et al., 2021). Although microbial biomass only represents approximately 1% of total soil carbon (Fierer et al., 2009), it is the interface which mediates soil organic carbon pools. The total soil carbon pool is massive, with 1500 Gt carbon, surpassing the carbon bound in the above-ground terrestrial biosphere and the atmosphere combined (Scharlemann et al., 2014).

Soil is, however, a challenging environment to live in. It has a heterogeneous structure with varying physical and chemical properties, which affect the spatial accessibility of resources for microbes. Soil consists of solid particles of varying size classes, with pore spaces between them that are filled with water or air (Young and Crawford, 2004). Plant roots and mycorrhizal hyphae release labile compounds and mucilage into spatially restricted soil volumes, influencing the physico-chemical environment of microorganisms (Jansa et al., 2013; Gorka et al., 2019; Benard et al., 2019; Canarini et al., 2019). The interplay of the different structural components facilitates strong gradients of moisture, pH, and oxygen levels, which even persist within soil aggregates at the microscale (Sexstone et al., 1985; Hinsinger et al., 2003; Young and Crawford, 2004; York et al., 2016). This heterogeneity creates relatively isolated micro-habitats where the distribution of nutrients and organic matter can significantly influence microbial activity and interactions (Nunan et al., 2020).

2. Environmental fluctuations and microbial perturbations

Soil microbes inhabit not only a structurally very heterogeneous habitat, they are also continuously exposed to fluctuations which change the physical and chemical characteristics of their immediate environment. These fluctuations can be of biological origin, such as growing plant roots or earthworms, which move through the soil and leave behind a trail of low- and high-molecular-weight compounds (exudates and mucilage). This spatial and temporal variability affects microbial activity as well as physical soil characteristics such as water retention, creating spatially and temporally restricted ‘hotspots’ and ‘hot moments’ of microbial

activity (Kuzyakov and Blagodatskaya, 2015; Hoang et al., 2017; Benard et al., 2019; Canarini et al., 2019; Guhra et al., 2022). Environmental fluctuations can also be of abiotic origin, as simple as diurnal or annual changes in temperature and moisture, which are usually realised on a macroscopic level. For instance, soils in temperate ecosystems are frequently exposed to extremes in moisture (dry-wet cycles) or temperature (freeze-thaw cycles), which can have large-scale repercussions on microbial community structure, and microbial carbon and nitrogen cycling (Thomson et al., 2010; Mooshammer et al., 2017; Miura et al., 2019; Meisner et al., 2021)

The degree to which a microbial community responds to environmental changes or stressors can be defined as its stability (Shade et al., 2012). Stability consists of two quantifiable metrics: resistance and resilience (Fig. 1). Resistance is the degree of environmental change needed to have an effect on a microbial community. Resilience is the rate at which a microbial community returns to its initial state (Rykiel, 1985; Allison and Martiny, 2008; Shade et al., 2012). Studies on the stability of soil microbial communities often investigate the effects of strong disturbances which are rare, intrinsically destructive, and often of anthropogenic origin (Griffiths and Philippot, 2013; Holden and Treseder, 2013). These can include heavy metal contaminations, heat treatments, application of toxic or destructive chemicals, or physical disturbances (Zelles et al., 1997; Griffiths et al., 2001; Müller et al., 2002; Banning and Murphy, 2008; Deng et al, 2009; Schnoor et al., 2011). Although it is known that pulsed shifts in environmental conditions can lead to drastic short-term changes in microbial activity and community composition, research is lacking on long-term responses (Skogland et al., 1988; Schimel and Clein, 1996; De Nobili et al., 2001; Sharma et al., 2006; Cleveland et al., 2007; Mau et al., 2015). Indeed, most studies investigating long-term responses apply repeated pulses or continuous stressors, whereas the impacts of a single short-term perturbation have rarely been explored (Allison and Martiny, 2008; Shade et al., 2012).

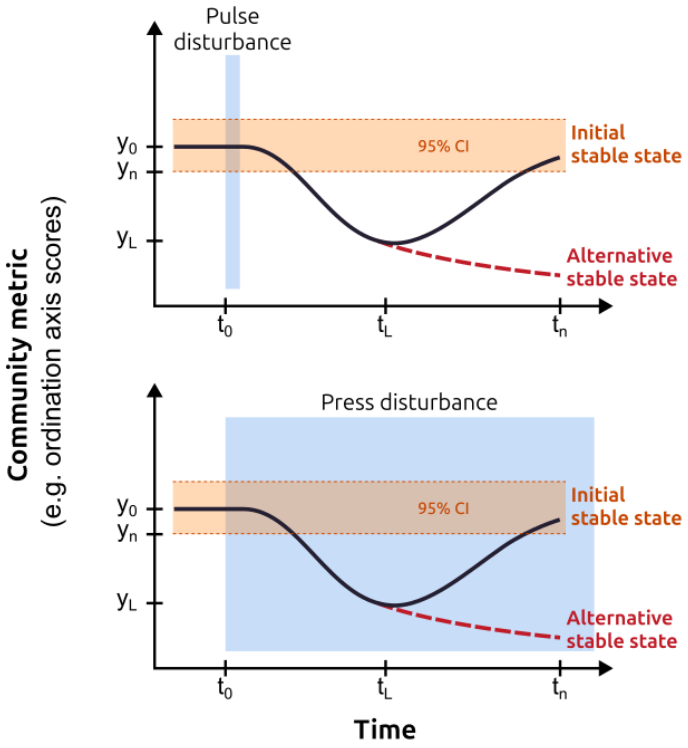

Figure 1 Resistance and resilience of microbial communities in response to changes in their environment. A microbial community parameter (red line, y-axis) reacts to a single-pulse (top) or continued (bottom) stressor. Resistance is defined as the degree to which the parameter is unaffected by the stressor, and resilience as the rate at which the parameter returns to its original value. A disturbance ends (pulse) and begins (press) at t_0 with a community metric at the initial state y_0. After a lag time t_L, the metric has changed to y_L. After time t_n, the parameter (y_n) may return to pre-disturbance values (initial stable state) or stay changed (alternative stable state). Figure adapted from Shade et al. (2012).

Since environmental changes that are characteristic in soils happen regularly, soil microbial communities have long histories of exposure to them. Microbial communities should therefore be physiologically or genetically adapted to certain types of pulse events and consequently show high resistance (i.e., the community does not change after perturbation) or high resilience (i.e., the community recovers from the perturbation to its initial state) towards such perturbations. Additionally, microbes exhibit traits like physiological flexibility, dormancy to overcome periods of low resource availability, and fast growth rates which should allow them to recover quickly from perturbations, especially if they are subjected to them regularly (Sorensen and Shade, 2020). However, the response of soil microbial communities to sudden, yet characteristic changes in their fluctuating environment, is not well known.

3. Soil microbial carbon storage

Microbial biomass growth is frequently understood synonymously with cellular replication. However, microorganisms can synthesise and store surplus carbon in the form of intracellular compounds. This allocation of carbon into storage compounds is an alternative pathway for biomass growth. It can play a crucial role in microbial survival, particularly under conditions where carbon is abundant but other growth factors, such as nitrogen or phosphorus, are limiting (Olukoshi and Packter, 1994; Alvarez and Steinbüchel, 2002; Butler et al., 2023). Storage compounds allow microbes to store carbon in a form that can be readily mobilised when external resources are limited.

Bacteria have the ability to store carbon using a diverse array of compounds, reflecting the adaptive strategies of microorganisms to different environmental pressures. These include polyhydroxyalkanoates (PHAs), glycogen, trehalose, wax esters, and TAGs. PHAs and wax esters are unique bacterial storage compounds, with PHAs primarily found in Proteobacteria and wax esters in Actinobacteria (Dalal et al., 2010; Murphy, 2012; Sehgal and Gupta, 2020). Glycogen, trehalose, and TAGs are also present in fungi and therefore not exclusive to bacteria (Preiss, 1984; Genet et al., 2000; Rangel-Castro et al., 2002; Elbein et al., 2003; Wilson et al., 2010; Mason-Jones et al., 2021). For instance, glycogen was found to accumulate in ectomycorrhizal *Lactarius* species in winter, but was almost non-existent during summer and became replenished in autumn (Genet et al., 2000). Unlike glycogen and trehalose, TAGs are hydrophobic, allowing for compact storage within lipid droplets with a high energy density. Although TAGs have been identified in various bacterial species, particularly within Actinobacteria (Alvarez and Steinbüchel, 2002), their prevalence in soil bacterial communities remains uncertain.

Microorganisms employ different storage strategies to optimise their survival in soil environments with high fluctuations in nutrient availability. These strategies can be broadly categorised into surplus storage and reserve storage (Mason-Jones et al., 2021). In surplus storage, microorganisms accumulate storage compounds during periods of resource abundance. Surplus storage allows microorganisms to rapidly sequester resources that can be utilised later during periods of scarcity, enabling them to maintain metabolic activity and growth. It also allows effective stoichiometric buffering and use of carbon resources, which can be particularly advantageous in environments of fluctuating resources (Manzoni et al., 2021). Reserve storage is a more strategic approach, where microorganisms continuously allocate resources into storage compounds at the expense of other metabolic demands. Although this direct cost impedes other physiological functions, it can be

a valuable investment for survival in scarce conditions and future reproductive success. Storage compounds can also fulfill additional functions and act against other stressors. For instance, trehalose was found to accumulate in response to heat, drying, and oxidative stress, and PHA can protect against high or low temperature, freezing, oxidative, and osmotic pressure (Elbein et al., 2003; Obruca et al., 2020).

4. Microbial necromass

The vast majority of carbon that enters soil comes from plants, either via dead plant material or root exudation (Fig. 2). Root exudates are simple carbon compounds and are readily metabolised by the microbial community, but dead leaf or root litter is mostly composed of chemically recalcitrant polymers like lignin, cellulose, and hemicellulose. Physical and bio-chemical transformation processes convert the plant material into a form which is aggregated and stabilised in soil, forming intricate associations with soil minerals, which compose the majority of soil organic matter (Miltner et al., 2012).

It was long assumed that these transformations form large organic polymers termed ‘humic substances’. However, in recent years this paradigm has been challenged by new insights on the chemical nature of soil organic matter, where soil microbes play a pivotal role in its creation and stabilisation. The emergent view is that plant residues are not inherently stable and continuously degraded by microbes (Lehmann and Kleber, 2015; Lehmann et al, 2020). Plant-derived organic matter is initially decomposed by microbes, which convert it into microbial biomass. When microbes die, the newly formed microbial necromass is stabilised and contributes to soil organic matter.

Soil microbial necromass is an important component of soil organic matter, constituting roughly half of the total organic carbon present in the soil (Liang et al., 2017; Liang et al., 2019; Wang et al. 2021). This includes intact or burst cells, fragmented cell walls, cytoplasmic components (monomers and polymers), and extracellular polymeric substances (Liang et al., 2019; Buckeridge et al., 2022). Necromass stabilises by interaction with mineral surfaces as well as other organic matter, and by occlusion in intra-aggregate pores at the sub-micron scale (Buckeridge et al., 2020).

Microbial necromass is frequently quantified by analysing hydrolyzed amino sugars, which stem from the chitin and peptidoglycan found in bacterial and fungal cell walls (Kästner et al., 2021; Salas et al., 2024). In addition to the chemically recalcitrant cell wall fragments, dead microbial cells release a wide variety of cytoplasmic and membrane molecules into the surrounding environment (Angst et

al., 2021; Camenzind et al., 2023). These molecules undergo continuous decomposition, breaking down from complex polymers into simpler monomers. The stabilisation of these decomposition products is intricately connected to the spatial heterogeneity and temporal variability of the soil matrix, factors that play a crucial role in determining how accessible they are to soil microorganisms (Lehmann and Kleber, 2015; Lehmann et al., 2020). The stabilisation process is further influenced by the chemical characteristics of the decomposition products themselves, such as their polarity and degree of ionisation, as well as their molecular diversity, which can affect how they interact with the soil environment and contribute to the long-term storage of organic matter (von Lützow et al., 2006, Lehmann et al., 2020).

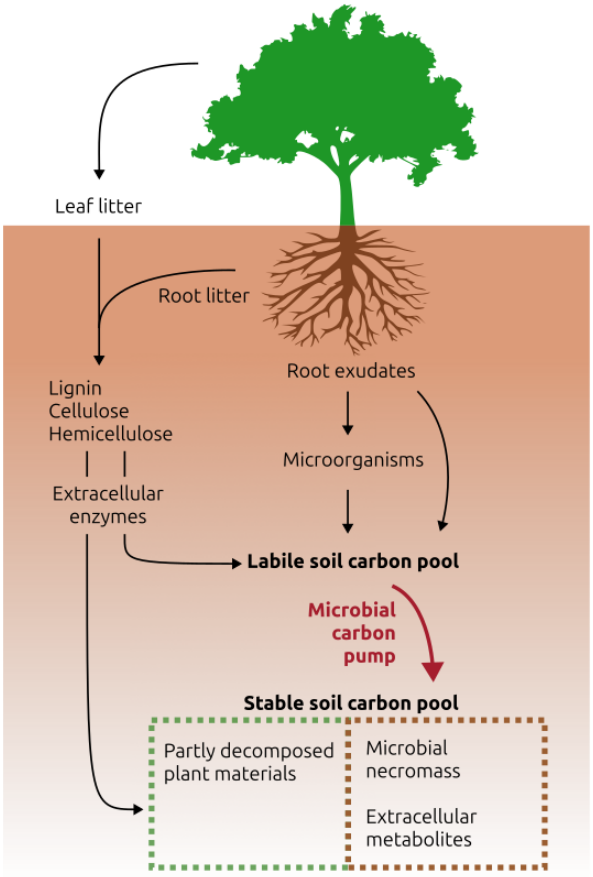

Figure 2 Carbon enters the soil primarily through plant-derived inputs, and is stabilised through microbial transformations. A portion of plant litter and exudates is converted into soil microbial biomass. When microbes die, fragmented microbial remains are stabilised on mineral surfaces or within soil aggregates. This 'microbial carbon pump' couples microbial growth with the formation of stable soil organic matter. Figure adapted from Liang et al. (2017).

5. Lipid biomarkers in soil microbial research

To effectively measure the composition and physiological traits of microbial communities, it is essential to identify marker compounds that can sensitively track their dynamics. These markers should be predominantly present in living biomass and exhibit a diverse range of structural forms, enabling the distinction of various taxonomic groups. Lipids are a diverse class of hydrophobic molecules, which serve various physiological roles in microorganisms (Ratledge, 1997). As such, they can act as reliable indicators of key functions like cellular growth, carbon storage, and stress responses (Shi et al., 2017; Ding et al., 2020; Mason-Jones et al., 2021; Willdigg and Helmann, 2021).

A widely used lipid-based method for analysing soil microbial communities is the measurement of phospholipid-derived fatty acids (PLFAs). Since phospholipids are present only in living cells, PLFA profiling enables quantification of viable microbial biomass across coarse taxonomic groups, as well as sensitive tracing of shifts in microbial community structure (Frostegård et al., 2011; Willers et al., 2015). This method is particularly powerful when coupled with stable isotope probing (SIP), enabling the quantification of carbon fluxes (^{13}C-SIP; Fuchslueger et al., 2014; Kaiser et al., 2015) or microbial growth (^{2}H-SIP; Canarini et al., 2023; Caro et al., 2023).

While DNA- or RNA-based methods offer higher taxonomic resolution, the PLFA method shows similar sensitivity in detecting changes in microbial community composition, while also enabling absolute quantification of microbial biomass (Ramsey et al., 2006; Orwin et al., 2018). It allows simultaneous quantification of bacterial and fungal biomass, whereas DNA/RNA approaches require separate analyses. Moreover, PLFAs are measured directly, which is particularly advantageous in SIP approaches where undiluted signals can be quantified, providing higher sensitivity compared to methods which require PCR amplification (Bengtson et al., 2009). DNA/RNA-SIP also increases the number of samples to analyse after density centrifugation—complicating analysis of large sample sets—while the PLFA method allows for high sample throughput (Buyer and Sasser, 2012; Hungate et al., 2015).

Apart from phospholipids, the PLFA method has the potential to be expanded to other lipid fractions. Neutral lipid-derived fatty acids (NLFAs) are particularly intriguing, as they may serve as valuable indicators of microbial carbon storage. Fungi are known to store neutral lipids, and fungi-specific NLFAs have been used to quantify fungal carbon storage in soils (Bååth, 2003). In contrast, soil bacteria are often assumed to be unable to synthesise neutral lipids and to rely on other

storage compounds. Instead, bacterial NLFAs have been suggested to originate from recently turned-over phospholipids (Bååth, 2003; Rinnan et al., 2005). This implies that they indicate bacterial necromass, but this interpretation lacks substantial evidence. Indeed, recent evidence suggests that bacterial NLFAs are derived from storage lipids (Mason-Jones et al., 2021). Bacterial NLFAs potentially provide sensitive information on important soil carbon pools, but it remains unclear whether they are markers for carbon storage or necromass.

5.1 Diversity of soil lipids

Lipids are ubiquitous compounds in all organisms as they are key components in the lipid bilayer of the cell membrane and intracellular storage compounds. Most lipids consist of a glycerol backbone, to which fatty acids are attached, forming a hydrophobic 'tail' (Fig. 3). Phospholipids contain two fatty acids, while the third glycerol moiety attaches to a hydrophilic 'head' which contains the phosphate group. This head group can vary in chemical composition, for instance incorporating ethanolamine, choline, serine, or inositol (Ding et al., 2020). The most common phospholipids in soil appear to be phosphatidylethanolamine and phosphatidylcholine (Mangelsdorf et al., 2009; Warren, 2018, 2019, 2020). However, microbes can use other lipids to substitute phospholipids when phosphorus availability is limited. Warren (2019) found that while phospholipids constituted over 90% of the total polar lipid abundance in soils where phosphorus was not limiting, the betaine lipid diacylglyceryl-N,N,N-trimethylhomoserine (DGTS) constituted 40% under phosphorus limitation. In accordance, Benning et al. (1995) found that *Rhodobacter sphaeroides* accumulates glycolipids and betaine lipids under phosphorus limitation. Glycolipids occur in many bacterial groups, and are abundant in chloroplasts, cyanobacteria, and arbuscular-mycorrhizal arbuscules and spores (Hölzl and Dörmann, 2007; Grigera et al., 2007).

Triacylglycerols (TAGs) have three fatty acids attached to their glycerol backbone and are electrostatically neutral (Fig. 3). They are important carbon storage compounds and are stored in energy-rich lipid droplets (Wältermann and Steinbüchel, 2005; Dalpé et al., 2012; Gao and Goodman, 2015). Their synthesis is widespread in eukaryotic organisms, although certain bacterial species have also been shown to produce TAGs (Alvarez and Steinbüchel, 2002; Wältermann and Steinbüchel, 2005; Murphy, 2012).

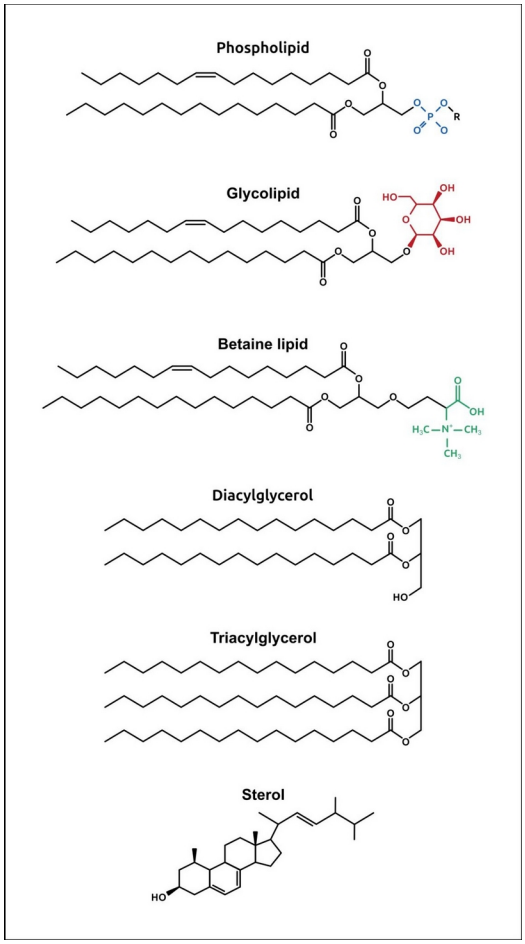

Figure 3 Example structures of microbial lipid classes which may be extracted with the PLFA method.

Diacylglycerols (DAGs) potentially have two sources in soils. They may stem from dead bacteria, as one of the first steps in phospholipid degradation is the cleavage of the charged phosphate head (Titball, 1993, Zhang et al., 2019). DAGs are also the anabolic precursors of both phospholipids and TAGs in cellular lipid synthesis (Eichmann and Lass, 2015; Alvarez, 2016) and can occur in substantial intracellular amounts (Ejsing et al., 2009).

Sterols are unique membrane lipids structurally based on cyclopentanes and -hexanes, which completely lack a fatty acid tail (Fig. 3). Phytosterols, such as β-sitosterol, have been used as markers for identifying plant-derived organic matter, and cholesterol as a marker for soil micro- and mesofauna (Puglisi et al., 2003). Fungi produce various sterols that are specific to certain taxa and clades, with ergosterol being the most common (Weete et al., 2010). Other fungal sterols include cholesterol, lanosterol, and brassicasterol, as well as some sterol biomarkers that may be specific to certain phyla, like 24-ethyl cholesterol for

Glomeromycota (Weete et al., 2010). Ergosterol, in particular, has been widely used as a biomarker for living fungal biomass due to its strong taxonomic specificity and high correlation with fungal hyphal length and the fungi-specific PLFA 18:2w6,9, making it a useful marker for quantifying fungal biomass (Stahl and Parkin, 1996; Klamer and Bååth, 2004; Wallander et al., 2013).

5.2 Fatty acid diversity and nomenclature

Most lipids, except sterols, contain fatty acids. Microbial fatty acids can vary in their length, consisting of 14–22 carbon atoms. In most microorganisms, the predominant lengths are 16 and 18 carbon atoms (Ratledge, 1997). They can branch off with methyl or hydroxyl groups, and can be saturated or unsaturated with varying amounts of double bonds. The structure of fatty acids is represented by the nomenclature A:B, where A is the total number of carbon atoms and B is the number of double bounds. The position of double bonds in unsaturated fatty acids is determined by counting from the carboxyl group towards the first carbon atom in the double bond, and designated by the greek letter ω. Thus, 18:0 is a saturated fatty acid with 18 carbon atoms, and 18:2ω6,9 is an unsaturated fatty acid with two double bonds on the sixth and ninth position when counting from the carboxyl terminus. The isomer can also be specified by adding *c* (for *cis)* or *t* (for *trans*) as a suffix. Terminal iso- and anteiso branching are specified by the suffix *i* and *a*, cyclopropane fatty acids are specified by the prefix *cy*, and methyl-branches on the tenth carbon atom are specified by the prefix *10Me*.

5.3 Analysis of phospholipid fatty acids

The extraction of lipids is commonly achieved by using a monophasic mixture of methanol, chloroform, and water. Dilution of the extract with chloroform and water results in a phase separation, where the lower chloroform layer contains a purified total lipid extract. This procedure is based on the seminal paper by Bligh and Dyer (1959), who aimed for a purified lipid extract of biological tissues. This method was later adjusted for extracting soils by replacing water with citrate buffer at pH = 4 (Frostegård et al., 1991). Further purification of lipid classes is achieved by fractionation on silicic acid solid phase extraction, where lipids are eluted with solvents of increasing polarity, which consecutively yield neutral lipids (chloroform), glycolipids (acetone), and phospholipids (methanol; Frostegård et al., 1991; Frostegård et al., 1993; Frostegård and Bååth, 1996). Finally, lipids are derivatised into fatty acid methyl esters (FAMEs) via mild alkaline methanolysis. FAMEs are then quantified with gas-chromatographic methods (GC-FID, GC-MS).

The structural characteristics of phospholipid-derived fatty acids (PLFAs) can be used to specify broad taxonomic groups (Zelles, 1997; Frostegård et al., 2011;

Willers et al., 2015). While straight-chain saturated fatty acids are common across microbial groups, the fatty acids 18:2ω6,9 and 18:1ω9 are abundantly found in fungi, other monounsaturated and cyclopropyl saturated fatty acids are often found in gram-negative bacteria (e.g. 16:1ω9, 18:1ω5, 19:1ω9, cy17:0, cy19:0), and terminally-branched fatty acids are often found in gram-positive bacteria (e.g. i14:0, i15:0, a15:0). Methyl-branched fatty acids, but not terminally-branched fatty acids, are often found in Actinobacteria, and the argument has been made that therefore terminally-branched fatty acids specify the phylum Firmicutes (Joergensen, 2022).

Complete soil PLFA profiles provide a 'fingerprint' of the microbial community composition, and multivariate statistical analyses can give an idea whether the microbial community was affected by a specific treatment. Due to their taxonomic specificity, PLFAs can also be used to discern if the abundance of specific microbial groups is affected by a treatment, or if it differs between soils. PLFAs are sensitive indicators of microbial biomass, and can achieve comparable results as 16S rRNA gene metabarcoding and other PCR-based approaches (Ramsey et al., 2006; Orwin et al., 2018). The analysis of $^{13}C/^{12}C$ or $^{2}H/^{1}H$ isotope ratios is easily integrated into the method by an additional measurement on GC-IRMS, which makes PLFAs particularly powerful to investigate carbon fluxes or microbial growth when coupled with SIP approaches (Gorka et al., 2019; Canarini et al., 2021; Schnecker et al., 2023).

Changes in PLFA patterns can also implicate an adaptive response to environmental stressors by restructuring the composition of the cell membrane, thereby maintaining membrane fluidity (de Carvalho and Caramujo, 2018). Thus, ratios of saturated to unsaturated fatty acids, trans- to cis configurations, and anteiso- to iso branching have been used to indicate physiological stress (Wixon and Balsner, 2013; Willers et al., 2015).

A disadvantage of the PLFA method is that, while it captures microbial community dynamics of soil bacteria and fungi, Archaea are neglected. This is because archaeal cell membranes consist of unique polar lipids where the 'tail' is attached by ether bonds to the glycerol backbone (ether phospholipids), while eukaryotic and bacteria use ester bonds (Gattinger et al., 2003). The PLFA method also captures a reduced lipid diversity by the derivatisation to FAMEs, which disregards the structural variation in the lipid head groups as well as the combinations of fatty acid chains. Indeed, while usually 10–18 PLFAs are found in soil samples, modern lipidomics approaches based on LC-MS/MS or LC-HRMS can identify several hundred complete lipids (Zhang et al., 2022). However, modern lipidomic

approaches also come with caveats, as a large fraction up to the majority of MS spectra can remain uncharacterised, the position of double bonds in unsaturated fatty acids cannot be elucidated, and only few taxonomic marker compounds are yet identified (Ding et al., 2020; Couvillion et al., 2023).

5.4 Expanding the PLFA method to other lipid fractions

The PLFA method potentially yields two additional lipid fractions which are often disregarded: glycolipids and neutral lipids. Glycolipids are membrane lipids and can be utilised as substitutes for phospholipids (Hölzl and Dörmann, 2007). They can also increase microbial resistance against heat or oxygen radicals (Nakata, 2000), but their definite function is not fully understood. In contrast, neutral lipids are generally assumed to be derived from TAGs, which are used for intracellular carbon storage. Since fungi are known for producing TAGs, fungi-specific NLFAs can be used to estimate fungal carbon storage (Bååth, 2003). In particular, the neutral lipid-derived fatty acid (NLFA) 16:1ω5 is frequently collected to quantify the biomass of arbuscular-mycorrhizal fungi (Olsson et al., 1995). While PLFA 16:1ω5 is also highly specific to arbuscular-mycorrhizal fungi, it is also found in gram-negative bacteria (Olsson, 1999). Bacteria however only produce negligible amounts of neutral lipids containing the fatty acid 16:1ω5, suggesting that they don't produce TAGs to the same extent as fungi. This makes the NLFA 16:1ω5 a reliable marker for arbuscular-mycorrhizal fungal biomass (Olsson and Lekberg, 2022).

NLFAs that are specific to bacteria are rarely analysed, even though they can be found in high abundance in soils extracts. Bacterial species have been shown to produce TAGs for carbon storage, and bacterial NLFAs have indeed been interpreted this way (Alvarez and Steinbüchel, 2002; Mason-Jones et al., 2023). However, some authors argue that the synthesis of bacterial TAGs is not widespread, and that soil bacterial NLFAs are actually derived from recently degraded phospholipids (e.g. Bååth, 2003; Rinnan et al., 2005; Amir et al., 2008). This would mean that bacterial NLFAs originate from dead cells and are indicative of bacterial necromass. This interpretation comes with certain assumptions on phospholipid degradation pathways, as not all phospholipases produce DAGs (Titball, 1993; Fisher and Jain, 2009; Ramrakhiani and Chand, 2011; Barman et al., 2018). It is also unclear whether neutral lipids can persist for long periods in soil, although they could be stabilised via hydrophobic interactions or occlusion in intra-aggregate pores (von Lützow et al., 2006; Hafida et al., 2007; Totsche et al., 2018). Furthermore, if bacterial phospholipids are degraded to DAGs, the same would apply to fungal phospholipids, which would suggest that a part of fungal NLFAs may be derived from dead fungal cells. Nonetheless, either interpretation (carbon

storage or necromass) indicates that bacterial NLFAs can be powerful markers of an important soil carbon pool.

6. Thesis outline and main goals

Understanding how soil microbial communities respond to environmental changes is crucial for predicting ecosystem resilience and function. Soils are dynamic environments, constantly influenced by natural perturbations that can significantly alter microbial activity and community composition. In addition, refining methods to accurately characterise these microbial communities is essential for advancing our knowledge of soil ecology. This thesis addresses these challenges by exploring the responses of soil microbes to common environmental perturbations and evaluating the potential for extending existing lipid analysis techniques to provide deeper insights into microbial community structure and function.

The main goals of this PhD thesis were (i) to examine how soil microbes respond to environmental perturbations that are common in soils, and (ii) to evaluate the PLFA extraction method to analyse other lipid fractions apart from phospholipids, assess if taxonomic specificities of fatty acids persist across all lipid fractions, and discuss their ecological meaning as marker compounds, and (iii) understand whether soil bacterial NLFAs represent bacterial storage compounds or microbial necromass.

Chapter 2 focuses on the short- and long-term response of a soil microbial community to single-pulse environmental perturbations which occur naturally in soils, but have disparate effects on microbial growth and activity. For this, beech forest soil was incubated under constant temperature and water content. The soil was subjected to either a one-time freeze-thawing event or a single-pulse glucose addition. I subsequently monitored changes in microbial community composition via PLFA and NLFA analysis, as well as soil carbon and nitrogen stoichiometry for a duration of 160 days. I found that both single-pulse perturbations influenced the microbial community structure, microbial biomass nitrogen, and dissolved nitrogen pools for up to 160 days. This indicates that even environmental changes which are common in soils and the microbial community should be well adapted to experience can have long-lasting effects on multiple soil parameters.

In a methodological approach, **Chapter 3** explores the expansion of the PLFA extraction method to other lipid fractions. Specifically, I tested (i) whether the PLFA method can be expanded to concurrently extract complete NLFA and GLFA profiles, as well as sterols, (ii) whether fatty acid profiles are retained across the three lipid fractions in pure culture strains, and (iii) whether NLFAs and GLFAs

allow fingerprinting of microbial community functions to the same extent as PLFA analysis. Addition of 2% ethanol to chloroform for silica solid phase extraction resulted in a complete separation of pure neutral, glyco-, and phospholipids. A high recovery of pure sterols was found in the neutral lipid fraction, albeit soil extracts only yielded miniscule amounts of sterols. Taxonomic specificities of fatty acid markers were consistent across PLFAs, NLFAs, and GLFAs in microbial pure cultures. However, fatty acid profiles from soil extracts showed stronger differences between the three lipid fractions than between soil types. The results indicate that the extraction of PLFAs, NLFAs, and GLFAs provide estimates of different properties of soil microbial communities, and that their combined measurement is a low-effort but potentially information-rich addition to the PLFA extraction method.

Chapter 4 addresses the question whether bacterial NLFAs found in soils represent bacterial storage compounds or microbial necromass. NLFAs specific to bacteria can be abundant in soil, and some bacteria are known to produce neutral TAGs for carbon storage. However, some authors assume that soil bacteria are incapable of producing TAGs, and bacterial NLFAs are rather derived from degraded phospholipids and therefore markers for bacterial necromass. I discuss that neutral lipids may only be intermediate compounds in merely two of several phospholipid degradation pathways. In contrast, I find that a significant portion of soil bacterial isolates have the genetic potential to synthesise TAGs, and labile carbon addition leads to rapid allocation into bacterial NLFAs, which indicates storage of excess carbon. Overall, it is likely that bacterial NLFAs are mostly derived from storage compounds, with a possible marginal contribution from degraded phospholipids. Soil bacterial NLFAs thus have the potential to be sensitive markers for bacterial carbon storage.

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Chapter 2

One-time freeze-thawing or carbon input events have long-term legacies in soil microbial communities

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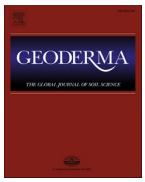

One-time freeze-thawing or carbon input events have long-term legacies in soil microbial communities

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ABSTRACT

Soil microbial communities are regularly exposed to sudden changes in environmental conditions, such as root exudation pulses or freeze-thaw events. As microbial communities have a high potential to adapt to changing conditions, they are expected to be resilient towards this kind of short-term perturbations and return to their pre-perturbed state quickly. Here, we conducted a lab incubation experiment to evaluate the resilience of soil microbial communities to single-pulse perturbations.

We incubated temperate forest soil at constant temperature (20 °C) and water content, and exposed it to strong single-pulse perturbations, which nonetheless mimic common pulse-events in temperate soils (glucose addition at 4 mg g^{-1} soil, or freeze-thawing overnight at −20 °C). We subsequently measured microbial community composition and microbial storage compounds via phospho- and neutral lipid fatty acid (PLFA and NLFA) profiling, as well as C/N stoichiometry of microbial biomass and dissolved organic carbon and nitrogen in the soil solution shortly after (0.4, 1, 4, and 6 days) and after longer time periods (84 and 160 days) following the perturbations.

Transferring the soils from their natural environment to the laboratory and incubating them under controlled conditions led to a continuous change of microbial community structure over time, along with an increase in microbial biomass and dissolved N in both perturbed and control soils over the time of the experiment. Against the background of this 'press-disturbance', caused by the permanently changed conditions, we see immediate and long-lasting effects of the single pulse events on microbial community composition, C storage and C/N stoichiometry. Both perturbations significantly influenced the microbial community structure (based on PLFA profiles), microbial biomass N and dissolved N up to 160 days, as well as fungal and bacterial biomass and storage (based on absolute PLFA and NLFA concentrations) up to 84 days. Both perturbations increased microbial N (+59.6 μg g^{-1} dw) and decreased dissolved N (−40.3 μg g^{-1} dw) after 160 days, and significantly altered C/N ratios in microbial and dissolved pools (particularly in the first 6 days of the experiment).

Our results demonstrate that single-pulse perturbations can have long-term legacies in soil microbial ecosystems. In our experiment they led to alternative system states which differed from the unperturbed control in multiple parameters even after 160 days. This indicates that soil microbial communities exhibit a low resistance and resilience towards single-pulse perturbations, and may easily be pushed on alternative trajectories by short but strong environmental pulses.

1. Introduction

Soil environmental conditions often change rapidly as a result of temporally distinct, pulsed events. These changes act as perturbations on soil microbes, potentially inducing adaptive responses of the microbial community which may shift it towards a different, alternative state (Kuzyakov and Blagodatskaya, 2015; Nguyen et al., 2020; Yang et al., 2008). Pulse events can be characteristic to the ecological system in which they occur, perturbing the soil microbial community regularly and at various spatial scales. Common examples include changes in temperature or water content, or sudden bursts of carbon (C) and nutrient availability. For instance soil surface drying and rewetting after

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rainfall events is experienced in most terrestrial ecosystems. In temperate forests specifically, freeze-thawing in spring can kill a large portion of the microbiome, which can have large-scale repercussions on microbial C and nitrogen (N) cycling (Kaiser et al., 2011; Mooshammer et al., 2017). Moreover, growing roots exude labile C compounds into a spatially restricted soil volume for a limited amount of time, creating hotspots of microbial activity and growth (Kuzyakov and Blagodatskaya, 2015).

Since these pulse events happen regularly, soil microbial communities have long histories of exposure to them. Each community should therefore be physiologically or genetically adapted to certain types of pulse events and consequently show high resilience (i.e., the community recovers from the perturbation to its initial state; (Allison and Martiny, 2008; Rykiel, 1985; Shade et al., 2012) towards such perturbations. Additionally, microbes exhibit traits like physiological flexibility, dormancy to overcome periods of low resource availability (Sorensen and Shade, 2020), and fast growth rates which should allow them to recover quickly from perturbations, especially if they are subjected to them regularly.

Pulsed shifts in environmental conditions can lead to drastic short-term changes (hours to days) in microbial activity and community composition (Allison and Martiny, 2008; Shade et al., 2012). For instance, repeated freeze-thaw cycles can induce immediate changes in microbial community structure (Ji et al., 2022; Sharma et al., 2006) and reduce fungal biomass (Feng et al., 2007) – particularly at lower frost temperatures (Schmitt et al., 2008). However, the effects of freeze-thawing may not last long; for instance, Koponen et al. (2006) found the microbial community structure and biomass unaffected three weeks after four freeze-thaw cycles. Similar to freeze-thaw events, labile C inputs have immediate effects on soil microbial communities. Strong shifts in microbial community composition have been observed within hours to days after labile C addition (Cleveland et al., 2007; Langer and Rinklebe, 2011). Additionally, the effect of C addition also changes when repeated: Mau et al. (2015) observed a strong decrease in bacterial diversity a week after a single glucose addition, while subsequent repeated pulses increased diversity.

While most studies analyse the short-term effect on microbial communities, long term responses beyond a few days or weeks are rarely investigated. Moreover, studies on long-term responses usually apply multiple pulses or continuous stressors (Allison and Martiny, 2008; Shade et al., 2012). Such studies investigate shifts in community structure as an adaptive response to repeated pulses which depend on the amount, intensity and frequency of the perturbations (Allison and Martiny, 2008; Nguyen et al., 2020; Shade et al., 2012). In contrast, exposing microbial communities to only a single pulse perturbation allows to study the resilience of the microbial community to the single-pulse perturbation, which is conditioned by their history of interactions with environmental fluctuations. The long-term response of soil microbial communities to single-pulse perturbations, which represent characteristic changes in their environment, is however lacking observations and not well known.

Here, we aimed to determine how soil microbial communities, as well as their stoichiometric environment (i.e. C and N in the microbial biomass and in the soil solution) respond to single-pulse environmental changes which occur commonly under natural conditions, but have disparate effects on microbial growth and activity. Specifically, we incubated a temperate beech forest soil under constant environmental conditions, and monitored changes in its microbial community composition and biomass, and soil environmental C and N stoichiometry, after (i) a one-time freeze-thawing event, and (ii) a single-pulse addition of glucose for a duration of 160 days. Since we additionally expected the soil system to slowly change in such a lab incubation experiment in a controlled environment (i.e., a transient state change), we additionally incubated (iii) untreated control soil which allowed us concurrent comparisons of perturbed vs. control soil as a measure of resilience. Our aim was to investigate if microbial communities are resilient against single-pulse perturbations, and if so, how long it would take for them to return to the state of a non-perturbed community. We expected that the community responds quickly to the perturbations, but is ultimately resilient to the perturbations; however, we were uncertain how long it takes the communities to return to its initial state (days or months). This experiment explicitly entailed a controlled setting of isolated soil mesocosms to reduce effects of microbial community dispersion/immigration from unperturbed regions, which allowed us to observe community dynamics after the pulse events in isolation.

2. Material and methods

2.1. Sampling and experimental setup

Soil was collected from a mature beech (*Fagus sylvatica* L.) forest site close to Hagenbach Valley (Hagenbachklamm) in the Vienna Woods near Vienna, Austria in January 2018. The top 5 cm of the soil were sampled after aboveground biomass had been removed with a knife. Soil organic C and total N content comprised 13.0 % and 0.78 % of dry mass, respectively. As the aim of this study was to trace the response of the soil microbial community to environmental perturbations in a controlled, artificial setting (and not to capture the ecological variablity of the forest soil), we aimed for high consistency across replicates at the start of the experiment. Consequently, all soil was pooled and homogenised by sieving (2 mm mesh size), and 25 g each were subsequently added to 90 glass jars in total. Aluminium caps used to close the jars had a hole in the middle (1 cm diameter) filled with cotton wool, which allowed gas exchange but prevented excessive water loss.

After one week of acclimatisation at 20 °C, we applied two treatments: (i) freeze-thawing, where 30 glass jars were frozen at −20 °C overnight, and (ii) addition of 100 mg glucose to 30 glass jars each (4 mg glucose g^{-1} soil). We chose the treatments to resemble strong to extreme natural perturbations. The freeze-thawing event was designed to simulate alpine temperature fluctuations (Wundram et al., 2010) to induce a strong response of the microbial community. For the glucose treatment we aimed to simulate the extremely high concentration of labile C compounds occurring in a restricted soil volume around exuding root tips. Phillips et al. (2011) estimated root exudation rates at around 1.5 μg C cm^{-1} fine root length per hour based on *in situ* field measurements of 15–20 cm long pieces of fine roots of loblolly pine trees. As root exudation is thought to be heterogeneously distributed along fine roots we assumed that root tips, which are thought to exhibit the highest rates of root exudation (Canarini et al., 2019; Cardon and Gage, 2006), will exude up to a 10× higher rate compared to the average. Assuming that root exudates diffuse about 1 mm away from their point of origin (Finzi et al., 2015), a cylinder with a radius of 1 mm around a 1 mm long root tip (3.1416 mm^3) would thus increase its concentration of labile C by 477.5 μg C $cm^{-3}h^{-1}$ (15 μg C $cm^{-1}h^{-1}$ × 0.1 cm/0.0031415 cm^3). A root exudation pulse of around 8 h would thus resemble a cumulative input leading to a final concentration of around 4000 μg C cm^{-3} in this small soil volume, which approximately resembles the 5 mg g^{-1} soil we applied (at a bulk density of 1 g soil cm^{-3}). We believe that our estimates are likely at the upper limit of possible C input during a root exudation event. However, not much knowledge exists on root exudation rates, which are difficult to measure *in situ* (Oburger and Jones, 2018). We upscaled this hypothetical 'hotspot' root tip C input to the whole soil sample of 25 g, to allow us to sample enough volume for measurements. Another 30 glass jars did not receive any treatment but served as unperturbed control samples. The glucose was applied in powder form as the addition dissolved in water would influence the water content of the soils which would potentially act as another perturbation. Glucose was added at the same time the soil from the freeze-thaw treatment was thawed at room temperature. To provide homogeneous distribution of the added glucose, jars were sealed with a lid and rigorously shaken for 30 s. Shaking was done for all soil samples regardless of treatment to ensure equivalent conditions. Soil samples from both treatments, and

untreated controls, were subsequently stored at 20 °C until harvest. The soil was re-wetted weekly to ensure constant water content. Five replicates of each treatment were destructively harvested at six timepoints: 4 h, 1 d, 4 d and 6 d (henceforth collectively referred to as 'short term'), as well as 84 d and 160 d (henceforth referred to as 'long term') after treatment.

2.2. Carbon and nitrogen in microbial biomass and dissolved pools

To determine dissolved organic C, total dissolved N (TDN) and C and N in microbial biomass, 1.5 g of fresh soil were extracted with 15 ml of 0.5 M K_2SO_4 before and after chloroform fumigation extraction (CFE). Aliquots of the extracts were measured on a TOC/TN analyser (TOC-V CPH/TMN-1, Shimadzu, Japan). Microbial biomass was calculated as the difference between fumigated and unfumigated samples. We did not include biomass conversion factors into our calculations.

2.3. Fatty acid analysis

We used phospholipid fatty acid (PLFA) profiles as a fingerprinting method for microbial community composition. Although PLFA profiles have less taxonomic resolution than sequencing based approaches, they provide comparable results for tracking changes in microbial communities (Ramsey et al., 2006), sometimes have even higher power to differentiate treatment effects (Orwin et al., 2018), and allow quantification of absolute biomass of broad microbial groups.

Lipids were extracted with a monophasic mixture of chloroform, methanol and citrate buffer (v:v:v = 1:2:0.8) from lyophilised soil samples (Bligh and Dyer, 1959; Frostegård et al., 1991). After extracting overnight (>8h), phase separation was induced by addition of chloroform and citrate buffer. The lower phase was transferred to new vials and dried under a constant stream of N_2. The total lipid extracts were then loaded on silica columns, and fractionated based on lipid polarity with subsequent elution with chloroform, acetone and methanol. The chloroform and methanol fractions were collected, yielding neutral lipids (containing triacylglycerids) and phospholipids, respectively. These lipid fractions were then derivatised to fatty acid methyl esters via mild alkaline methanolysis (Frostegård et al., 1991), and measured in 100 µl *iso*-octane on a gas chromatograph (Trace GC Ultra, Thermo Scientific, Germany) coupled to a mass spectrometer (ISQ, Thermo Scientific, Germany). Methyl nonadecanoate was added to samples prior to methylation and used as an internal standard for quantification. We thus differentiated between neutral lipid derived fatty acids (NLFA), which indicate C storage in fungi (Bååth, 2003), and PLFA, which are indicators for viable microbial biomass. Bacterial NLFA can be used as C storage markers (Alvarez and Steinbüchel, 2002; Mason-Jones et al., 2021), but may also reflect bacterial necromass (Amir et al., 2008; Malosso et al., 2004) and should therefore be intepreted with caution.

As indicators for microbial groups, we assigned the fatty acids 18:1ω9 (*cis* and *trans* form) and 18:2ω6,9 to fungi, and other unsaturated fatty acids (16:1ω5, 16:1ω7, 17:1ω7), straight-chain saturated fatty acids (14:0, 15:0, 17:0), terminally branched fatty acids (a15:0, i15:0, i16:0, a17:0, i17:0) and cyclopropyl fatty acids (17:0cy, 19:0cy) to bacteria. The fatty acids 16:0 and 18:0 were used as markers for general microbial biomass. We removed the NLFA 16:1ω5 from our analysis on the broad phyla of fungi and bacteria as it is also known to occur in arbuscular-mycorrhizal tissue and spores (Olsson et al., 1995), and analysed it separately.

2.4. Statistics

All statistical analyses were done using R (v4.20). We visualized the temporal dynamics of the microbial community composition via correspondence analysis (CA, with symmetric scaling; package *vegan* v2.6–2; (Oksanen et al., 2020) of the full PLFA dataset, as well as separately for the short- and long-term. Differences in community composition were analysed via two-sample t-tests after testing for normality (via Shapiro-Wilk test) and homogeneity of variances (via Levene's test) on scores of the first and second ordination axes (CA1, CA2). When the assumptions of the *t*-test were violated, Mann-Whitney tests (function *wilcox.test* with *paired* = *FALSE*) were used. We additionally tested the same dataset for general patterns of clustering with PERMANOVA with Bray-Curtis distances via the function *adonis2* from the *vegan*-package. Significant differences to control treatment values of univariate data were calculated via t-tests or Mann-Whitney tests. Differences were considered significant when $p < 0.05$ for all analyses. All figures were created with the package *ggplot2* (v3.3.6; Wickham, 2016).

3. Results

3.1. Experimental conditions during laboratory incubation influenced the microbial community composition

PLFA profiles from control samples indicate restructuring of the microbial communities throughout the duration of the experimental incubation (Fig. 1), most likely due to a permanent change in several environmental parameters (e.g. lack of input of C and nutrients, constant temperature, no influence of plant roots and animals). Consequently, the control soils were not in a steady state, but rather changed slowly within a transient state. Notably, we observed the strongest restructuring of the community in the first week of tracking, and comparatively minor changes in the long term between three and five months (Fig. 1A). Specifically, the first week is characterised by high inter-sample variability (Fig. 1B). In the long term, however, all control samples became more similar to each other (see states at 84 and 160 days in Fig. 1C and Table S1).

The time evolution of the control system can also be seen in changes in microbial biomass based on PLFA, and microbial and dissolved C and N, which varied considerably over the incubation time (Fig. 2, Fig. 3). In particular, fungal and bacterial biomass showed a 25 % and 33 % average increase during the total period (Fig. 2). We also note the accumulation of dissolved N which, although it can be considered negligible in the first week, showed a steady overall increase up to 200 % of the initial value, with an average rate of 1 $\mu g\ g^{-1}$ DW day^{-1}. Thus, the experimental controlled environment had a strong effect on the overall soil system, including PLFA community profiles, microbial biomass, and dissolved C and N pools.

3.2. Single-pulse perturbations had short- and long-term effects on the microbial community

Both single-pulse perturbations affected the microbial community composition in the short term. Multivariate analysis of the PLFA data shows that in the short term freeze-thawed communities deviated more strongly from the control community compared to the communities that received glucose addition (Fig. 1B and Fig. S1, Table 1). Additionally, PERMANOVA results indicate significant effects of the single-pulse perturbations as well as experimental duration on the microbial community composition (Table S1).

Furthermore, we observed legacy effects of both perturbations, as communities were still significantly altered 84 and 160 days after freeze-thawing and glucose addition, respectively (Fig. 1C, Table 1). Specifically, glucose addition was well separated from the control on the first CA axis which explains most of the variance (38.27 % when only analysing long-term timepoints, Fig. 1C; 65.65 % when analysing all timepoints together, Fig. 1A). The composition of the glucose amended community was still significantly separated from control values in the long term (both at 84 and 160 days) on the first CA axis ($p < 0.001$, Table 1). At the same time it also showed a clear tendency to very slowly evolve towards the composition of the control community, being visually closer (but still different) to it after 160 days than it was after 84 days on the CA biplot (Fig. 1A and C). The freeze-thawed community

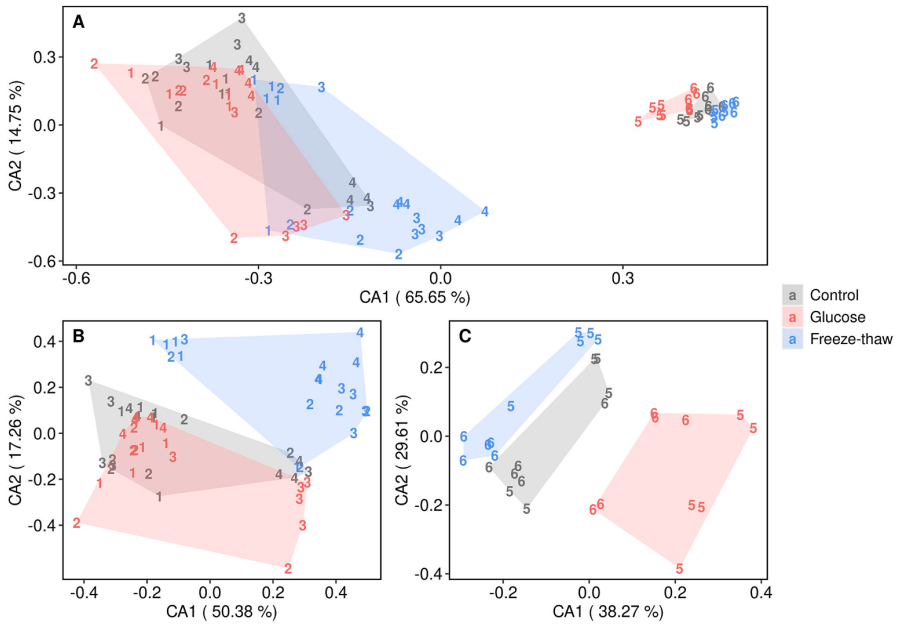

Fig. 1. Short- and long-term effects on microbial community composition after one-time events of glucose addition and freeze-thawing. Shown are correspondence analysis (CA) ordination biplots of the microbial community based on PLFA biomarkers. The community structure is depicted in (A) both short and long term, (B) only the short term and (C) only the long term after treatment application. Effects of glucose addition (red) and freeze-thawing (blue) on the community composition compared to the control (grey) are still evident after 160 days. Differences between treatments were tested on the full dataset (plot A) via two-sample tests on CA axis scores (Table 1, Fig. S1) and PERMANOVA (Table S1). There was a strong decrease in compositional variance in the long term (timepoints 5 and 6 in plot A) compared to short-term timepoints (timepoints 1–4). The variance explained by each CA axis is given in parentheses. Numbers correspond to ranked sampling timepoints: 1 – 0.4 d, 2 – 1 d, 3 – 4 d, 4 – 6 d, 5 – 84 d, 6 – 160 d.

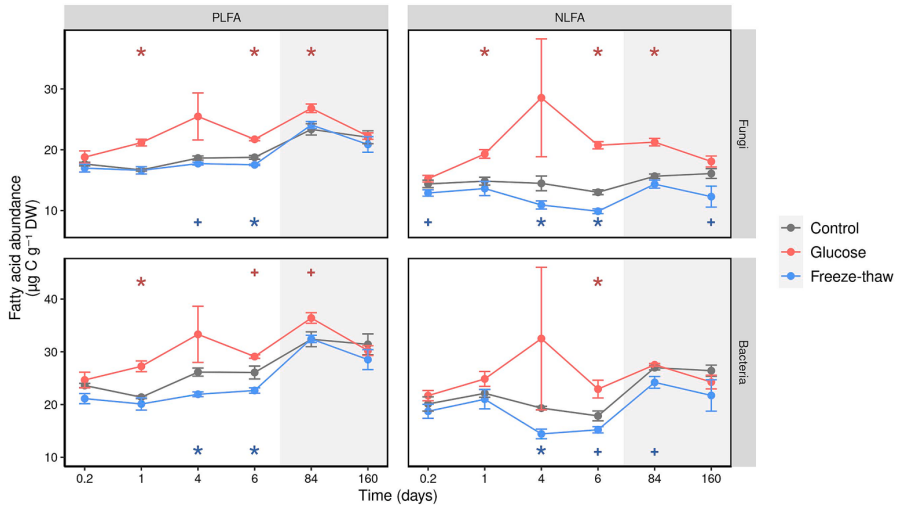

Fig. 2. Effects of one-time glucose-addition (red) and freeze-thawing (blue) on abundances of fatty acids specific for fungi and bacteria. PLFA represent microbial biomass, and NLFA represent microbial C storage. Bacterial NLFA should be interpreted with caution, as they may also reflect bacterial necromass. While glucose addition increased microbial fatty acid abundances, freeze-thawing decreased them. Points represent means; error bars represent standard errors. Note that the x-axis shows discrete time values; long-term timepoints are highlighted with a grey background. Significant differences from the control were tested via t-tests or Mann-Whitney tests (Table 1). Asterisks and plus-signs represent significant differences from control values (* $p < 0.05$, + $p < 0.1$).

was still significantly different from the control after 84 and 160 days on the first CA axis of the all-timepoint analysis (Fig. 1A, Table 1). When only analysing long-term timepoints, separation of control and freeze-thaw communities was less strong, but still significant (Fig. 1C).

3.3. *Single-pulse perturbations had long-term effects on microbial biomass and carbon storage*

Viable microbial biomass (based on PLFA) and microbial storage (based on NLFA) were affected by the perturbations. Specifically, PLFA of both fungi and bacteria rapidly increased immediately after glucose addition, and continued growing over the first four days at an average rate of 1.67 and 2.16 µg C g^{-1} DW day^{-1} respectively (red data in left panels of Fig. 2). Fungal and bacterial NLFA increased similarly within the same time frame at a rate of 3.33 and 2.71 µg C g^{-1} DW day^{-1} (red data in right panels of Fig. 2). After reaching the maximum growth after four days, we observed a decrease in biomass markers at almost the same rate. However, there was no return to the initial or to the control state in the first week. Instead, the biomass remains above the control values for at least 84 days, especially for the fungal fatty acid markers (Fig. 2 and Fig. S2).

There was a delayed reaction of microbial biomass to the freeze-thawing, with no changes in PLFA and NLFA after the first day. The difference between the treatment and control only became significant in this case after four days (blue data in Fig. 2). Our fatty acid measurements indicate that the microbial biomass is not significantly different from the control three months after freeze-thawing. Nevertheless, contrary to the glucose treatment, the system showed deviations from the control in the storage markers, with a deviation which persists even after three months (blue data in right panels of Fig. 2). Note that after the first week all treatments followed the same trend observed in the control system, which showed a slow increase of the viable biomass (as

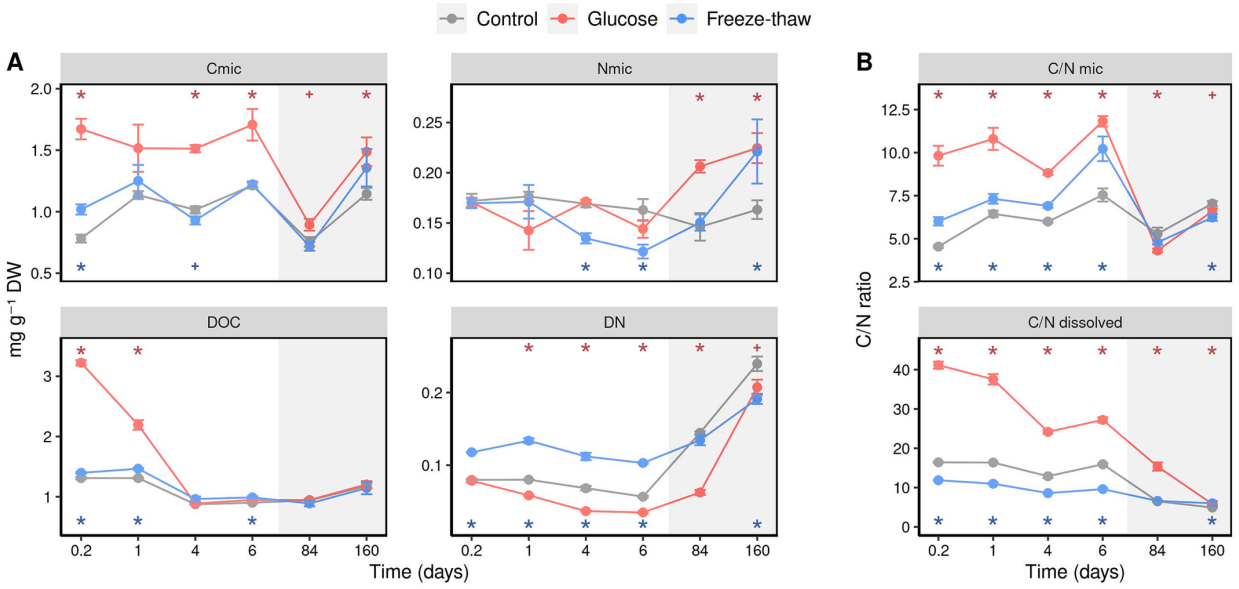

Fig. 3. Effect of one-time events of glucose addition (red) and freeze-thawing (blue) on temporal dynamics of microbial and dissolved C and N pools. Both single-pulse perturbations had significant long-term effects on soil C/N stoichiometry. Shown are C and N in microbial biomass (Cmic and Nmic), and dissolved organic C and total dissolved N (DOC and DN; plot A), as well as their respective ratios (plot B). Note that the x-axis uses discrete scaling; long-term timepoints are highlighted with a grey background. Points represent mean values; error bars represent standard errors. Significant differences from the control were tested via t-tests or Mann-Whitney tests (Table 1). Asterisks and plus-signs represent significant differences from control values (* $p < 0.05$, + $p < 0.1$).

Table 1
Effects of single-pulse perturbations on microbial biomass and community composition, and soil C/N stoichiometry. Shown are the results (p-values) of t-tests and, if its assumptions were violated, two-sample Mann-Whitney tests between each perturbation treatment and control. The tested data were fatty acid abundances (PLFA and NLFA) specific for fungi and bacteria, the scores of the first two ordination axes (CA1, CA2) of the ordination of all timepoints based on the PLFA dataset (Fig. 1C and Fig. S1), and soil microbial and dissolved C and N pools and their respective ratios (Fig. 3 and Fig. S3). Significant differences ($p < 0.05$) to the control are marked in bold.

Treatment	Time since perturbation (days)	PLFA		NLFA		CA (PLFA)		Cmic	Nmic	C/N mic	DOC	DN	C/N dissolved
		Fungi	Bacteria	Fungi	Bacteria	axis 1	axis 2						
Glucose	0.2	0.548	0.530	0.326	0.372	1.000	0.949	**0.008**	0.880	**0.008**	**0.000**	0.711	**0.008**
	1	**0.000**	**0.008**	**0.002**	0.137	0.473	0.841	0.118	0.156	**0.008**	**0.000**	**0.000**	**0.008**
	4	0.151	0.151	0.151	0.310	0.242	**0.016**	**0.008**	0.592	**0.008**	0.398	**0.000**	**0.000**
	6	**0.000**	0.095	**0.000**	**0.037**	**0.016**	0.69	**0.008**	0.310	**0.008**	0.107	**0.000**	**0.000**
	84	**0.032**	0.095	**0.000**	0.309	**0.000**	0.36	0.057	**0.008**	**0.008**	0.893	**0.000**	**0.008**
	160	0.878	0.608	0.139	0.237	**0.001**	0.83	**0.040**	**0.011**	0.078	0.719	0.057	**0.008**
Freeze-thaw	0.2	0.378	0.151	0.095	0.458	**0.008**	0.421	**0.002**	0.785	**0.003**	**0.005**	**0.000**	**0.008**
	1	0.929	0.335	0.394	0.593	**0.013**	0.056	0.690	1.000	**0.037**	**0.008**	**0.008**	**0.000**
	4	0.061	**0.003**	**0.008**	**0.004**	**0.016**	**0.016**	0.089	**0.008**	**0.008**	0.151	**0.000**	**0.000**
	6	**0.012**	**0.047**	**0.000**	0.050	**0.008**	**0.008**	0.769	**0.008**	**0.008**	**0.006**	**0.000**	**0.000**
	84	0.310	0.421	0.134	0.065	**0.008**	0.533	0.421	0.795	0.222	0.387	0.218	0.613
	160	0.497	0.322	0.096	0.197	**0.002**	0.226	0.222	**0.032**	**0.011**	0.310	**0.005**	**0.008**

described in Section 3.1).

The overall patterns in fatty acid abundances (increase after glucose addition, decrease after freeze-thawing) are reflected in our measurements of microbial biomass C based on CFE (Fig. 3A), although methodological differences between the two measurements inevitably produce discrepancies. Microbial biomass N decreased shortly after freeze-thawing, but increased with both treatments in the long term compared to the unperturbed control. Both perturbations also significantly altered microbial C/N ratios, which increased in the short term and decreased in the long term relative to the control (Fig. 3B). The strongest effects on C/N ratios took place in the short term, and we note that although the long-term differences are statistically significant, they were only marginal at the end of the experiment and converged towards control values.

3.4. Single-pulse perturbations altered dissolved carbon and nitrogen pools in the long term

In addition to microbial community profiles and C and N in biomass, dissolved C and N were altered by the single-pulse perturbations (Fig. 3). There was a strong difference in the concentration of the dissolved C pool compared to the control in the first week (as we had expected, especially after the glucose addition). However, all values seemed to converge in the long term. The concentration of dissolved N showed the strongest differences, although both treatments followed the general trend of long term accumulation of N (lower panels of Fig. 3A), which is

often observed in lab incubations of soil or litter (e.g. Mary et al., 1993; Mooshammer et al., 2012). Interestingly, 160 days after the pulse, N was significantly lower in the dissolved matter, but higher in the microbial biomass in both perturbation treatments (compared to the unperturbed samples), with consequently altered C/N ratios of dissolved matter and microbial biomass.

4. Discussion

4.1. Overall transient state changes

The results of our incubation experiment show how isolated microbial communities react to short-term environmental changes commonly experienced in soil. By design our isolated system of soil microorganisms had to adapt to the incubation conditions and all the responses to the pulse events were evaluated in comparison to an unperturbed control system's state. For this reason, before describing the effect of perturbation we first discuss the slow accumulative transformation of the system in response to the incubation. The changes observed here are a common consequence of putting a field-adapted system into an artificial environment, which lacks most of the influences and matter inputs the soil communities were exposed to before. A notable sign of this evolution is the slow increase in dissolved N over time (Fig. 3) which is often measured in soil incubation studies that miss constant C input and N uptake by roots. In addition, we also observed a restructuring of the microbial communities themselves. Our results show high initial variability in the soil microbial communities which we see as a consequence of the unintended perturbations caused by our experimental procedure of sieving and shaking, and abruptly changing environmental conditions by exposing them to a constant temperature, which likely were major stressors. As we did not include no-sieve or pre-incubation controls, we cannot discern whether sieving, shaking or incubation bore the stronger influence. Observations of N recovery suggest that sieving may amplify the mineralization of free amino acids to inorganic N forms (Inselsbacher, 2014; Inselsbacher et al., 2014), which could induce short-term changes in microbial community functioning. Calderón et al. (2000) found significant effects of sieving on the microbial community composition within two weeks, but did not include a no-sieve control and incubated at ca. 20 °C. In contrast, Petersen and Klug (1994) found no effect of sieving within three weeks, but found incubation at 25 °C to influence the microbial community composition. Thus, while microbial communities seem to recover quickly from sieving, the persistent influence of incubation can have strong, long-term effects.

The high inter-sample variability of microbial communities in the short-term resembles the 'Anna Karenina' principle, where external stressors lead the microbial community to change in a stochastic, rather than a deterministic way (a concept usually applied to host-dependent microbiomes; (Zaneveld et al., 2017). Although the initial community variability reflects instability due to stress, our communities stabilized after three months, as shown by replicates of each treatment collapsing into a distinct, less variable cluster in the ordination space (Fig. 1A). This indicates that the community overcame the initial stressors and deterministic processes governed the long-term community development.

4.2. Long-term legacies and resilience

From the description above it is important to highlight that our experiment involves processes occurring at very different time scales: on one hand the time scale of the slow accumulative effect to the closed incubation environment; on the other the time scale of the single-pulse perturbations applied. The first one is observed to take place on a pace much slower than the second, which allows us to assume that it is possible to treat the control as a reference state. Our hypothesis therefore was that after pulse events, the self-regulation of microbial communities would be sufficiently strong and fast to be able to bring the system back to the reference state, since similar events happen regularly in temperate soils. Note that we assume that the reference state can self-regulate and attract any deviations back to itself, regardless of the transient changes occurring within it. This may differ from theoretical definitions of resilience where the reference state is assumed to have simple properties, such as for example be constant in time (equilibrium state), or change in a predictable periodic way (periodic orbit; Hodgson et al., 2015; Schoenmakers and Feudel, 2021). Nevertheless, this framework is useful since natural systems are often subjected to external drivers and the assumption of a steady state does not always apply.

The general trends described above for the control samples were also present in the time evolution of perturbed ones. We observed them, for example, in the initial inter-sample variability of microbial communities and the dynamics of slow accumulation of dissolved N. In addition to these trends, the short perturbations clearly drive the communities away from the reference state, as recorded by fatty acid abundances (Fig. 1 and Fig. 2). Our measurements also show restoring tendencies present in the system in the first week. However the reference state is not fully recovered. Our results show that the consequences of the shift observed in microbial community composition one day after freeze-thawing and six days after glucose addition were still observed for up to 160 days after the pulses (Fig. 1, Table 1 and Table S1). In addition, microbial and dissolved C and N significantly deviate from control values not only in the short, but also in the long term. Short-term changes in C and N pools likely reflect direct effects of the different pulse events. For example, glucose addition will increase C availability, while freeze-thawing breaks up microbial cells releasing labile cell compounds into the soil solution (Morley et al., 1983). Although abiotic mechanisms like binding to mineral surfaces (Kleber et al., 2015; Lehmann and Kleber, 2015) can also influence C and N stoichiometry, the observed long-term deviations from control values can also be derived from sustained changes in microbial process rates and metabolism, which ultimately relate to the biochemical functioning of the community. For instance, the concurrent loss of dissolved N and increase in microbial N at 160 days after the perturbations (Fig. 3) suggests that both glucose addition and freeze-thawing led to enhanced microbial N uptake and storage in the long term. Functional redundancy, i.e. the ability of different microbes to provide the same function, theoretically promotes functional resilience in microbial communities (Allison and Martiny, 2008; Biggs et al., 2020; Louca et al., 2018). Thus, although our measurements only indirectly relate to community functions, the results suggest that the community shows low functional resilience towards the perturbations.

Another theoretical possibility to explain the observed long-term effects is a perturbation-induced time-lag of natural oscillations within the microbial community. Soil environments are known to intrinsically fluctuate due to their heterogeneous structure of aggregates and pores with varying size classes (Bronick and Lal, 2005; Nunan et al., 2003; O'Donnell et al., 2007), which facilitate strong chemical gradients (Sexstone et al., 1985), disparate water flows (Pachepsky and Rawls, 2003), and varying proportions of water and air (Luo et al., 2008). If the perturbations caused a deviation in microbial community oscillations, but ultimately returned to the oscillating path of the control soils, they may have re-entered this path at a set point which still differed from the control. Thus, the perturbed communities theoretically could have had already returned to the path of the control community, so that perturbed soils appear shifted but are actually off-set measurements of the same oscillating metrics. However, we think this explanation is not applicable in our case. First, many soil-intrinsic fluctuations occur on small spatial scales and are expected to average out when larger soil volumes are sampled, like in our experiment. Second, this explanation assumes that the oscillations would be synchronised across all replicate samples, which is unlikely in isolated communities without external drivers. The variance of our measurement will also capture any fluctuating dynamics, so that if the perturbations caused only a shift of the period of microbial community oscillations, the average of all samples at a single time point would stay the same.

The long-term legacy effects on both the microbial community

composition (Fig. 1, Table 1), and microbial and dissolved C and N pools (Fig. 3), demonstrate that single-pulse perturbations can have lasting effects on soil microbial ecosystems. This can be asserted regardless of the other unintended influences of our experimental design, as differences between soils which received the perturbations and control soils were maintained in the long term. The one-time perturbations left a significant mark not only on the trajectory of the soil communities but also on storage compounds as well as pool sizes and stoichiometries of C and N in microbial biomass and soil solution through time. The fact that soil microbial communities and their biogeochemical environments are strongly influencing each other may explain why the system, contrary to our hypothesis, did not return to the reference state during the relatively long incubation period. While the perturbation may have initially just altered one or more components of the system (i.e. community composition, C and N availability and stoichiometry, C and N storage in microbes), the strong internal feedbacks between these parameters could have reinforced the change, lowering the system's tolerance to get back to the reference state and shifting it to an alternative trajectory (Schoenmakers and Feudel, 2021).

In summary we have identified two orders of state changes within the experimental soils: an overall transient change due to the controlled experimental conditions (which includes the unperturbed soil), and a change in response to the short-term perturbations (which happens within the bounds of the transient change). Although our experiment was carried out in an artificial setting, it resembles some aspects of microbes in natural soils: they undergo long-term changes, e.g. driven by seasonally changing environmental conditions, but are at the same time exposed to pulse-perturbations, such as rapid weather events, freeze-thawing or, at a very small scale, root exudations. Indeed, natural communities likely never reach full stability and rather occupy transient states (Holling, 1973). The observed long-term community changes in our experimental soils demonstrate that the history of pulse events may be an important factor in causing soil microbial ecosystems to irreversibly deviate from the dynamics set initially. On the other hand, we note that migration and transport from unperturbed areas could in principle be another type of mechanisms which regulates the system, bringing it back to initial state (Sorensen and Shade, 2020). This mechanism is intentionally left out from the current study, but in natural systems coupling with unaffected areas could be a mechanism which plays a central role in resilience.

5. Conclusions

Given the ubiquity of short-term environmental changes which influence microbial biomass and activity in natural soil systems, the presented results highlight the complex coupling of microbial community structure and functions with soil resources. Based on our observations, we conclude that pulsed perturbations which are intrinsic, recurring properties of the environment can leave substantial imprints on microbial communities. The fact that pulse events which one may be tempted to call 'environmental fluctuations' can sustainably alter community structure and soil C and N pools highlights the importance of the history of interactions between the soil microbial community and its environment. Given that perturbations or environmental fluctuations often occur at small scales in the soil (e.g. root exudation happens at small 'hotspots', freezing may affect specific, small soil spots more than others, depending on their position and water content), this opens the interesting question whether individual soil spots may bear unique legacy profiles of past disturbances, and how this may contribute to the high biodiversity and small-scale spatial heterogeneity of soil microbial communities.

Declaration of Competing Interest

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

Data availability

The data presented in this study, and R code reproducing statistical analysis and figure plotting, are openly available on Zenodo at https://zenodo.org/record/7669777 (Gorka et al., 2023).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.geoderma.2023.116399.

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SUPPLEMENTARY FIGURE & TABLE

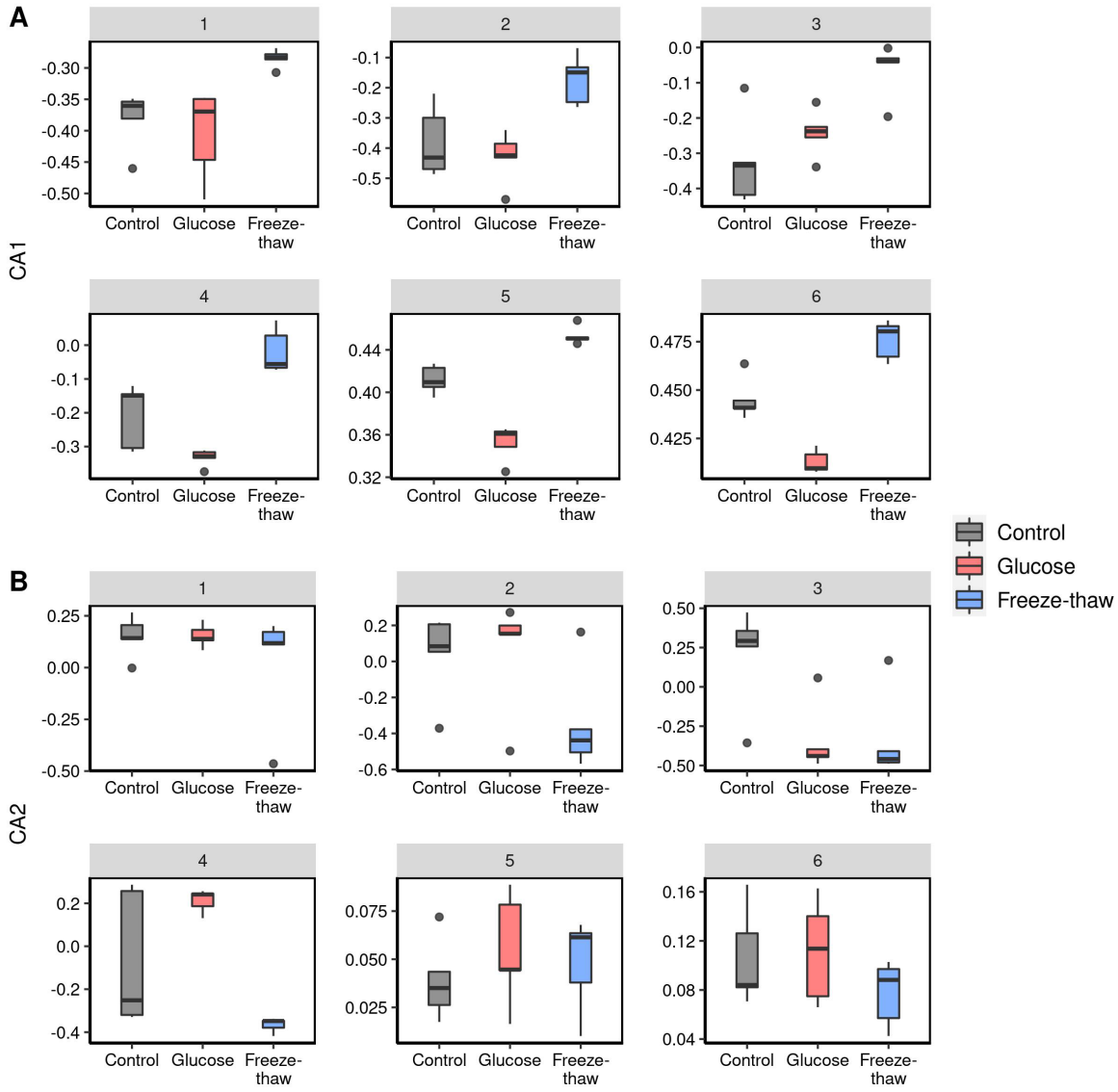

Figure S1 Boxplots of scores of the first (A) and second (B) ordination axis (65.65 % and 14.75 % explained variation, respectively) from the CA based on PLFA data of all timepoints (see Fig. 1A). Facet plot titles correspond to the harvest timepoints as visualised in Fig. 1 (1 – 0.4 d, 2 – 1 d, 3 – 4 d, 4 – 6 d, 5 – 84 d, 6 – 160 d).

Table S1 Effect of single-pulse perturbations on microbial community composition. Shown are the results of *adonis* (PERMANOVA) with Bray-Curtis distances. Significant differences ($p < 0.05$) are marked in bold.

	All timepoints				
	df	Sums of Sqs	R^2	F Model	Pr (>F)
Treatment	2	0.15468	0.14140	15.994	**0.0001**
Short/Long term	1	0.52335	0.47843	108.229	**0.0001**
Residuals	86	0.41586	0.38017		
Total	89	1.09388	1.00000		

	Short term				
	df	Sums of Sqs	R^2	F Model	Pr (>F)
Treatment	2	0.16654	0.35807	18.6697	**0.0001**
Time point	1	0.04985	0.10718	11.1764	**0.0001**
Treatment:Time point	2	0.00787	0.01691	0.8817	0.4390
Residuals	54	0.24085	0.51784		
Total	59	0.46511	1.00000		

	Long term				
	df	Sums of Sqs	R^2	F Model	Pr (>F)
Treatment	2	0.014774	0.14013	3.1406	**0.0451**
Time point	1	0.026253	0.24902	11.1619	**0.0018**
Treatment:Time point	2	0.007952	0.07543	1.6905	0.1928
Residuals	24	0.056449	0.53543		
Total	29	0.105428	1.00000		

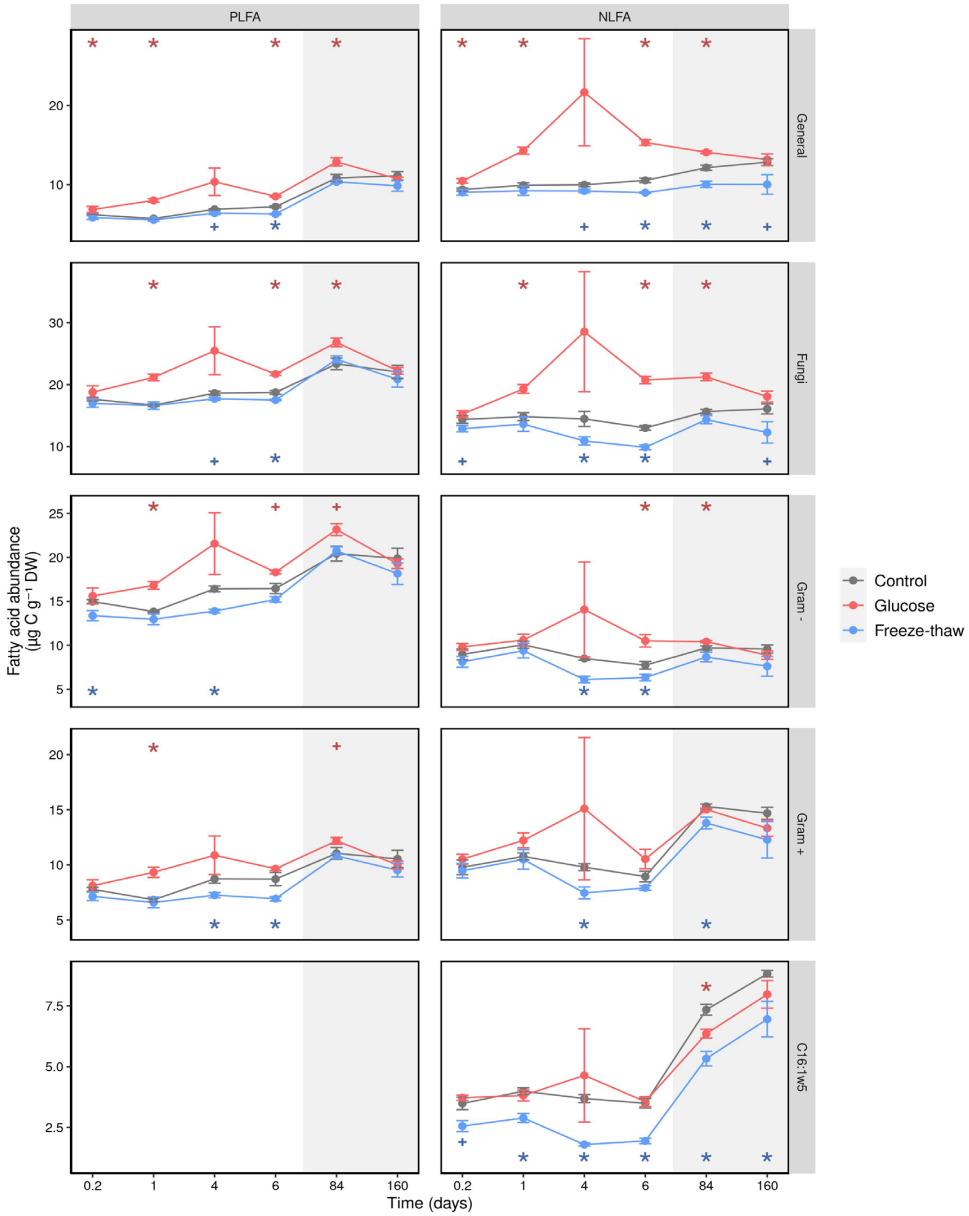

Figure S2 Effects of one-time glucose-addition (red) and freeze-thawing (blue) on abundances of fatty acids. Here, we assigned the straight-chain saturated fatty acids 16:0 and 18:0 to general microbial biomass, the fatty acids 18:1ω9 (cis and trans form) and 18:2ω6,9 to fungi, other unsaturated fatty acids (16:1ω7, 17:1ω7) and cyclopropyl fatty acids (17:0cy, 19:0cy) to Gram- bacteria, terminally branched fatty acids (a15:0, i15:0, i16:0, a17:0, i17:0) to Gram+ bacteria. For the fatty acid 16:1ω5, the PLFA was assigned to Gram- bacteria, but the NLFA is plotted separately as it is highly specific to arbuscular-mycorrhizal fungi and may be a proxy for arbuscular-mycorrhizal spores in our system. Points represent means; error bars represent standard errors. Note that the x-axis shows discrete time values; long-term timepoints are highlighted with a grey background. Significant differences from the control were tested via t-tests or Mann-Whitney tests (Table 1). Asterisks and plus-signs represent significant differences from control values (* $p<0.05$, + $p<0.1$).

Chapter 3

Beyond PLFA: Concurrent extraction of neutral and glycolipid fatty acids provides new insights into soil microbial communities

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Beyond PLFA: Concurrent extraction of neutral and glycolipid fatty acids provides new insights into soil microbial communities

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ABSTRACT

The analysis of phospholipid fatty acids (PLFAs) is one of the most common methods used to quantify the abundance, and analyse the community structure, of soil microbes. The PLFA extraction method can yield two additional lipid fractions—neutral lipids and glycolipids—which potentially hold additional, valuable information on soil microbial communities. Yet its quantitative sensitivity on complete neutral lipid (NLFA) and glycolipid fatty acid (GLFA) profiles has never been validated. In this study we tested (i) if the high-throughput PLFA method can be expanded to concurrently extract complete NLFA and GLFA profiles, as well as sterols, (ii) whether taxonomic specificities of signature fatty acids are retained across the three lipid fractions in pure culture strains, and (iii) whether NLFAs and GLFAs allow soil-specific fingerprinting to the same extent as PLFA analysis. By adjusting the polarity of chloroform with 2% ethanol for solid phase extraction, pure lipid standards were fully fractionated into neutral lipids, glycolipids, and phospholipids. Sterols eluted in the neutral lipid fraction, and a betaine lipid co-eluted with phospholipids. We found consistent taxonomic specificities of fatty acid markers across the three lipid fractions by analysing pure culture extracts representative of soil microbes. Fatty acid profiles from soil extracts, however, showed stronger differences between PLFAs, NLFAs, and GLFAs than between soil types. This indicates that PLFAs and NLFAs signify different community properties (biomass vs. carbon storage, putatively), and that GLFAs are sensitive markers for community traits which behave differently than PLFAs. Although we consistently found high abundances of characteristic sterols in fungal extracts, the PLFA extraction method only yielded miniscule amounts of ergosterol from soil extracts. We argue that concomitant measurement of fatty acid profiles from all three lipid fractions is a low-effort and potentially information-rich addition to the PLFA method, and discuss its applicability for soil microbial community analyses.

1. Introduction

The analysis of phospholipid fatty acids (PLFAs) derived from microorganisms is widely used to quantify soil microbial biomass based on coarse phylogenetic groups (Frostegård et al., 2011; Willers et al., 2015). It also allows to trace changes in the state of microbial communities, including shifts in community composition (Ramsey et al., 2006; Orwin et al., 2018) or adaptive modifications of lipid membranes in response to varying environmental conditions (Sinensky, 1971; Heipieper et al., 1992; Los and Murata, 2004; Wixon and Balser, 2013). The method is especially powerful when coupled with stable isotope probing to quantify ecologically relevant processes (such as transfer of ^{13}C from plant to microorganisms; Fuchslueger et al., 2014; Kaiser et al., 2015; Gorka et al., 2019). The analysis typically consists of four consecutive steps (Fig. 1). First, lipids are obtained from soil with a two-phase extraction method (Bligh and Dyer, 1959), which are subsequently fractionated by silicic acid solid-phase extraction. This is achieved by eluting the lipids with solvents of increasing polarity (chloroform, acetone, and methanol; Vorbeck and Marinetti, 1965), yielding neutral lipids, glycolipids and phospholipids. The obtained lipid fractions are

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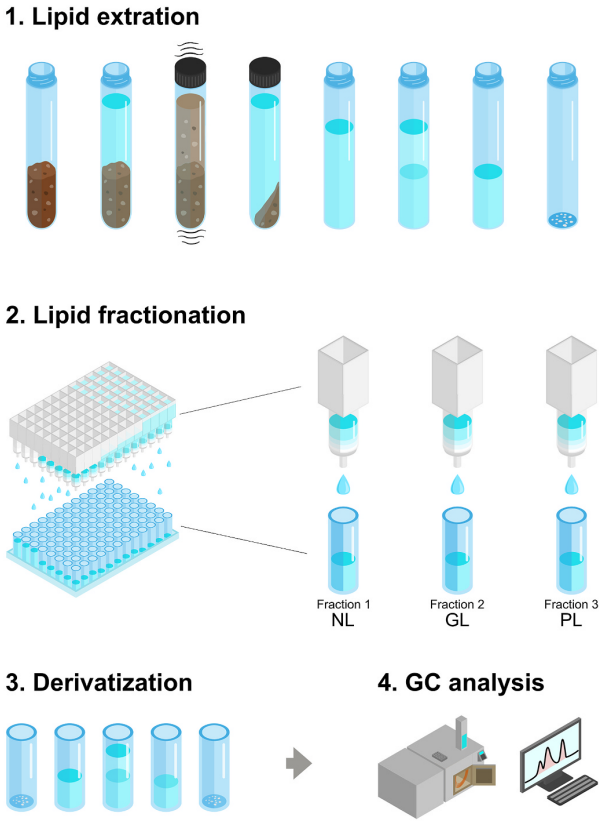

Fig. 1. Visual summary of the main steps of the high-throughput PLFA extraction protocol. (1) Total lipids are extracted with a monophasic mixture of chloroform, methanol and citrate buffer, followed by phase separation and retrieval of the chloroform phase. (2) The lipid fractionation step via silicic acid solid-phase extraction potentially yields three lipid fractions: NL (neutral lipids), GL (glycolipids), and PL (phospholipids). (3) The obtained lipid fractions are cleaved and derivatised to fatty acid methyl esters, which are (4) measured by gas-chromatography.

then derivatised into their respective fatty acid methyl esters (FAME): neutral lipid fatty acids (NLFAs), glycolipid fatty acids (GLFAs), and PLFAs. These are finally analysed by gas chromatography-mass spectrometry (GC-MS) or isotope ratio mass spectrometry (GC-IRMS). Many studies measure PLFAs as microbial biomarkers because they are considered to originate from viable cell membranes (White, 1993), quickly decompose after cellular disintegration (Zhang et al., 2019), and have strong taxonomic specificity (Zelles, 1999).

Fewer studies have analysed neutral lipids, yet they represent important lipid classes such as the energy storage compounds triacylglycerols (Waltermann and Steinbu; chel, 2005; Gao and Goodman, 2015). The NLFA 16:1ω5 was shown to be a reliable biomarker for energy storage lipids or spore production (using milled material) of arbuscular mycorrhizal fungi (Olsson, 1999; van Aarle and Olsson, 2003; Sharma and Buyer, 2015; Lekberg et al., 2022; Olsson and Lekberg, 2022). Although existing protocols to extract and measure PLFAs have been used to measure NLFAs in soil (Olsson et al., 1995; Sharma and Buyer, 2015), to our knowledge no proper evaluation of NLFA purity and quantification during solid phase extraction has been done. In a recent study it was found that changing the polarity of the eluent has a strong effect on neutral and glycolipid separation (Drijber and Jeske, 2019). Chloroform is often stabilised with ethanol in varying quantities, but insufficient amounts of ethanol can lead to losses of neutral lipids in the intermediate glycolipid fraction. Specifically, Drijber and Jeske (2019) found that significant amounts of neutral lipids are lost to the glycolipid fraction at ethanol contents below 1%, leaving concerns on the precision of published NLFA quantifications.

Sterols, membrane lipids based on cyclohexane and -pentane units but lacking fatty acid chains, can also be found in the neutral fraction (Breil et al., 2017). Phytosterols, especially β-sitosterol, can be used as markers for plant-derived organic matter, and cholesterol for soil micro- and mesofauna (Puglisi et al., 2003). Fungi produce a variety of taxon- and clade-specific sterols, the most-widespread being ergosterol, but also other sterols such as cholesterol, lanosterol, or brassicasterol, including even some potentially phylum-specific sterol biomarkers, such as 24-ethyl cholesterol for Glomeromycota (Weete et al., 2010). Ergosterol in particular has been widely used as a biomarker of living fungal biomass and has shown good correlation with other estimation methods (Wallander et al., 2013), making it a valuable target for fungal biomass quantification. Although ergosterol is usually extracted separately (e.g. Klamer and Bååth, 2004; Potthoff et al., 2006; van der Wal et al., 2006; Demoling et al., 2008; Moeskops et al., 2012; Grosso et al., 2018), the PLFA extraction and fractionation protocol has been previously used to quantify ergosterol in soils in the neutral lipid fraction (McKinley et al., 2005; Amir et al., 2008). However, to our knowledge no study has assessed the efficiency of the PLFA extraction protocol to concurrently quantify ergosterol.

The glycolipid fraction has rarely been considered in previous studies (Rinnan et al., 2005; Welc et al., 2012; Mattsson et al., 2015). Yet glycolipids may represent an important pool in arbuscular-mycorrhizal structures, as they were shown to be the primary lipid type in mycorrhizal arbuscules and spores (Jabaji-Hare et al., 1984; Nemec, 1981; Grigera et al., 2007). It can, however, not be ruled out that some of these earlier studies identified spill-overs from the neutral lipid fraction as glycolipids by using purified chloroform with very low concentration of ethanol (Jabaji-Hare et al., 1984; Grigera et al., 2007; Drijber and Jeske, 2019). Glycolipids also occur in many bacterial groups (Hölzl and Dörmann, 2007; Sohlenkamp and Geiger, 2016). They are membrane lipids and have therefore been suggested to be measured combined with the PLFA fraction (Heinzelmann et al., 2014; Drijber and Jeske, 2019). However, not much is known about their abundance patterns and specific functional roles in microbial communities, hence their potential value for soil microbial research has yet to be determined.

About 10 years ago a high-throughput variation of the PLFA extraction protocol was introduced, allowing a faster sample preparation by using smaller solvent volumes combined with 96-well solid phase extraction plates (Buyer and Sasser, 2012). This protocol has been successfully adopted by an increasing number of studies. The PLFA extraction protocol presents the potential to be expanded to lipid classes other than phospholipids; however it has never been systematically evaluated for the high-throughput method.

The aim of this study was to evaluate and expand the high-throughput PLFA extraction protocol to enable the concomitant analysis of fatty acid profiles of different classes of lipid biomarkers, including PLFAs, NLFAs, GLFAs, and sterols, and to assess its potential application to soil microbial research. We first evaluated the efficiency of the high-throughput PLFA method (Buyer and Sasser, 2012) to recover multiple lipid classes simultaneously and then applied this protocol to multiple microbial pure cultures and soils. Specifically, we tested: (i) the purity and recovery of the PLFA, NLFA, GLFA, and sterol fractions during the solid phase extraction of the high-throughput protocol, (ii) how an adjusted polarity of the first (neutral) lipid fraction affects separation between the lipid fractions and of sterols, (iii) whether the taxonomic specificities of the fatty acid profiles are consistent across PLFAs, NLFAs and GLFAs in pure culture strains, and (iv) whether NLFAs and GLFAs allow soil-specific fingerprinting to the same extent as PLFA analysis.

2. Materials and methods

2.1. Lipid fractionation and analysis

All solvents used were HPLC- or GC-grade (min. 99.8% purity). Particular attention was paid on the quality of the chloroform, which showed 99.8% purity and was stabilised with amylene (Drijber and Jeske, 2019). All glassware used was muffled at 500 °C for >4 h to oxidise and therefore remove potential organic contaminants. Glassware was used throughout the extraction protocol. Plastic was used only in two steps: during solid phase extraction fractionation (plastic walls of the silica gel columns) and during transesterification (plastic pipette tips).

For lipid fractionation, we used 96-well plates filled with 50 mg silica gel per well (Phenomenex, Strata SI-1 Silica, 55 μm, 70 A), which were conditioned by washing with 3 × 1.5 ml methanol and 3 × 1.5 ml chloroform before loading with samples. After elution of the three lipid fractions into 1.5 ml glass vials, all fractions were dried under a gentle stream of N_2.

Each lipid fraction was subsequently transesterified by adding 0.1 ml methanol:toluene (v:v = 1:1) and 0.1 ml of approximately 0.07 M methanolic KOH (3 pellets KOH in 50 ml methanol). The vials were sealed with a PTFE-coated silicon cap mat and incubated for 15 min at 37 °C in a water bath. The transesterification was stopped by adding 0.4 ml 0.075 M acetic acid, followed by 0.4 ml chloroform to induce phase separation. The two resulting phases were mixed for 20 s by shaking vigorously and allowed to separate for 5 min. The bottom 0.3 ml (chloroform phase) were transferred with a multichannel pipette into clean 1.5 ml vials. This step was repeated with a second addition of 0.4 ml chloroform and transferring 0.4 ml of the lower phase. The resulting samples were dried under a gentle stream of N_2.

All final extracts were dissolved in 100 μl iso-octane (2,2,4-trimethylpentane, Sigma-Aldrich) and transferred to GC-vials with inserts. Samples were subsequently injected on a GC (7890 B Agilent Technologies, USA) connected to a time of flight (TOF) MS (Pegasus BT, LECO, USA). The instrument was equipped with a split/splitless injector and a DB-5 column (60 m; Agilent, USA).

For identification of FAME we used two external mixed standards, i.e. the fatty acid methyl ester mix (FAME mix, Supelco, USA), and the bacterial fatty acid methyl ester mix (BAME mix, Supelco, USA). Unknown FAME and sterol peaks were identified via mass spectra comparisons with the NIST libraries *mainlib* and *replib*. For pure culture and soil extractions, methyl nonadecanoate was added to samples before transesterification, and used as an internal standard for fatty acid quantification via total ion chromatograms. Ergosterol peak area was normalised by the internal standard 5-α-cholestane and quantified via an external standard row, using extracted-ion chromatograms (XIC) of the masses 396.34 ± 0.3 for ergosterol and 372.38 ± 0.3 for 5-α-cholestane.

FAME extracts were injected in split mode at an injector temperature of 280 °C. FAME were eluted at a constant flow of 1.5 ml min^{-1} by slowly ramping from 80 to 200 °C at 2 °C min^{-1}, then immediately to 230 °C at 4 °C min^{-1}. The oven was then kept at this temperature for 15 min. As sterols are less volatile than FAME (e.g. ergosterol boiling point = 250 °C), higher GC temperatures are needed for their successful separation and elution when injecting them in an underivatised form (Ai, 1997; Kramer et al., 2012). Here, we tested two temperature programs: (i) combined measurement of FAME and sterols by running isothermally at 300 °C for 30 min following the FAME program, and (ii) quickly ramping from 160 to 300 °C, after which the temperature was kept isothermal for 20 min. Details of all GC programs are presented in Table S1.

2.2. Assessing lipid fractionation with pure lipid standards

First, we tested the recovery of different pure lipid and sterol compounds which are representative of lipid classes commonly found in soil. These included the glycolipid monogalactosyl diacylglycerol (MGDG), the betaine lipid diacylglyceryl trimethylhomoserine (DGTS), neutral triacylglycerols (glyceryl trimyristate, glyceryl tripentadecanoate), sterols (ergosterol, dehydrocholesterol), and phospholipids (phosphatidylcholine, phosphatidylglycerol). Each lipid (except for sterols) contained unique fatty acids which allowed definitive identification after derivatisation to their respective methyl esters (Table S2). All lipids were purchased from Avanti Polar Lipids (USA).

Lipids were prepared at 100 μg ml^{-1} and added directly to silica gel columns in 1 ml of the eluent for the first fraction. They were eluted with 2 ml chloroform (including the volume added with the lipid loading), 1 ml acetone, and 0.5 ml methanol-chloroform-water (v:v:v = 5:5:1) following the high throughput protocol by Buyer and Sasser (2012). We tested two adjustments of the eluents. (i) We aimed for full separation and improved recovery of the three lipid fractions by eluting the first fraction with the addition of 2% ethanol (1.5 ml chloroform:ethanol v:v = 98:2) and the second fraction with 1.5 ml acetone ("adjusted eluents"). (ii) Additionally, we tested for a simplified protocol for sole PLFA analysis by eluting both the first and the second fraction with 1.5 ml acetone ("acetone", Table 1), aiming for a full separation of the phospholipid fraction. Compared to the high throughput protocol after Buyer and Sasser (2012), we reduced the volume to 1.5 ml in the first fraction, as this allows collection into single 1.5 ml glass vials and thus improves lab workflow. This reduction in eluent volume did not change the recovery of NLFAs (Fig. S1). We additionally tested for improved recovery of phospholipids in the "adjusted eluents" treatment by increasing the third fraction volume to 1 ml (Table 1).

2.3. Specificity of fatty acid profiles across lipid fractions in microbial pure cultures

We analysed lipid profiles of 6 bacterial and 7 fungal strains (Table S3). Selected gram-positive bacterial strains belonged to Actinobacteria (*Solirubrobacter soli* DSM 22325, n = 4; *Streptosporangium roseum* DSM 43021, n = 2) and Firmicutes (*Paenibacillus alginolyticus* DSM 5050, n = 3), and gram-negative species to Proteobacteria (*Labilithrix luteola* DSM 27648, n = 3; *Chelativorans multitrophicus* DSM 6780, n = 5; *Paraburkholderia xenovorans* DSM 17367, n = 3). Selected fungal species belonged to arbuscular-mycorrhizal Glomeromycota (*Rhizophagus irregularis*, n = 1), Ascomycetes (*Periconia macrospinosa* DSM 62880, n = 2; *Trichoderma virens* DSM 3500, n = 3), and Basidiomycetes of which we extracted saprotrophic (*Armillaria gallica* DSM 3732, n = 3; *Psilocybe cyanescens* DSM 4999, n = 3) and ectomycorrhizal (*Lactarius subdulcis,* n = 3; *L. quietus*, n = 3) strains. We additionally extracted fungal sporocarps of *Amanita muscaria* and an *Agaricus* species, but only included them in sterol analyses as we focused only on pure culture strains for the fatty acid profiling.

Pure culture strains were washed by centrifugation in Milli-Q water, except for the *Lactarius* strains which were scooped from the agar growth medium with a sterile spatula. Samples were subsequently lyophylised, and stored at −20 °C until extraction. We extracted total lipids from 3 to 20 mg of lyophilised samples with 6 ml of an adjusted Bligh-Dyer extractant made of chloroform, methanol and citrate buffer (CMB, v:v:

Table 1
Eluents and volumes used to elute the three lipid fractions during silica SPE. Chloroform-ethanol v:v = 98:2; Methanol-chloroform-water v:v:v = 5:5:1.

Fraction	Classic eluents	Adjusted eluents	Acetone
1 (NL)	Chloroform 2 ml	Chloroform:ethanol 1.5 ml	Acetone 1.5 ml
2 (GL)	Acetone 1 ml	Acetone 1.5 ml	Acetone 1.5 ml
3 (PL)	Methanol-chloroform-water 0.5 ml	Methanol-chloroform-water 1 ml	Methanol-chloroform-water 0.5 ml

v = 1:2:0.8, citrate buffer pH = 4; Frostegård et al., 1991). The samples were subsequently sonicated for 10 min, vortexed for 30 s and extracted overnight. After centrifugation at 3500 rpm, the supernatant was collected and transferred to new glass vials. We re-extracted the remaining pellet with 2.6 ml CMB, repeating the same steps of vortexing, centrifugation, and transferring the supernatant. Phase separation was induced by adding 1.5 ml of each chloroform and citrate buffer, whereupon the lower phase was collected and weighed to account for losses. The final extract was then dried under a constant stream of N_2. Lipids were then fractionated with the adjusted method by addition of 2% ethanol to chloroform for neutral lipid elution (Table 1) and transesterified the same way as the pure lipid standards as described above.

2.4. Concomitant extraction of PLFAs, NLFAs, GLFAs and sterols from soils

We applied the adjusted lipid fractionation method with chloroform: ethanol (v:v = 98:2) elution of the NLFA fraction to soil samples, and tested the validity of the method for the concomitant extraction of PLFAs, NLFAs, GLFAs, and sterols from soils. We used three soil types: a beech forest soil, a grassland soil, and an agricultural field soil. The sites were located in Klausen-Leopoldsdorf (Lower Austria, Austria), Gumpenstein (Styria, Austria), and Wieselburg (Lower Austria, Austria; Schnecker et al., 2022), respectively. From each site, we harvested four soil cores of 5 cm depth and 10 cm diameter (beech forest and grassland, n = 4) or 2 cm diameter (agricultural soil, n = 4). Soil from each soil core was subsequently homogenised by sieving (2 mm), lyophilised, and stored at −20 °C until extraction.

Soil carbon and nitrogen content was measured with an elemental analyser (EA 1110 elemental analyzer, CE, Italy). Soil pH was determined by suspending soil in Milli-Q water (w:v = 1:5), shaking for 30 min, and measuring the pH after 10 min to allow soil particles to settle.

Extractions of soil PLFAs, NLFAs, GLFAs, and sterols were performed as described above. In short, 2 g dry soil per sample were extracted with 6 ml CMB, the resulting total lipid extracts were separated with the adjusted lipid fractionation method, and transesterified the same way as the pure culture microbial strains. We assigned the fatty acids identified in the soil extracts to the following taxonomic groups: General microbial biomass (14:0, 15:0, 16:0, 17:0, 18:0), unspecified biomass (16:1ω6i, 16:0Me, 17:0Me, 18:1ω9trans), gram-negative bacteria (14:0-3OH, 14:1ω5, 15:1ω5, 16:1ω7, 16:1ω9, 17:0cy, 17:1ω8, 18:1ω5, 18:1ω7, 19:0cy(ω9), 19:1ω9, and additionally 16:1ω5, being aware that it is also highly specific to arbuscular-mycorrhizal fungi, particularly in the neutral lipid fraction and in systems dominated by this mycorrhizal type), gram-positive bacteria (14:0i, 15:0a, 15:0i, 16:0i, 17:0a, 17:0i, 18:0i), Actinomycetes (10Me17:0, 10Me18:0), and fungi (18:1ω9cis, 18:2ω6,9, 18:3ω6,9,12).

As the soil extractions did not recover ergosterol in measurable amounts (data not shown, as no ergosterol was observed), we tested a modified version of our lipid extraction protocol to improve ergosterol recovery in soil extracts. We increased the extractant volume while reducing the soil dry weight, which resulted in an increase of the extractant:soil ratio. Our reasoning was that a well-cited ergosterol extraction method which achieved 92–101% recovery of an ergosterol spike (Djajakirana et al., 1996) used a much higher extractant:soil ratio, albeit with ethanol as the extraction solvent. We proceeded with two tests: first, we extracted the total lipids from a beech forest soil from the Vienna woods (Austria), omitting the subsequent steps of lipid fractionation and alkaline methanolysis as they do not influence the ergosterol yield (see results section 2). Specifically, we increased the extractant:soil ratio from 3 to 10 and 20 (Table S4). This test resulted in higher ergosterol yields with increasing extractant:soil ratio (Table S4). We then applied the higher volume test to the full method of total lipid extraction, fractionation and mild alkaline methanolysis (Table S4). Here, we freshly harvested three soil types: a beech forest soil from Klausen-Leopoldsdorf, and a spruce forest and a grassland soil from Ausserneuwald. All sites were located in Lower Austria, Austria. Each test was compared to a published method for extracting ergosterol from soils with ethanol (Djajakirana et al., 1996); in short, we extracted with 96% ethanol, shook at 250 rpm for 30 min, centrifuged at 3500 rpm and dried the transferred extract under a constant stream of N_2 (for volumes and weights, see Table S4). Carbon and nitrogen contents, and pH of all soils are summarised in Table S5.

2.5. Calculations and statistical analyses

The recovery of pure lipid standards after solid phase extraction was calculated as $area_{sample}/area_{Ext.Std.} \times 100$, where $area_{sample}$ refers to the total ion chromatogram peak area of pure lipid standard samples after fractionation and derivatisation, and $area_{Ext.Std}$ to peak areas of derivatised pure lipid standard samples without preceding fractionation. Fatty acid abundances of pure culture strains and soil extracts were calculated as mol%, that is the fraction of each fatty acid compared to the sum of all fatty acids in nmol C in each sample, as well as normalised per gram of strain or soil dry weight. PLFAs with less than 0.5 mol% abundance were excluded in soil samples (Quideau et al., 2016). The fatty acid 18:0 occurred in almost all lipid fractions in all analysed microbial pure culture strains (Fig. S2). In addition, this fatty acid is a common contaminant in the NLFA fraction following solid phase extraction with presence of high-density polyethylene (Russell and Werne, 2007), and showed high variability in blank samples. We consequently removed it from all analyses. Its exclusion did not influence the distribution of fatty acid profiles in multivariate space (Fig. S3).

Data analysis was done in R (v4.2.1; R Core Team, 2022). Data transformations and plotting were done with the *tidyverse* package bundle (v1.3.2; Wickham et al., 2019), and multivariate statistics were calculated with the package *vegan* (v2.6-2; Oksanen et al., 2020) with Bray-Curtis dissimilarity measures. Non-metric multidimensional scaling (NMDS) models were calculated with the function *metaMDS*, and the multivariate null hypothesis of no differences between groups was tested via PERMANOVA (function *adonis2* in package *vegan*).

3. Results

3.1. Ethanol addition to chloroform improves lipid fractionation

Adding 2% ethanol to chloroform improved the fractionation of pure lipid standards. It allowed the complete recovery of triacylglycerols in the first (neutral lipid) fraction (Fig. 2), where otherwise about 35% would be lost to the second (glycolipid) fraction. The glycolipid showed no response to the ethanol addition and 90% was recovered in the glycolipid fraction, while around 5% was found in the phospholipid fraction. The betaine lipid (DGTS) mostly eluted in the phospholipid fraction (78%), while about 10% eluted in each the neutral lipid and the glycolipid fraction, respectively. Phospholipid recovery was not affected by the ethanol addition. However, by eluting phospholipids with 0.5 ml of the 5:5:1 methanol:chloroform:H_2O eluent (Buyer and Sasser, 2012), phospholipids showed recovery rates of 70–80% (Fig. S1). By increasing the elution volume of the third (phospholipid) fraction to 1 ml, the recovery increased to 90–95% (Fig. 2, Fig. S1), although this also extended the drying time considerably (up to 10 h under cautiously low N_2 flow, which we left to dry overnight).

Using acetone as the sole eluents for the first two fractions resulted in the simultaneous elution of neutral lipids and glycolipids (Fig. 2). Both eluted in the first fraction with high recoveries (triacylglycerols, 91%; sterols, 95%; glycolipid, 82%). The majority of the betaine lipid again co-eluted in the phospholipid fraction. The recovery of phospholipids was not affected by using acetone for neutral and glycolipid elution (*t*-test, P = 0.573).

The addition of 2% ethanol allowed full recovery of sterols in the neutral lipid fraction (Fig. 2). Without this adjustment (i.e. using solely pure chloroform to elute the neutral lipid fraction), about 60% of the

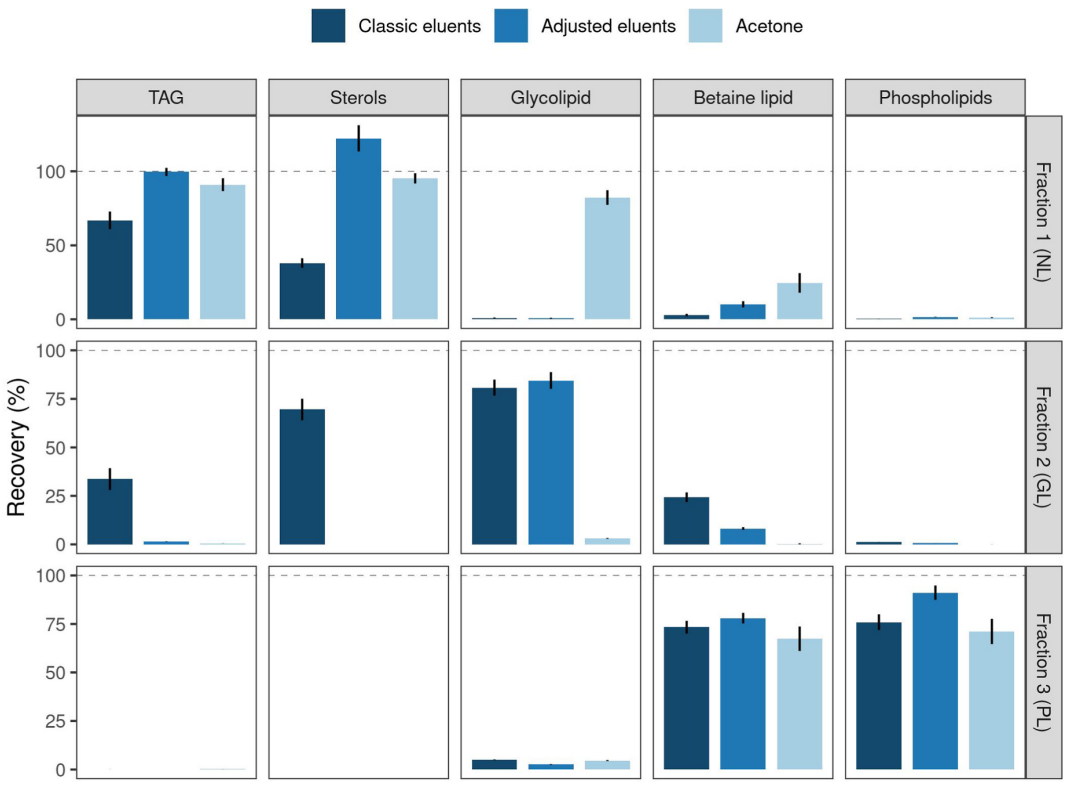

Fig. 2. Results from the fractionation of lipid classes during solid phase extraction, comparing different eluents as shown in Table 1. Barplots are arranged per lipid class and fraction collected from the solid phase extraction step: fraction 1 (expected to collect neutral lipids, NL), fraction 2 (expected to collect glycolipids, GL) and fraction 3 (expected to collect phospholipids, PL). Fractionation with the "Adjusted eluents" (i.e. addition of 2% ethanol to chloroform in fraction 1 and using 1 ml for the recovery of phospholipids) achieved an improved separation between neutral triacylglycerols (TAG) plus sterols and the glycolipid, and increased the recovery of phospholipids to 90–95%. Using acetone as eluent in both fraction 1 and 2 resulted in the combined collection of TAG, sterols and glycolipids in the first fraction. Bars represent mean values (n = 3) and error bars represent standard errors.

sterols were recovered in the neutral lipid fraction, while the remaining were lost to the glycolipid fraction. Gas chromatography measurements of ergosterol gave excellent standard row linearity on GC-MS and GC-IRMS ($R^2_{adj} > 0.99$, data not shown). Sterol recovery was also not affected by the derivatisation step of mild alkaline methanolysis (*t*-test, P = 0.85, n = 3; data not shown).

3.2. Pure culture extracts show consistent fatty acid profiles across lipid fractions

Analysis of pure culture fatty acid profiles revealed species-specific fatty acid patterns which were consistent across PLFA, NLFA and GLFA fractions of the respective strain (Fig. 3, heatmap). This is additionally indicated by NMDS analysis, which shows primary clustering of species, and only secondary clustering by lipid class (Fig. 4). PERMANOVA results confirm this, with significant differences between the microbial strain's fatty acid profiles ($P < 0.001$, $R^2 = 0.74$), which explain a substantially higher proportion of the variance compared to differences between lipid classes ($P < 0.001$, $R^2 = 0.04$).

Both actinobacterial strains (belonging to gram-positive bacteria) contained the iso-branched fatty acid 16:0i (36.5 mol% on average across lipid fractions), which is, like other terminally branched short-chain fatty acids, a known biomarker for gram-positive bacteria. The Actinobacterium *Solirubrobacter soli* additionally contained 18:1ω9cis (22.5 mol% averaged across the three lipid fractions), a fatty acid otherwise specific to fungi. Only *Streptosporangium roseum* contained the Actinobacteria-specific methyl-branched fatty acids 10Me17:0 and 10Me18:0 (17.8 and 7.3 mol% on average respectively). The Firmicutes strain *Paenibacillus alginolyticus* (gram-positive bacteria) had significant amounts of the gram-positive-specific fatty acid 15:0a (69.7 mol% on average), but also contained several other terminally branched, gram-positive-specific, fatty acids with abundances below 10 mol% (14:0i, 15:0i, 16:0i, 17:0a). Proteobacterial (i.e. gram-negative) strains showed high specificity in the monounsaturated fatty acids 18:1ω9trans (18.3–48.3 mol%), 19:1ω9 (8.5–53.9 mol%), 17:0cy (1.3–19.8 mol%), and 16:1ω7 (1.3–13.9 mol%), all known biomarkers for gram-negative bacteria. The arbuscular-mycorrhizal fungus *Rhizophagus irregularis* contained the specific fatty acid 16:1ω5 in all three lipid fractions (24.5 mol% on average), although other fatty acids were also present in substantial amounts above 5 mol% (18:ω9trans, 20.8 mol%; 16:0, 15.0 mol %; 18:1ω9cis, 13.9 mol%; 18:1ω5, 5.8 mol%). The other fungal strains generally showed high abundances in the polyunsaturated fatty acid 18:2ω6,9 (26.5–68.5 mol% averaged across the three lipid fractions per strain), a widely used biomarker for fungi. They additionally had high abundances in 18:1ω9cis, especially Ascomycetes (up to 36.3 mol% on average across the three lipid fractions in *Trichoderma virens*), but showed low abundances in 18:1ω9trans (<3 mol%).

Absolute abundances (per g dw) of PLFAs, NLFAs and GLFAs varied significantly between the lipid fractions and strains (Fig. 3, barplot). PLFAs were highest in bacterial strains (up to 1650 nmol g^{-1} dw on average in *Paenibacillus alginolyticus*), while the highest NLFA values were found in the Ascomycete *Trichoderma virens* (3520 nmol C g^{-1} dw on average) and the arbuscular-mycorrhizal fungus *Rhizophagus irregularis* (6780 nmol C g^{-1} dw). Otherwise, fungi and bacteria showed

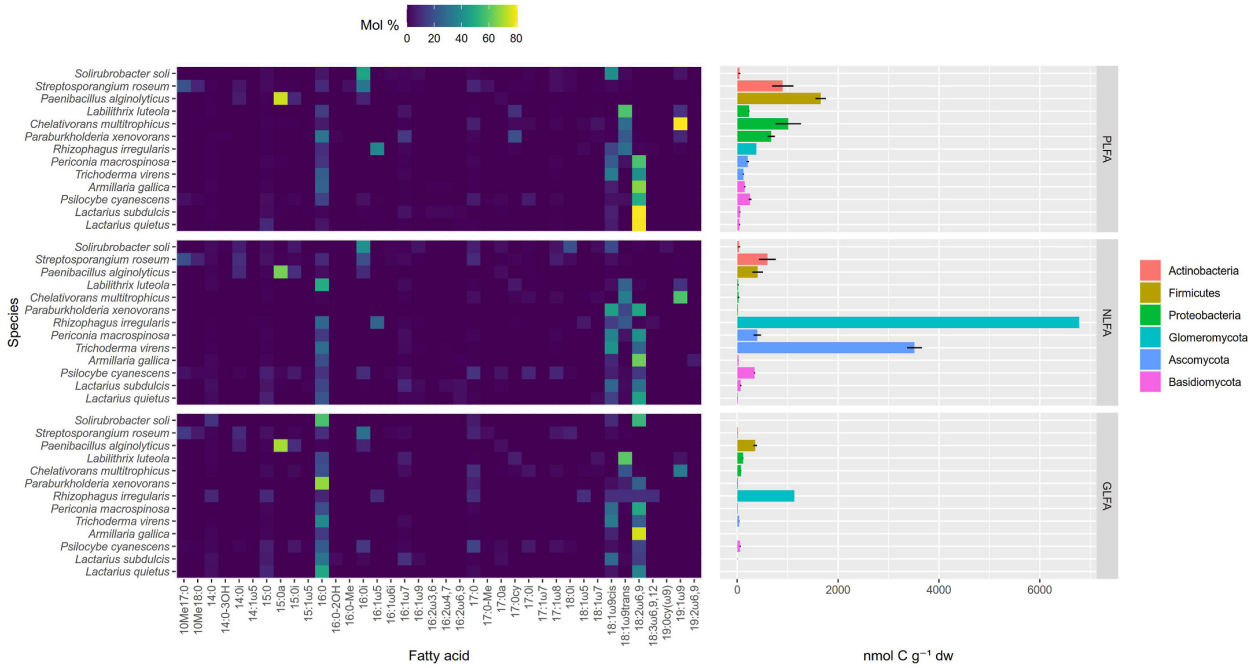

Fig. 3. Microbial pure culture fatty acid profiles of the three lipid fractions. The taxonomic specificity of marker fatty acids is similar across PLFAs, NLFAs and GLFAs. Values in the heatmaps (left graphs) are expressed in mol%, that is the percentage of each fatty acid from the total of each lipid fraction (mean of technical replicates). The bar charts (right graphs) show the sum of all fatty acids per lipid fraction and species in nmol C per gram of dry weight. Error bars represent standard errors.

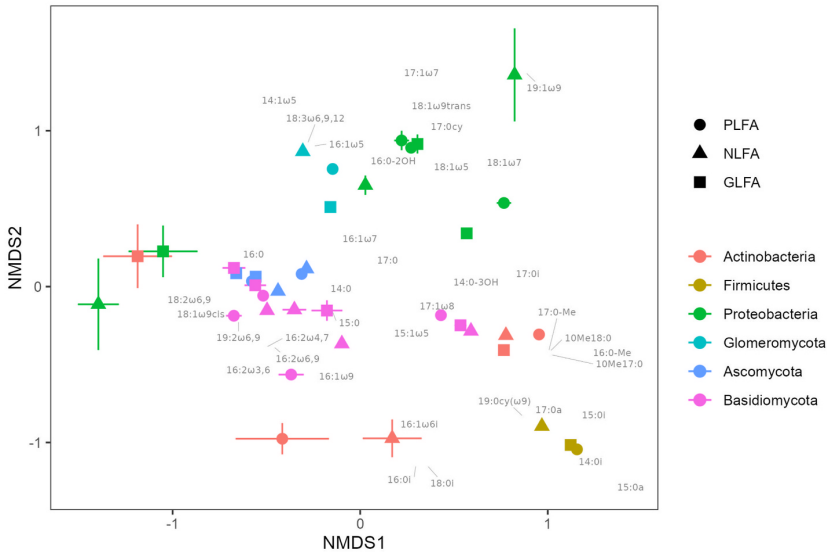

Fig. 4. Non-metric multidimensional scaling (NMDS, stress = 0.153) plot of the microbial pure culture fatty acid profiles. PERMANOVA analysis shows that pure culture strains explain a much larger portion of the variance ($R^2 = 0.73$, $P < 0.001$) compared to fatty acid lipid class ($R^2 = 0.03$, $P < 0.001$). Points represent average site scores across technical replicates of each microbial strain, coloured by phylum and separated by shape for each fatty acid lipid class (PLFA/NLFA/GLFA). Error bars represent standard errors. The plotted data are the same as in Fig. 3.

similar NLFA abundances, with high variability (13–600 nmol C g^{-1} dw). Ascomycetes and Basidiomycetes consistently had the lowest absolute abundances in the GLFA fraction (1–61 nmol C g^{-1} dw). *Rhizophagus irregularis* was the only fungus to show the lowest abundances in the PLFA fraction (390 nmol C g^{-1} dw). Gram-negative bacteria showed the highest abundances in the PLFA fraction (235–1010 nmol C g^{-1} dw), with comparatively low amounts of NLFAs (10–30 nmol C g^{-1} dw) and GLFAs (10–120 nmol C g^{-1} dw). In contrast, within individual strains, gram-positive bacteria showed relatively similar abundances of PLFAs (50–1650 nmol C g^{-1} dw) and NLFAs (40–600 nmol C g^{-1} dw), and the lowest amounts in GLFAs (3–360 nmol C g^{-1} dw).

3.3. Soil fatty acid profiles are more strongly separated by lipid class than by soil types

In soils, we found the majority of total lipid-derived fatty acids in the PLFA and NLFA fractions (combined ca. 88%, Fig. 5a). These were distributed unequally between the three analysed soils: Agricultural and forest soils showed ca. 50% in the PLFA fraction, and grassland soils ca. 50% in the NLFA fraction due to a substantial influence of the arbuscular

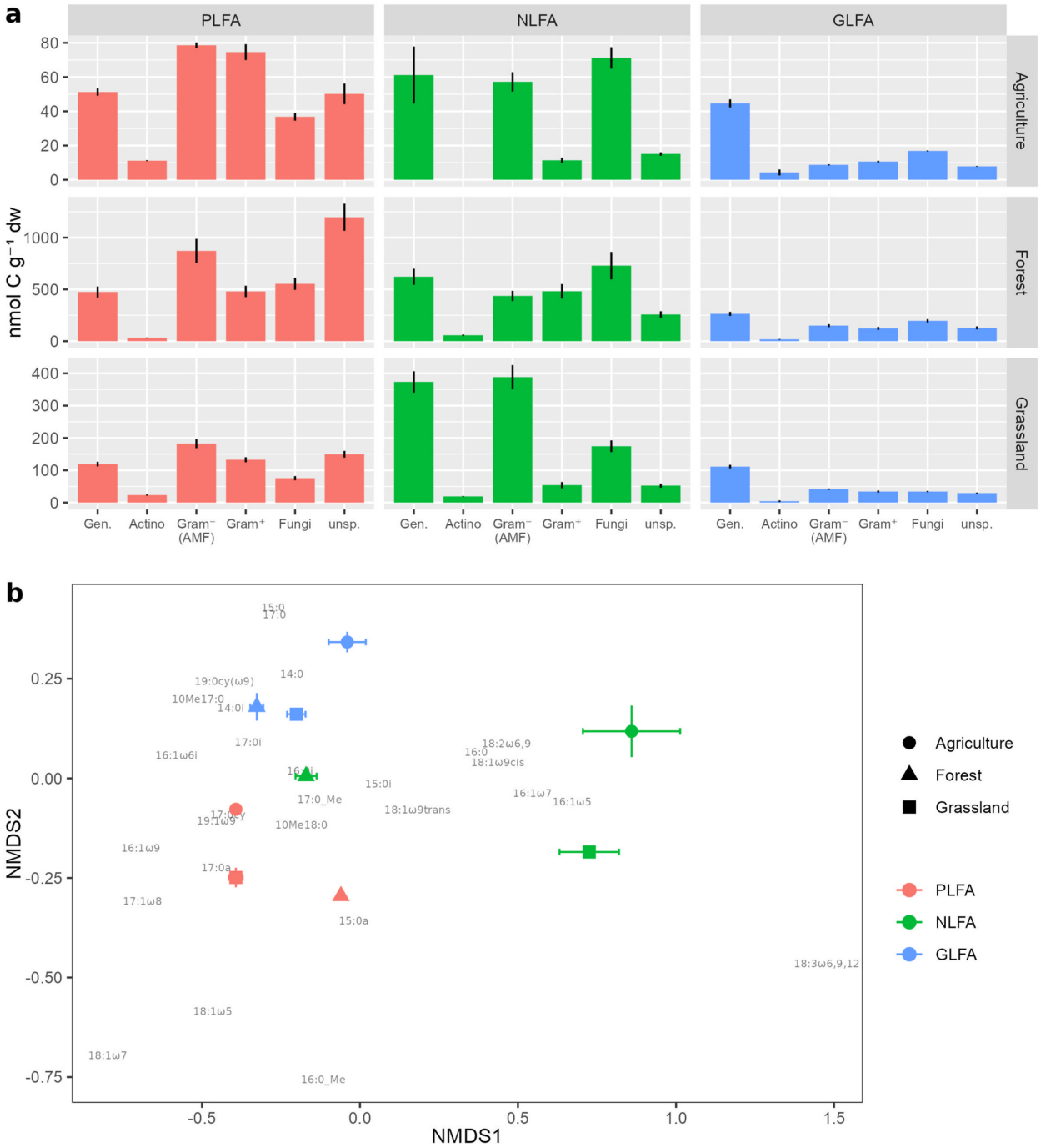

Fig. 5. **(a)** Fatty acid abundances of the three lipid fractions in soil extracts. Barplots depict the sum of fatty acids specific to different microbial groups: general (General), Actinomycetes (Actino), gram-negative bacteria (Gram$^-$) including the fatty acid 16:1w5 which is highly specific to arbuscular-mycorrhizal fungi (AMF, particularly in the neutral lipid fraction), gram-positive bacteria (Gram$^+$), fungi, and unspecified (unsp.). Error bars represent standard errors. Note the strong differences in total fatty acid abundances between soils on the y-axes. **(b)** Non-metric multidimensional scaling (NMDS; stress = 0.084) plot of the same fatty acid profiles as in (a). Profiles are primarily separated by fatty acid lipid class (PERMANOVA $P < 0.001$, $R^2 = 0.47$), but also by soil type ($P < 0.001$, $R^2 = 0.21$).

mycorrhizal fungal marker 16:1ω5 (Lekberg et al., 2022). The GLFA fraction consistently contained ca. 14% of the total fatty acids. If included, the fatty acid 18:0 held less than 1% of the total in the PLFA and GLFA fraction respectively, and 15–57% in the NLFA fraction (Fig. S4). Across all soils, fungal NLFAs were more abundant than fungal PLFAs.

Multivariate NMDS analysis of the soil profiles showed distinct clustering of the three lipid fractions (Fig. 5b; PERMANOVA $P < 0.001$, $R^2 = 0.47$). Additionally, within each lipid fraction cluster, soils were significantly separated (PERMANOVA $P < 0.001$, $R^2 = 0.21$). Each lipid class was associated with specific fatty acids across the three analysed soils. For instance, PLFA profiles were strongly influenced by the straight-chain fatty acids 14:0, 15:0, and 17:0. NLFA profiles were strongly influenced by the fungal markers 18:1ω9cis, 18:2ω6,9, 18:3ω6,9, as well as the fatty acids 16:0, 16:1ω5, and 16:1ω7, particularly in agricultural and grassland soils (Fig. 5b).

3.4. Sterol extractions allow quantification in pure cultures, but are inconsistent in soils

In Basidiomycete and Ascomycete mycelia (and sporocarps), we found variable ergosterol concentrations of 0.62–23.3 mg g^{-1} dw (Table S6). Interestingly, we found several sterols in the Ascomycete *Periconia macrospinosa* apart from ergosterol: squalene, dehydroergosterol (Ergosta-5,7,9(11),22-tetraen-3-ol, (3β,22 E)-), ergone (Ergosta-4,6,8(14),22-tetraen-3-one), and sitostenone (Fig. 6b). The sterol profile of the arbuscular-mycorrhizal fungus *Rhizophagus irregularis* (spores and mycelium) showed the sterols 24-ethylcholesterol and 24-methylcholesterol (Fig. 6c). In this case, although NIST library comparison returned β-sitosterol ((3β)-Stigmast-5-en-3-ol) and campesterol ((3β,24 R)-Ergost-5-en-3-ol) as the highest similarity matches, we identified the peaks in the structurally more general terms 24-ethylcholesterol ((3β,24ξ)-Stigmast-5-en-3-ol) and 24-methylcholesterol ((3β,24ξ)-Ergost-5-en-3-ol), respectively. This is because we could not determine the stereochemistry of the C-24 ethyl/methyl groups, and arbuscular mycorrhizal sterols are commonly assigned this way (Grandmougin-Ferjani et al., 1999; Fontaine et al., 2001, 2004; Weete et al., 2010; Dalpé et al., 2012; Wewer et al., 2014; Siebers et al., 2016). Both GC programs (elution of both FAME and sterols, and of only sterols) resulted in good chromatographic separation with well resolved peaks (Fig. 6, Fig. S5).

Ergosterol extraction from soil proved to be difficult, yielding only low amounts of ergosterol following the PLFA protocol (Table S4). Our first test on a beech forest soil improved the extraction yield approximately twofold by increasing the extractant:soil ratio (1 g on 20 ml CMB), performing better than the ethanol extraction (Djajakirana et al., 1996). However, later tests on different soils revealed a poor and highly variable performance of the PLFA method, yielding 2- to 9-fold lower values than the ethanol extraction (Table S4). While the quantification of ergosterol was done with its molecular ion mass spectra because of its

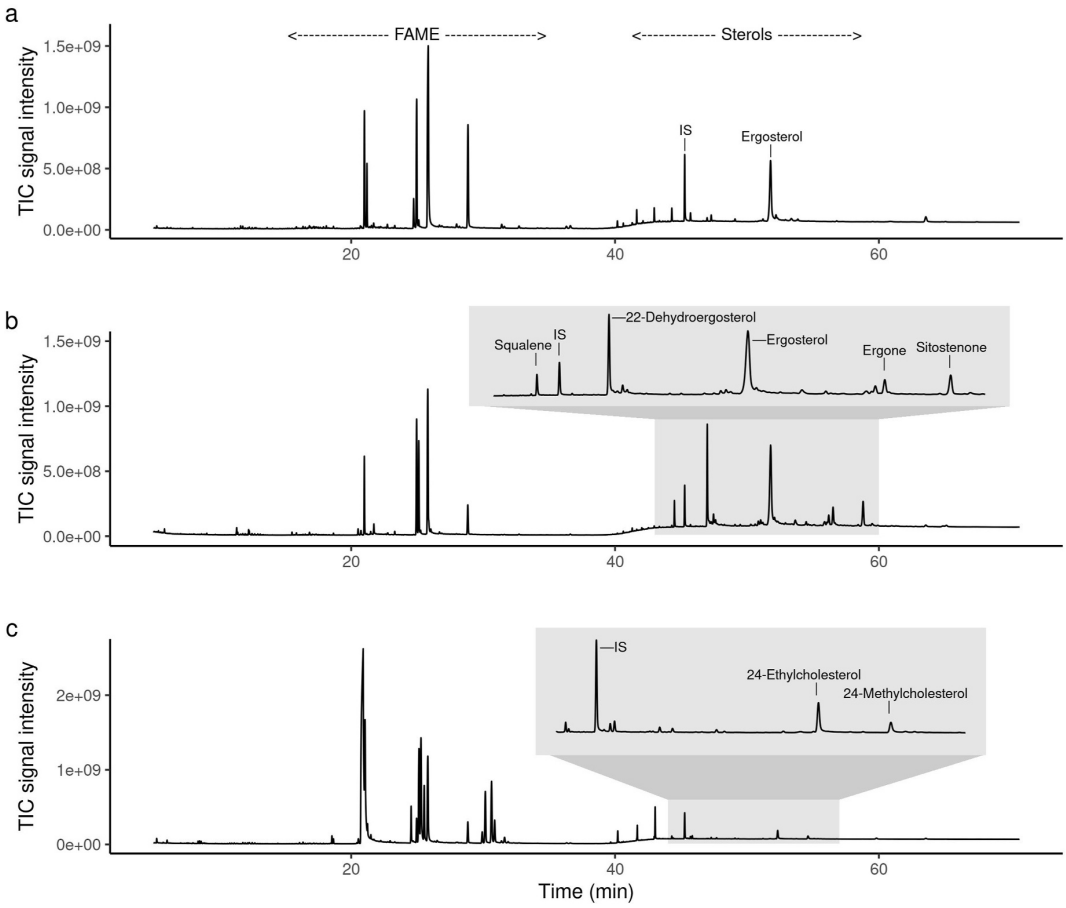

Fig. 6. Total ion chromatograms showing concomitant measurement of NLFA and sterols within a single GC run. Shown are fungal NLFA and sterol profiles of (a) an *Agaricus* sp. sporocarp extract, (b) a *Perigonia macrospinosa* pure culture extract, and (c) a *Rhizophagus irregularis* spores and mycelium extract. IS, internal standard (5-α-Cholestane).

low abundance in total ion chromatograms, we also identified the plant sterols brassicasterol, campesterol, sitosterol, stigmasterol and sitostenone, as well as cholesterol with well resolved peaks in the total ion chromatograms of soils (Fig. S5).

4. Discussion

Microbial lipids can provide detailed information on the biogeochemistry and microbial ecology of soil ecosystems. The PLFA method is a useful tool to quantify soil microbial biomass based on coarse taxonomic groups and to trace microbial community dynamics. Here, we tested the suitability of the PLFA method for other lipid fractions. We show that a simple modification (after Drijber and Jeske, 2019) allows satisfactory separation of the three lipid fractions (based on representative lipid standards), and that taxonomic specificities of marker fatty acids are retained across microbial pure culture PLFA, NLFA and GLFA profiles (Figs. 3 and 4). We further show that soil PLFA, NLFA, and GLFA profiles differ significantly (Fig. 5), which suggests that their combined analysis offers sensitive information on soil microbial communities beyond the scope of PLFAs alone. With the PLFA method having become a standard method in soil science, the analysis of NLFA and GLFA profiles is easily integrated and has the potential to provide valuable insights on soil microbial community dynamics and functions.

4.1. Purity of lipid fractions

Our results show that the addition of 2% ethanol to chloroform achieves suitable separation of the neutral and glycolipid fraction, while leaving the phospholipid fraction unaffected (Fig. 2). Specifically, triacylglycerols and sterols eluted in the neutral lipid fraction with negligible losses to the glycolipid fraction. The glycolipid used was unaffected by the ethanol addition and fully recovered in the GLFA fraction.

Incomplete fractionation was only observed with the betaine lipid DGTS (both with and without the 2% ethanol addition): approximately 80% were recovered in the phospholipid fraction, the rest being equally distributed between the neutral lipid and glycolipid fraction (Fig. 2). Similarly, recent critical assessments of the lipid fractionation protocol have shown contamination of the phospholipid fraction by betaine lipids (Heinzelmann et al., 2014; Warren, 2019). DGTS can be utilised by bacteria and fungi as a substitute for phospholipids under phosphorus limitation (Geiger et al., 2010; Riekhof et al., 2014; Warren, 2020). Thus, although these compounds are usually not abundant in soils, interpretive caution of the PLFA fraction has to be taken in low-phosphorus systems (Warren, 2020). This should however not influence the validity of quantified lipid data when using PLFAs to refer to biomass, as DGTS occurs in cell membranes together with phospholipids.

Similar to betaine lipids, bacterial glycolipids can act as substitutes for phospholipids under phosphorus limitation (Hölzl and Dörmann, 2007). It has therefore been suggested to combine PLFAs and GLFAs into a singular 'membrane-lipid fraction' for soil microbial community analyses (Heinzelmann et al., 2014). This would slightly increase the total fatty acid yield as our analyses showed only small contributions of GLFAs to the total lipid-derived fatty acid pool in both pure cultures and soils (Figs. 3 and 5a). However, our results on soil PLFA and GLFA abundances indicate that their combination could influence the relative distributions of the fatty acid profile compared to only PLFAs (Fig. 5b). In the case of studies interested in combining these two fractions, the 2% ethanol addition would also represent a critical factor to avoid contamination of the GLFA fraction by NLFAs.

4.2. Taxonomic specificity of fatty acids

Our pure culture analysis reflects, on a broad scale, known assignments of PLFA biomarkers to large microbial groups (such as gram-positive or gram-negative bacteria or fungi), but also demonstrates that certain combinations of individual fatty acids are typical for individual strains and that they correspond across the three lipid fractions. Although relative fatty acid abundances varied, the taxonomic specificity of marker fatty acids was retained between PLFAs, NLFAs, and GLFAs (Figs. 3 and 4). This makes sense as the intracellular diversity of *de novo* fatty acid synthesis is constrained by the respective species. Indeed, fatty acid synthesis is the first step in cellular lipid production (Kosa and Ragauskas, 2011), and functionally intermediate diacylglycerols serve as precursors in the synthesis of both triacylglycerols and phospholipids (Carrasco and Mérida, 2007; Alvarez, 2016). Although microbes can assimilate exogenous fatty acids, their majority acts as precursor for further transformations (e.g. desaturation, elongation, branching; Dippold and Kuzyakov, 2016). For instance, Khoomrung et al. (2008) found that the addition of the fatty acid 18:0 significantly increased the concentration of 18:3ω6,9,12 in a fungal pure culture of *Mucor rouxii* (Mucoromycota). Also, untransformed incorporation of assimilated fatty acids into bacterial biomass is likely negligible on a community level as the bacterial fatty acid synthase system inhibits this in most species (Yao and Rock, 2017).

The fatty acid specificities we found in the pure cultures are mostly in accordance with previous literature (e.g. Olsson et al., 1995; Zelles, 1997; Willers et al., 2015): Fungal strains (Basidiomycetes and Ascomycetes) exhibited high levels of the C18 unsaturated fatty acids 18:2ω6,9 and 18:1ω9cis, and the arbuscular-mycorrhizal fungus contained the characteristic fatty acid 16:1ω5. Gram-positive bacterial strains showed high concentrations of the anteiso-branched fatty acids 15:0a (Firmicutes) and 16:0i (Actinobacteria), while gram-negative strains showed the monounsaturated fatty acids 18:1ω9trans and 19:1ω9. Interestingly, we found the Actinobacteria-specific methyl--branched fatty acids 10Me16:0 and 10Me17:0 only in *Streptosporangium*, while *Soluribrobacter* showed high concentrations of 18:1ω9cis.

Although 18:1ω9trans has previously been used as a marker for bacteria (or gram-negative bacteria specifically; Tong et al., 2007; Schütz et al., 2009; Willers et al., 2015; Grossman et al., 2019; Abán et al., 2021), other authors used it for both fungi and bacteria (Spyrou et al., 2009; Sommer et al., 2017), or only for fungi (e.g. Jiang et al., 2018; Gorka et al., 2019; Singh et al., 2021). Similarly, 18:1ω9cis has been used as a marker for fungi (Willers et al., 2015; Schmidt et al., 2017; Gorka et al., 2019; Grossman et al., 2019; Singh et al., 2021), bacteria and fungi (Spyrou et al., 2009; Smith et al., 2015), and only bacteria (or gram-negative bacteria specifically; Gunina et al., 2014, Abán et al., 2021, Wang et al., 2021; Yang et al., 2022). Our data find 18:1ω9trans in gram-negative bacteria, but also in (albeit lower) relative amounts in all analysed fungal strains, suggesting it to be non-specific. In contrast, we found high abundances of 18:1ω9cis in fungi, but only in a single bacterial species (the Actinobacterium *Solirubrobacter*). The specificity of the *cis*-form towards fungi is supported by findings of high abundances of 18:1ω9cis, but no or low abundance of 18:1ω9trans in fungal sporocarps, pure cultures, and ectomycorrhizal morphotypes (Müller et al., 1994; Karliński et al., 2007; Pedneault et al., 2008).

4.3. Interpretation of NLFA and GLFA profiles for soil microbial ecology

Complete NLFA profiles of soil samples have rarely been considered previously (Hedlund, 2002; Rinnan and Bååth, 2009). NLFAs have mostly been utilised as storage markers for ascomycete and basidiomycete fungi (Bååth, 2003), or as a proxy for biomass as well as spore production of arbuscular mycorrhizal fungi (Olsson et al., 1995; Sharma and Buyer, 2015; Lekberg et al., 2022). Here we show that soils can also harbour significant amounts of bacteria-derived NLFAs, which ranged in similar concentrations as their PLFA counterparts (Fig. 5a). Soil NLFA and PLFA profiles also differed in their relative distributions (Fig. 5b), indicating that NLFAs reflect distinct community traits. It has to be noted that the literature is quite ambiguous on the origin of bacterial NLFAs. On the one hand, bacteria can produce neutral triacylglycerols

for carbon storage, in the same way as fungi (Alvarez and Steinbüchel, 2002). Indeed, a recent meta-analysis suggested that triacylglycerols are ubiquitous across eukaryotes and bacteria (Mason-Jones et al., 2021). On the other hand, one of the first steps in the degradation of phospholipids is the cleavage of the phosphate head, leaving a neutral diacylglycerol, which would be collected in the NLFA fraction. This way, bacterial NLFAs would be indicative of recently formed bacterial necromass (Bååth, 2003; Amir et al., 2008; Honvault et al., 2021). Additionally, intracellular diacylglycerols (intermediate neutral lipid compounds in lipid synthesis) could also be a potential source of bacterial NLFAs (Parsons and Rock, 2013; Röttig and Steinbüchel, 2013). To this date it is unknown which source they mostly originate from, and more direct approaches like shotgun lipidomics might resolve this uncertainty (Ejsing et al., 2009). Nevertheless, we also found substantial amounts of NLFAs in bacterial pure cultures (Fig. 3), which were thoroughly washed before extraction and thus contained only negligible concentrations of extracellular components like remains from dead cells. We therefore hypothesise that the majority of bacterial pure culture NLFAs was derived from intracellular triacylglycerols. Similarly, previous reports show bacterial triacylglycerols or lipid droplets production, especially in Actinobacteria (Wältermann and Steinbüchl, 2005; Hernández and Alvarez, 2010; Röttig et al., 2016; Mason-Jones et al., 2021). Our pure culture data therefore suggest that bacterial NLFAs may be indicative of investment into carbon storage, although, we cannot rule out that some fraction of soil bacterial NLFAs are derived from recently degraded bacterial phospholipids. Thus, when handled with appropriate caution in interpretation, complete NLFA profiles could provide sensitive measurements on both fungal and bacterial carbon storage dynamics. Especially when the analysis of NLFAs is combined with stable isotope tracing, simple substrates, as well as photosynthates from mycorrhizal hosts, can be used for tracing carbon flows into fungal storage compounds (Rinnan and Bååth, 2009; Drigo et al., 2010; Rinnan et al., 2013; Karlowsky et al., 2018). Recently produced bacterial triacylglycerols could be targeted in a similar manner, limiting the risk of potential confounding bacterial necromass remains—at least within short timeframes after labelled substrate application.

The definite functions of microbial glycolipids are not yet fully understood. In addition to functioning as substitutes for phospholipids (Hölzl and Dörmann, 2007), they were found to increase resistance against heat and chemical agents in *Microbacterium* sp. (Nakata, 2000). Glycolipids can also act as biosurfactants, which allows the adhesion of microorganisms to hydrophobic surfaces and can increase the bioavailability of insoluble nutrients (Kitamoto et al., 2002). We speculate that they could be used as markers for certain types of environmental stress, although further investigation is necessary.

The combined analysis of the fatty acid profiles obtained from the three lipid fractions thus has the advantage of quantifying not only microbial biomass based on membrane lipids (phospholipids), but also of carbon storage and possibly bacterial necromass formation (neutral lipids) and potential markers for environmental stress (glycolipids). Our results show that while largely maintaining fatty acid specificity (Figs. 3 and 4), they can differentiate soils in different ways along a multivariate environment (Fig. 5). This information is lost when using only PLFAs while discarding the other fractions, or omitting the lipid fractionation step for undifferentiated analysis of total ester-linked fatty acids (Miura et al., 2017). Analysis of all three lipid fatty acid profiles could thus be used to trace changes in the structure and functioning of microbial communities from multiple perspectives. This could be ideal for sensitive measurements of community functional states in experiments which apply environmental disturbances or stressors, or other treatments which influence soil microbial community dynamics. Especially combinations with stable isotope techniques (^{13}C or ^{2}H) to target recently produced lipids might reveal a powerful approach.

4.4. Extractability of sterols from pure cultures and soils

All Ascomycete and Basidiomycete strains showed high concentrations of ergosterol as the only sterol in the NLFA fraction (see example chromatograms in Fig. 6a and Fig. S5b), which were in the expected range when compared to previous reports (Antibus and Sinsabaugh, 1993; Djajakirana et al., 1996, and references therein). Only the Ascomycete *Periconia macrospinosa* contained several other compounds (Fig. 6b), most of which are precursors or derivatives of ergosterol. Squalene is a precursor in the sterol biosynthetic pathway (Spanova and Daum, 2011; Micera et al., 2020). Ergone and dehydroergosterol are likely metabolic derivatives of ergosterol (Atherton et al., 1972; Price and Worth, 1974) and have previously been identified in Ascomycetes (Heald et al., 1981; Jinming et al., 2001; Kwon et al., 2002; Fujimoto et al., 2004; Lee et al., 2007) and Basidiomycetes (Yaoita et al., 2001; Zhao et al., 2009; Senik et al., 2019). Dehydroergosterol has additionally been found in a Mucoromycete fungus (Atherton et al., 1972), and although its function is unclear, it was observed to accumulate under high temperature stress in the Ascomycete *Favolaschia manipularis* (Senik et al., 2019). Sitostenone is a phytosterol usually found in endophytic fungi (Carvalho et al., 2016; Li et al., 2019; Wu et al., 2019); we speculate that in our case it was not anabolised by the fungus itself, but incorporated into fungal biomass from corn meal used in the growth medium (Table S3). *Rhizophagus irregularis* contained 24-ethylcholesterol and 24-methylcholesterol, and no ergosterol (Fig. 6c). These sterols have been found to be characteristic for arbuscular-mycorrhizal fungi (Fontaine et al., 2001, 2004; Weete et al., 2010).

While the adjusted PLFA method extracted ergosterol from single strains, and phytosterols and cholesterol from soil, ergosterol yields from soils were variable, miniscule and in general significantly less than the reference extraction method with ethanol (Djajakirana et al., 1996; Table S4). The Bligh and Dyer lipid extraction only achieved higher ergosterol yields in one of four soils. In contrast, we found high values in fungal tissue extracts (Table S6) and excellent recovery of pure sterols subjected to lipid fractionation and mild alkaline methanolysis. Extractions from other matrices (mycorrhizal roots, sewage sludge and straw) also successfully quantified ergosterol from the neutral lipid fraction (Laczko et al., 2003; Amir et al., 2008). This suggests that the adjusted Bligh and Dyer lipid extraction method is not suitable for ergosterol extraction when a soil matrix is present. Other published soil extraction methods with ergosterol recoveries of 70–100% are more suitable (Davis and Lamar, 1992; Djajakirana et al., 1996; Eash et al., 1996; Montgomery et al., 2000; Laughlin and Stevens, 2002; Turgay and Nonaka, 2002; Plaza et al., 2015). While the reason why ergosterol could not be extracted following the Bligh and Dyer protocol was not the focus of this study, we speculate that soil organic matter interaction with ergosterol might be the primary reason. Because ergosterol is not electrically charged and because we did not find peaks indicative of ergosterol derivatives in soil samples, the non-extractability of ergosterol is unlikely due to binding to mineral surfaces (Angst et al., 2021) or oxidation processes. Instead we hypothesise that it could be stabilised via organo-organo interactions (Buckeridge et al., 2022). Indeed soil organic matter content can also influence ergosterol recovery (de Ridder-Duine et al., 2006), which could explain our observed differences in yields between soil types (Table S4). We thus recommend using a separate soil extraction method for ergosterol (e.g. Djajakirana et al., 1996; Gong et al., 2001). Nonetheless, our GC method should still be applicable to soil extracts from alternative extraction methods. The full extraction method can also be applied to other matrices, which additionally allows stable isotope probing approaches with GC-IRMS measurements of ergosterol- and other sterol-^{13}C enrichment.

5. Summary and conclusions

We show that the high-throughput PLFA method can be expanded for the simultaneous extraction and fractionation of NLFA and GLFA, along

with PLFA profiles. Chloroform with 2% ethanol guarantees proper elution of the different lipid fractions. Betaine lipids are concomitantly extracted in the PFLA fraction. They generally represent a minor fraction of the soil lipids except in phosphorus-limited environments, where caution should be taken. The combined analysis of the three lipid fractions potentially provides sensitive information about fungal and bacterial physiology (carbon storage and/or necromass formation, stress response) in addition to microbial biomass, which is of considerable interest for microbial community analysis. Our findings show high similarities between pure cultures fatty acid profiles, but contrasting results across soils, giving a first glimpse into the potential of using the three lipid fractions for fatty acid fingerprinting. We thus propose that the high-throughput PLFA method, especially in combination with stable isotopes, has the potential to provide a wider range of information on important ecological traits of soil microbes than its current use.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Christina Kaiser reports financial support was provided by Austrian Science Fund. Christina Kaiser reports financial support was provided by European Research Council.

Data availability

Open access to the dataset and R scripts to reproduce analyses and graphs in the main article is available at: https://doi.org/10.5281/zenodo.8410341

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2023.109205.

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Supplementary Tables

Table S1 GC programs used for separation of FAME only, combined measurement of FAME and sterols, and only sterols. For our measurements, we only employed the 'FAME' and 'Sterols' programs. The total measurement times per sample were 58.83 min ('FAME'), 70.67 min ('FAME + Sterols'), and 38.33 min ('Sterols').

GC program	Rate (°C min^{-1})	Target temp. (°C)	Hold duration (min)
FAME	initial	80	1
	30	150	1
	2	200	0
	4	230	15
	30	290	5
FAME + Sterols	initial	80	1
	30	150	0
	4	230	15
	30	300	30
Sterols	initial	160	1
	20	300	20
	30	310	10

Table S2 List of used lipid standards. Standards were mixed together and used with a final concentration of 100 µg ml^{-1}.

Class	Lipid	Fatty acids
Phospholipids	Phosphatidylcholine	19:0
	Phosphatidylglycerol	17:0
Neutral lipids (TAG)	Glyceryl trimyristate	15:0
	Glyceryl tripentadecanoate	14:0
Neutral lipids (sterols)	Ergosterol	n.a.
	Dehydrocholesterol	n.a.
Glycolipids	Monogalactosyl diacylglycerol (MGDG)	18:3, 18:2, 16:3, 16:1
Betaine lipids	Diacylglyceryl trimethylhomoserine (DGTS)	16:0

Table S3 Media used and incubation temperatures of the extracted microbial pure cultures.

Kingdom	Phylum	Species	ID	Medium	Incubation temp.
Bacteria	Actinobacteria	*Solirubrobacter soli*	DSM 22325[A]	830[a]	28°C
Bacteria	Actinobacteria	*Streptosporangium roseum*	DSM 43021[A]	65[a]	28°C
Bacteria	Firmicutes	*Paenibacillus alginolyticus*	DSM 5050[A]	1[a]	28°C
Bacteria	Proteobacteria	*Paraburkholderia xenovorans*	DSM 17367[A]	220[a]	28°C
Bacteria	Proteobacteria	*Chelativorans multitrophicus*	DSM 6780[A]	830[a]	28°C
Bacteria	Proteobacteria	*Labilithrix luteola*	DSM 27648[A]	1509[a]	28°C
Fungi	Ascomycota	*Periconia macrospinosa*	DSM 62880[A]	191[a]	25°C
Fungi	Ascomycota	*Trichoderma virens*	DSM 3500[A]	129[a]	25°C
Fungi	Basidiomycota	*Armillaria gallica*	DSM 3732[A]	90[a]	22°C
Fungi	Basidiomycota	*Psilocybe cyanescens*	DSM 4999[A]	90[a]	25°C
Fungi	Basidiomycota	*Lactarius subdulcis*	CBS 411.75[B]	CHA[b]	20°C
Fungi	Basidiomycota	*Lactarius quietus*	S23C[C]	MMN[c]	20°C
Fungi	Glomeromycota	*Rhizophagus irregularis*	QS81[D]	-	-

[A] https://www.dsmz.de/

[a] https://www.dsmz.de/collection/catalogue/microorganisms/culture-technology/list-of-media-for-microorganisms

[B,b] https://wi.knaw.nl/page/fungal_table

[C] https://mycocosm.jgi.doe.gov/Lacqui1/Lacqui1.home.html

[c] https://www.bio-world.com/microbiological-media/melinnorkrans-medium-modified-wo-agar-p-30627028

[D] https://inoq.de/

Table S4 Soil ergosterol yields varied between soils and extraction methods. Shown are mean values, followed by standard error in parentheses. The absolute quantities of ergosterol were calculated via an external standard row. Data were normalised by the internal standard 5-α-cholestane beforehand, and corrected for transfer losses during the extraction. CMB, chloroform-methanol-citrate buffer mix (v:v:v = 1:2:0.8).

Soil	Extraction method	Extractant vol. (ml)	Soil (g dw)	Extractant:soil ratio	Ergosterol (µg g^{-1} dw)	n
Beech forest[a]	6 ml CMB	6	2	3	4.17 (0.39)	3
	20 ml CMB	20	2	10	7.38 (0.44)	4
	20 ml CMB	20	1	20	11.8 (0.51)	4
	Ethanol	30	2	30	6.51 (0.59)	3
Beech forest[b]	6 ml CMB	6	1.5	4	0.48 (0.02)	5
	20 ml CMB	20	1	20	0.67 (0.03)	5
	Ethanol	40	0.5	80	2.17 (0.36)	5
Spruce forest[b]	6 ml CMB	6	1.5	4	0.68 (0.04)	5
	20 ml CMB	20	1	20	0.98 (0.04)	5
	Ethanol	40	0.5	80	9.31 (2.49)	5
Grassland[b]	6 ml CMB	6	1.5	4	0.61 (0.05)	5
	20 ml CMB	20	1	20	0.71 (0.02)	5
	Ethanol	40	0.5	80	1.57 (0.06)	5

Extraction methods: [a] Only total lipid extraction; [b] Total lipid extraction, fractionation and mild alkaline methanolysis (samples from the same soils were re-extracted with the 20 ml CMB lipid extraction, and the total lipids were directly measured as in [a], resulting in similar ergosterol yields (data not shown), excluding the possibility of an influence of lipid fractionation and alkaline methanolysis on ergosterol extractability)

Table S5 Carbon (C) and nitrogen (N) contents, and pH values, of all used soils. [a] Soil used for analysis of total fatty acid profiles (corresponding to Fig. 5); [b,c] Soil used only for further ergosterol extraction tests, where [b] was only analysed after total lipid extraction, and [c] was analysed after total lipid extraction, fractionation and mild alkaline methanolysis (see Table S4).

Soil	C (%)	N (%)	pH
Beech forest[a]	14.3	0.9	6.2
Grassland[a]	3.1	0.3	5.2
Agricultural[a]	0.9	0.1	5.3
Beech forest[b]	5.9	0.4	5.1
Beech forest[c]	5.6	0.4	4.9
Spruce forest[c]	28.4	1.2	4.1
Grassland[c]	9	0.8	4.1

Table S6 Ergosterol concentrations in fungal species, extracted with the adjusted PLFA method. Shown are mean values followed by the standard error in parentheses.

Species	**mg Ergosterol g^{-1} dw**
Agaricus sp.[a]	3.8 (0.98)
Amanita muscaria[a]	4.18 (0.18)
Armillaria gallica[b]	0.62 (0.1)
Lactarius quietus[b]	0.77 (0.02)
Lactarius subdulcis[b]	1.01 (0.18)
Periconia macrospinosa[b]	23.3 (3.64)
Psilocybe cyanescens[b]	0.71 (0.09)
Trichoderma virens[b]	6.68 (0.5)

[a] Sporocarps, [b] Mycelium

Supplementary Figures

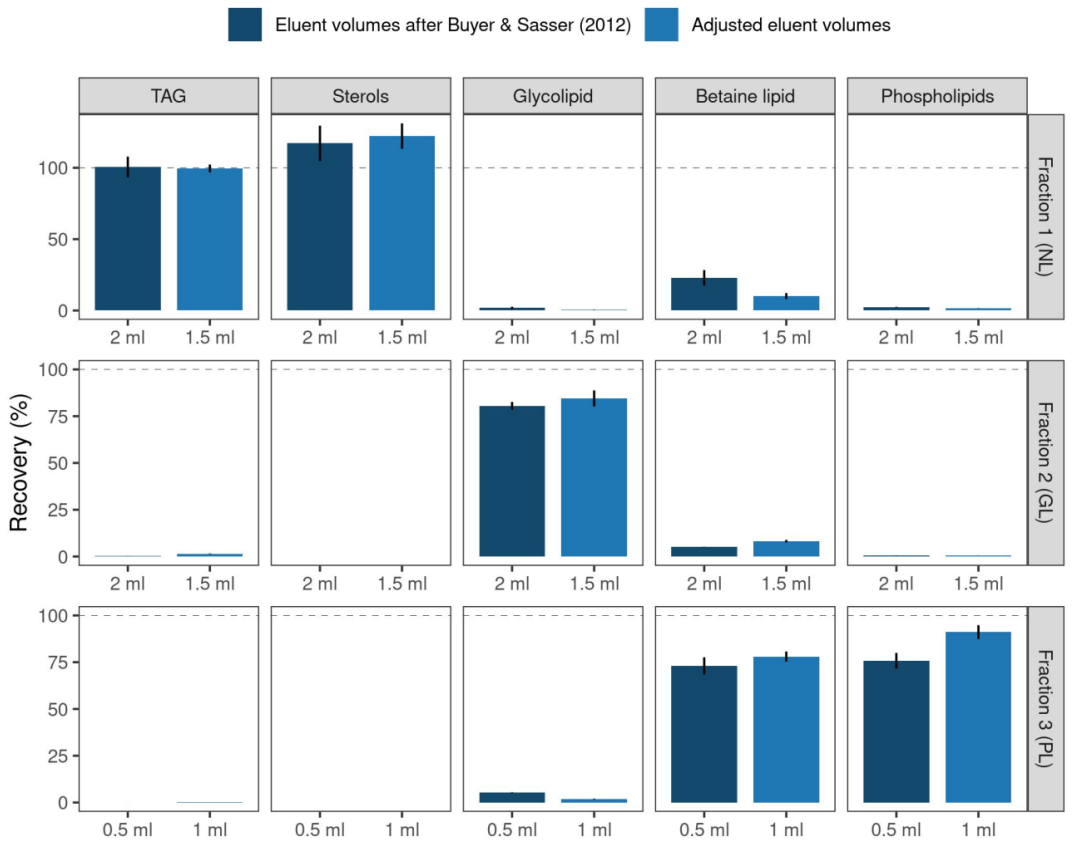

Figure S1 Lipid recovery after eluting with the adjusted eluents (see Table 1). Fraction 1 + Fraction 2: The reduction in eluent volumes from 2 ml to 1.5 ml did not result in reduced recoveries of neutral lipids and glycolipids. Fraction 3: The recovery of phospholipids (PL) improved on average from 76% to 91% by increasing the volume of the third fraction (methanol-chloroform-water, v:v:v = 5:5:1) from 0.5 to 1 ml.

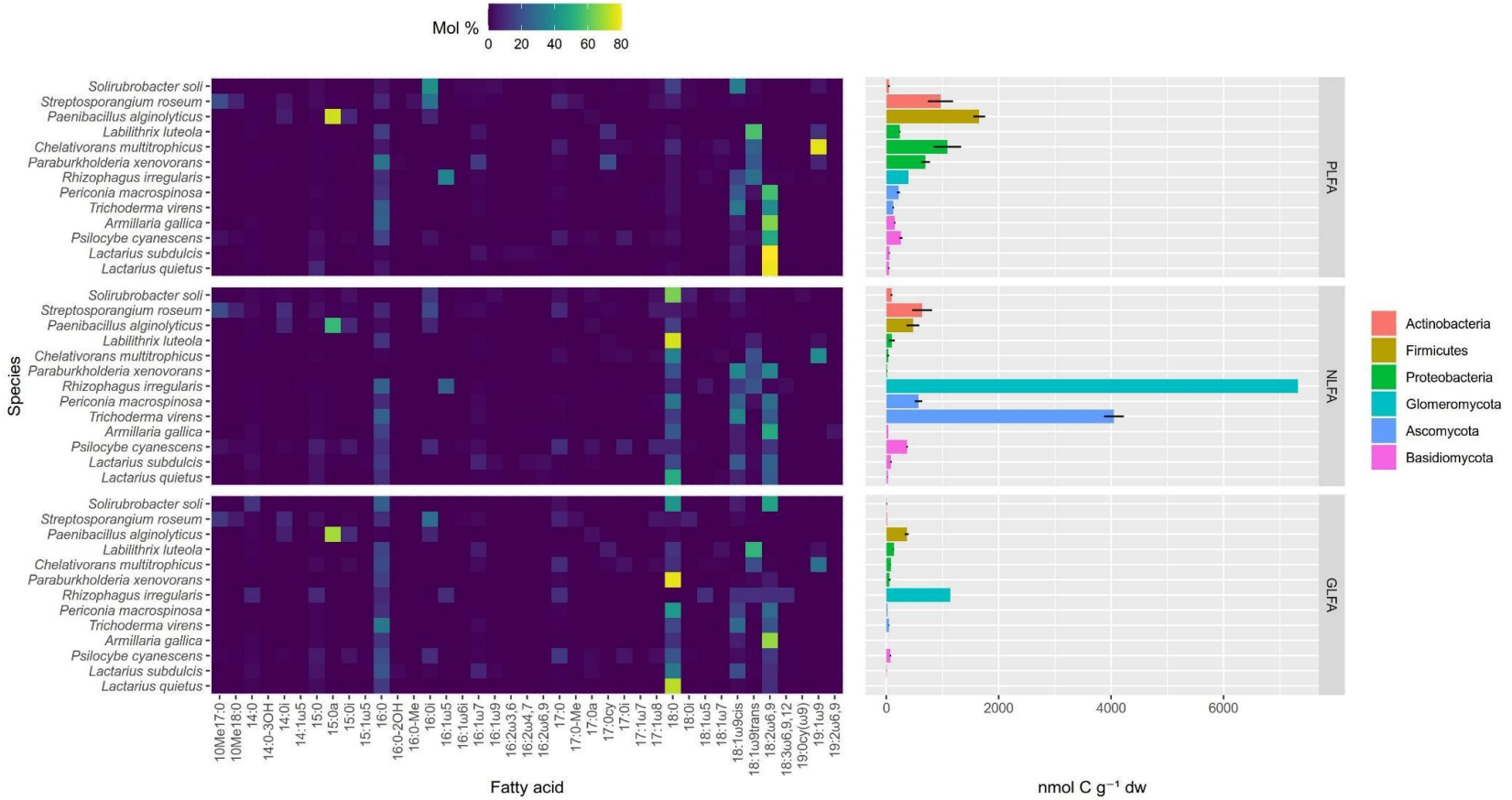

Figure S2 This figure shows the same data as Fig. 3, but includes the fatty acid 18:0. Values in the heatmaps (left graphs) are expressed in mol%, that is the percentage of each fatty acid from the total of each lipid fraction (mean of technical replicates). The bar charts (right graphs) show the sum of all fatty acids per lipid fraction and species in nmol C per gram of dry weight. Error bars represent standard errors.

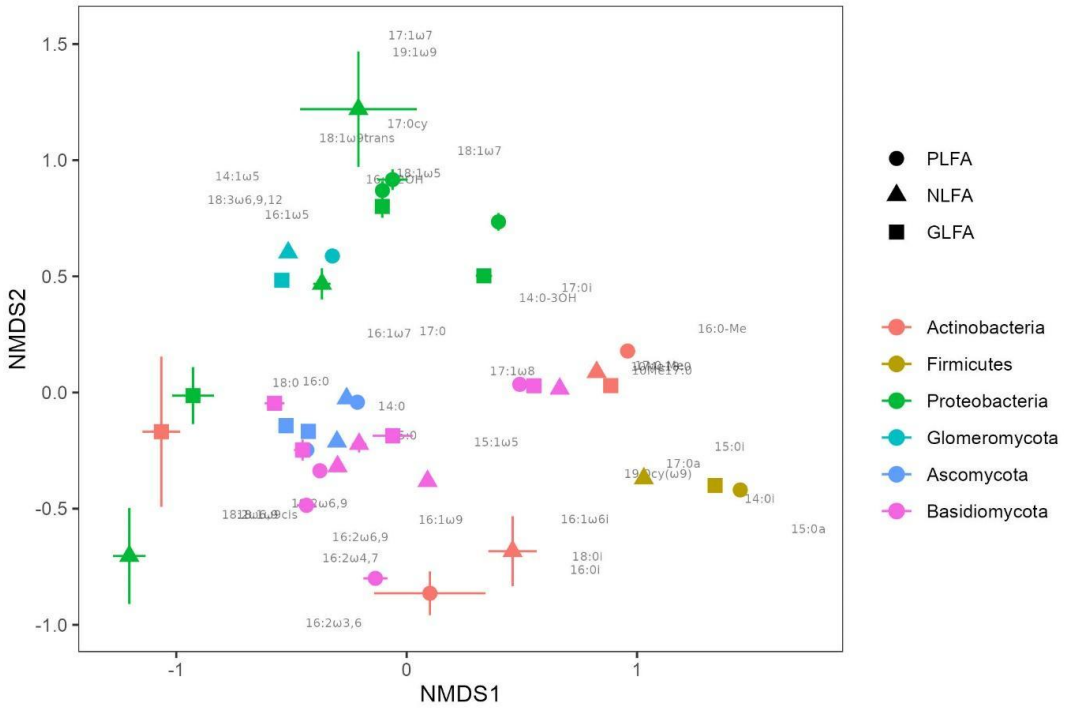

Fig. S3 This figure shows the same data as Fig. 4, but includes the fatty acid 18:0. Non-metric multidimensional scaling (NMDS, stress = 0.178) plot of the microbial pure culture fatty acid profiles.

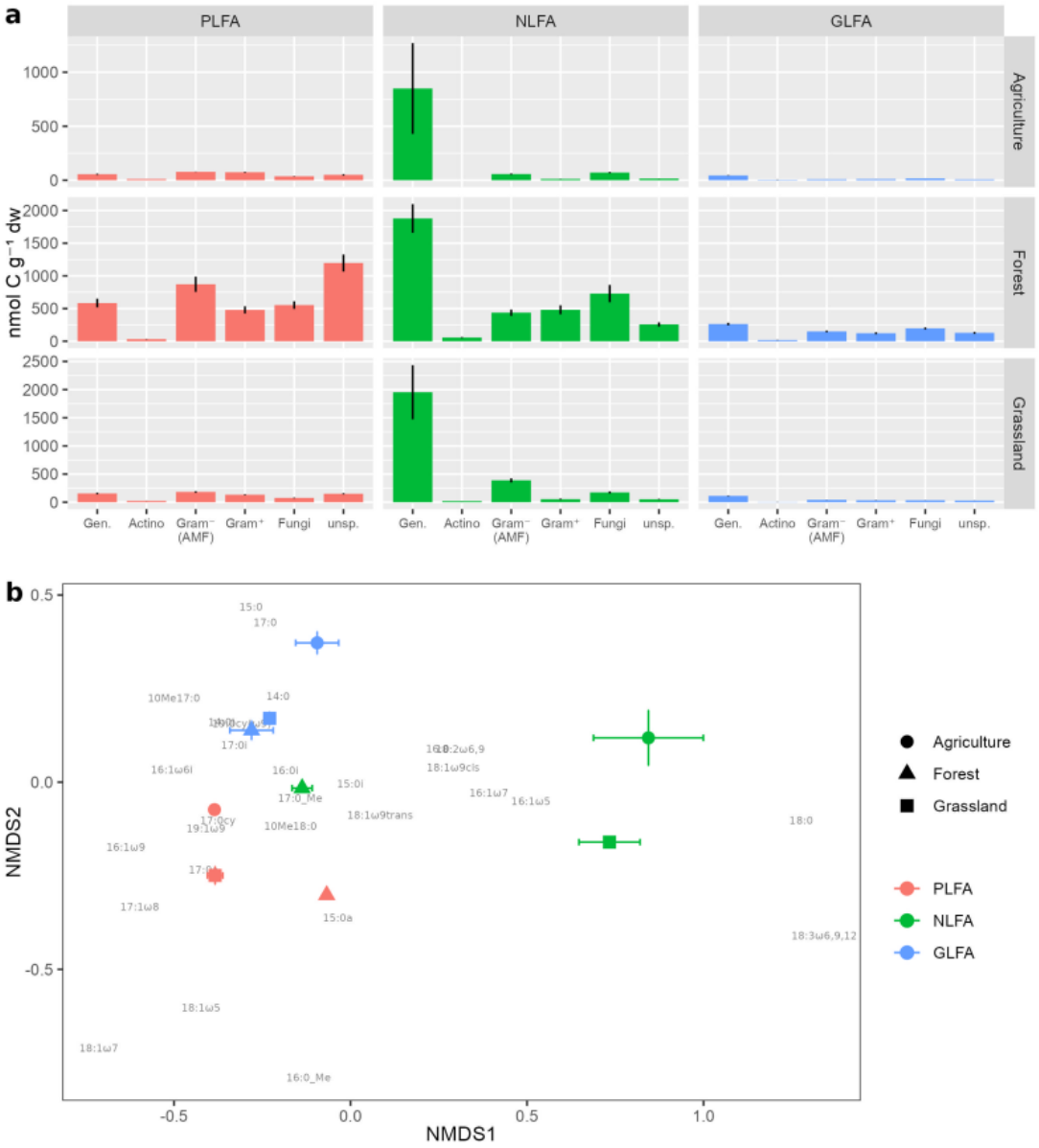

Figure S4 The figure shows the same data as Fig. 5, but includes the fatty acid 18:0. **(a)** Fatty acid abundances of the three lipid fractions in soil extracts. Shown are fatty acids specific to microbial biomass in general (Gen.), Actinomycetes (Actino), gram-negative bacteria ($Gram^-$) including the fatty acid 16:1ω5 which is highly specific to arbuscular-mycorrhizal fungi (AMF) in grassland soils, gram-positive ($Gram^+$) bacteria, fungi, and unspecified (unsp.). Error bars represent standard errors. **(b)** Non-metric multidimensional scaling (NMDS, stress = 0.083) plot of the same fatty acid profiles as in (a).

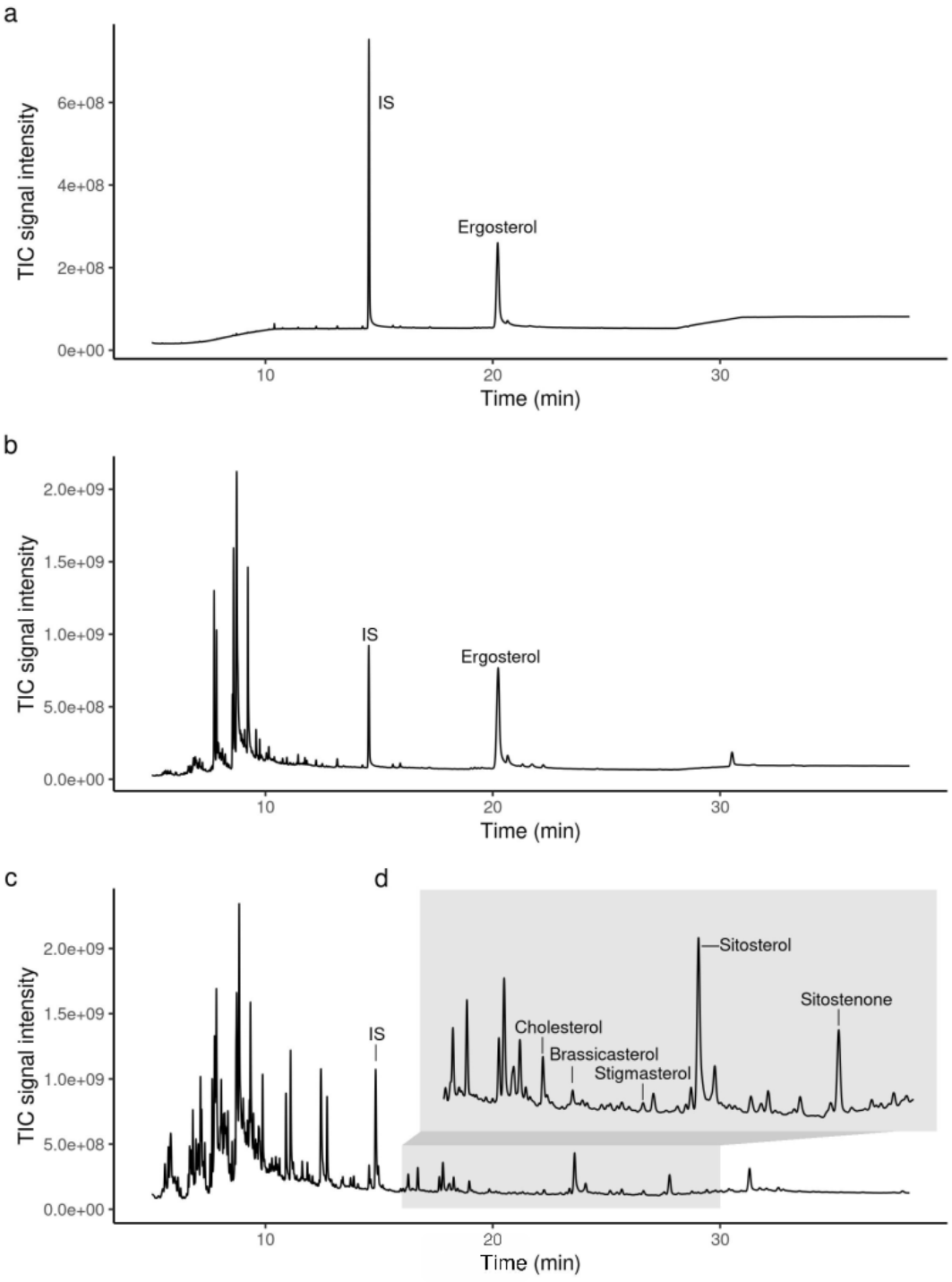

Figure S5 Total ion chromatograms of sterol profiles from (a) pure lipid standards, (b) a fungal (*Agaricus* sp.) sporocarp, and (c, d) a soil extract. IS, internal standard (5-α-Cholestane).

Chapter 4

Soil bacterial neutral lipid fatty acids: Markers for energy storage or necromass?

Soil bacterial neutral lipid fatty acids: Markers for energy storage or necromass?

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Keywords
NLFA, triacylglycerol (TAG), diacylglycerol (DAG), phospholipid turnover, bacterial carbon storage, WS/DGAT

Highlights

- Bacterial NLFAs may originate from triacylglycerols (TAGs) or degraded phospholipids
- Neutral lipids may only be an intermediate compound in phospholipid degradation
- Soil bacteria have the genetic potential to produce neutral storage lipids
- Excess labile carbon allocates into soil bacterial NLFAs, suggesting carbon storage
- Bacterial NLFAs likely reflect storage rather than necromass

Abstract

Carbon storage is a common strategy of soil microbes to deal with fluctuations in resource availability. Fungi are known to store neutral lipids (triacylglycerols, TAGs), and their derived fatty acids (NLFAs) can give valuable insights into the physiology of soil fungi. NLFAs specific to bacteria can also be abundant in soils, and several pure culture studies provide evidence for TAG production in selected bacterial isolates. However, soil bacteria are often assumed to be incapable of producing TAGs, and to rely on other means of storage like polyhydroxyalkanoates. Instead, bacterial NLFAs have been suggested to represent remains from degraded phospholipids. If so, they would be suitable markers for bacterial necromass. This interpretation, however, lacks substantial evidence. Thus, although bacterial NLFAs potentially provide sensitive information on important soil carbon pools, it is unclear whether they are markers for bacterial carbon storage or necromass.

Here, we synthesise knowledge on the origin of soil bacterial NLFAs. Enzymatic degradation of phospholipids from dead cells can result in neutral diacylglycerols (DAGs). Neutral lipids are thought to stabilise in organic patches or water-inaccessible microsites, which can potentially lead to an accumulation of NLFAs from necromass. However, only two of several phospholipid degradation pathways can lead to neutral lipid formation, and they may only be an intermediate compound during phospholipid degradation. Analysis of the JGI database of soil bacterial isolate genomes showed that wax ester synthase/diacylglycerol acyltransferase (WS/DGAT)—a protein which mediates the last step of TAG synthesis—is abundant in genomes from soil bacterial isolates, suggesting the ability to produce TAGs. Furthermore, we observed that glucose-^{13}C added to soil is rapidly allocated into bacterial NLFAs, indicating storage of excess carbon.

Overall, our synthesis and experimental results provide evidence that soil bacterial NLFAs are mainly derived from storage compounds, but with a possible contribution from degraded phospholipids, which requires further experimental evidence for proper validation. We suggest that if coupled with isotopic labelling this issue can be resolved, making NLFAs a valuable biomarker for storage compounds in soil microbes.

1. Introduction

Soil organic matter represents the largest pool of terrestrial carbon, and its turnover is primarily mediated by soil microbes. Biological marker compounds provide valuable insights into the processes and pools involved in soil organic matter cycling. For instance, profiling of lipid-derived fatty acid methyl esters (FAMEs) is an established method to analyse soil microbial communities. In the FAME extraction method, lipids are usually fractionated based on their polarity. Polar phospholipid fatty acids (PLFAs) are widely used as sensitive indicators of microbial biomass and community composition (Frostegård et al., 2011; Willers et al., 2015). Additionally, the FAME extraction method can yield neutral lipid fatty acids (NLFAs), which have been used as biological markers for fungal carbon storage (Bååth, 2003).

Fungi are known for producing triacylglycerols (TAGs), which are typically found in the neutral lipid fraction when using the FAME extraction method. TAGs are composed of three fatty acids attached to a glycerol backbone (Athenaki et al., 2018). They serve as common carbon storage compounds in eukaryotic organisms and are stored in energy-rich lipid droplets (Murphy, 2012). Thus, specific NLFAs associated with fungi can be used to estimate fungal carbon storage (Bååth, 2003).

Bacteria have also been shown to produce TAGs for carbon storage in culture studies (Alvarez and Steinbüchel, 2002; Wältermann and Steinbüchel, 2005), and NLFAs specific to bacteria can also be abundant in soil extracts. However, the origin of bacterial NLFAs has been interpreted in two different ways. Bacterial NLFAs have been used as markers for carbon storage, analogous to fungal NLFA (Ngosong et al., 2020; Mason-Jones et al., 2023). In contrast, some authors assume that the synthesis of TAGs in bacteria is either non-existent or not widespread, and instead suggest that bacterial NLFAs are derived from degraded phospholipids (Bååth, 2003; Rinnan et al., 2005; Amir et al., 2008; Rinnan and Bååth, 2009; Koyama et al., 2018; Honvault et al., 2021). This way, bacterial NLFAs originate from dead cells and are indicative of bacterial necromass. This interpretation not only comes with certain assumptions on phospholipid degradation pathways and extracellular lipid stabilisation, but also suggests that a certain portion of fungal NLFAs may be derived from dead fungal cells.

Both carbon storage and necromass biomarkers have recently received attention as important soil carbon pools (Liang et al., 2017; Kästner et al., 2021; Mason-Jones et al., 2021). Bacterial NLFAs may serve as a very powerful biomarker, but consensus is missing whether they are reliable indicators of carbon storage or necromass. Soil microbes invest in carbon storage compounds particularly in

situations of excess availability, and use them as important resources to overcome limiting conditions (Mason-Jones et al., 2021). Their analysis thus provides information on the physiological state of the microbial community. Microbial necromass has repeatedly been identified to form a large portion of soil organic carbon (Liang et al., 2019), and is usually measured as amino sugars derived from hydrolysed chitin and peptidoglycan (Joergensen, 2018; Salas et al., 2023; Salas et al, 2024).

Here, we aim to synthesise knowledge on the potential usage of soil bacterial NLFAs. We weigh the arguments for their application as carbon storage or necromass markers, discuss both interpretations critically and contrast them with our own empirical observations. We provide suggestions for future research to elucidate ambiguities in this matter, and argue that bacterial NLFAs have the potential to be an easily extractable yet powerful metric in soil science.

2. A short history of soil NLFA extractions and usage as fungal biomarkers

NLFAs are usually extracted concurrently with PLFAs in the FAME extraction method. It consists of total lipid extraction, and subsequent fractionation where lipids are separated based on their polarity. Lipid fractionation is generally achieved by successively eluting with chloroform (neutral lipids), acetone (glycolipids), and methanol (phospholipids) on silicic acid columns. The final step in the protocol is the derivatisation of the fractionated lipids into fatty acids via mild alkaline methanolysis. The resulting fatty acid methyl esters are usually measured via gas chromatography. This commonly used method is based on the lipid extraction method by Bligh and Dyer (1959), adapted for microbial profiling by White et al. (1979). It was modified for soil extractions by the pH-adjustment of the monophasic chloroform:methanol:water extractant by replacement of water with citrate buffer of pH=4 (Frostegård et al., 1991; Frostegård et al., 1993; Frostegård and Bååth, 1996), and later for high-throughput sample analysis by reducing the extractant volume and using 96-well silica column plates (Byer and Sasser, 2012). Recently it was found that the polarity of chloroform—which may be stabilised with small amounts of a more polar solvent like ethanol—is critical for the recovery of neutral lipids from silicic acid columns (Drijber and Jeske, 2019). Adding 2% ethanol to chloroform allows the complete recovery of TAGs in the neutral lipid fraction, but pure chloroform can lead to an incomplete fractionation (Gorka et al., 2023a). It is therefore important to note that previous studies which measured NLFAs in soil may have underestimated NLFA yields.

Soil NLFAs are most commonly extracted to quantify arbuscular mycorrhizal biomass via the specific NLFA 16:1ω5 (Olsson et al. 1995; Lekberg et al., 2022).

The use of NLFA biomarkers of other fungal groups (ascomycete- and basidiomycete-specific fatty acids) was popularised in the conclusive paper by Bååth (2003). In this study, a labile carbon source (glucose) was added to soils, which created conditions for storage compound production as other nutrients were limiting. In consequence, the fungi-specific NLFAs 18:2ω6,9 and 18:1ω9 increased 60-fold and 10-fold, respectively, while no increase was observed when glucose was added together with nitrogen and phosphorus. This strongly indicates that fungal NLFAs are derived from compounds used for carbon storage. Fungi-specific NLFAs can therefore be used as indicators of fungal carbon storage, and the corresponding NLFA/PLFA ratios infer the physiological state of fungi (Rinnan et al., 2005; Rinnan and Bååth, 2009).

3. The enigmatic origin of soil bacterial neutral lipids

Full fatty acid profiles (i.e., including bacteria- as well as fungi-specific NLFAs) are rarely considered in soil studies, even though the taxonomic specificities of marker fatty acids correspond across lipid fractions in the FAME method (Gorka et al., 2023a). We searched studies which analysed complete PLFA and NLFA soil profiles and found only 15 publications from 2002 to 2023. The search demonstrates that full NLFA profiles show significant amounts of fatty acids specific to bacteria (Table 1). We also extracted PLFA and NLFA profiles from three different soils, and found consistently high amounts of bacterial NLFAs (60-77% of nmol PLFAs g^{-1} dm) in a beech forest, spruce forest, and grassland soil (Fig. 1). In our extraction, the majority of fatty acids derived from phospholipids were also recovered in NLFA profiles, showing similar total NLFA counts compared to PLFAs (NLFA/PLFA count ratios of 0.95–1.05). Counts of bacteria-specific fatty acids were also similar between NLFAs and PLFAs (NLFA/PLFA count ratios of 0.91–1.08; Table1). Interestingly, while almost all fatty acids specific to gram-positive bacteria were found in both PLFA and NLFA profiles, some fatty acids specific to gram-negative bacteria were only present as PLFAs (16:1ω9, 18:1ω5) or as NLFAs (17:1ω8, 19:0cy(ω9); Table S1). The values we found are in the range of previously published soil fatty acid profiles, where bacterial NLFA abundances account for 9–82% of PLFA abundances (mean 39%), and 0.15–1.14 as NLFA/PLFA count ratios (mean 0.69; Table 1). As previously mentioned, recent studies have shown that classic chloroform extraction might lead to NLFA losses, therefore these numbers might represent an underestimation.

Table 1 Comparison of full PLFA and NLFA profiles extracted from soils or similar ecological matrices. Fatty acid (FA) counts refer to the number of observed or analysed fatty acids. Abundance data were always calculated relative to mass (e.g. nmol FAs g^{-1} soil). Asterisks indicate that authors noted the given usage of bacterial NLFAs with caution, and considered the alternative interpretation as well. See *Materials and Methods* section 9.1 for details.

		Counts of bacteria-specific FAs			NLFA/PLFA abundance ratios					
Reference	**Soil**	**PLFA**	**NLFA**	**NLFA/PLFA**	**Total**	**Fungi**	**Bacteria**	**Gram+**	**Gram-**	**Usage of bacterial NLFAs**
Hedlund et al. (2002)	Agricultural field	13	2	0.15	0.72	-	0.33	-	0.66	-
	Beech forest	13	2	0.15	0.27	-	0.09	-	0.17	
Bååth (2003)	Agricultural	14	14	1	0.58	1.95	0.27	0.34	0.22	Necromass
	Beech forest (A-horizon)	14	14	1	0.4	0.54	0.2	0.25	0.12	
	Forest humus	11	11	1	0.42	0.46	0.27	0.32	0.14	
	Grassland	13	13	1	0.48	0.38	0.11	0.16	0.09	
Malosso et al. (2004)	Antarctic soil	-	-	-	-	-	-	-	-	Necromass*
Rinnan et al. (2005)	Arctic heathland	13	13	1	0	2.15	0.31	-	-	Necromass
Bradley et al. (2007)	Grassland (sandy loam)	7	1	0.14	0.31	-	-	-	-	Necromass*
	Grassland (silt loam)	9	4	0.44	0.67	-	-	-	-	
	Grassland (clay loam)	9	4	0.44	0.77	-	-	-	-	
Rinnan and Bååth (2009)	Tundra heath	18	10	0.56	0.61	1.31	0.26	0.42	0.14	Necromass
Rinnan et al. (2013)	Tundra heath	17	8	0.47	0.68	-	-	-	-	-
Ferrari et al. (2015)	Agricultural soil (Summer)	-	-	-	9.79	-	-	-	-	-
	Agricultural soil (Winter)	-	-	-	5.25	-	-	-	-	
Koyama et al. (2018)	Nevada desert (covered vegetation)	10	8	0.8	0.36	0.55	0.14	-	-	Necromass
	Nevada desert (plant interspace)	10	8	0.8	0.48	0.72	0.14	-	-	
Grossman et al. (2019)	Temperate forest (experimental)	-	-	-	1.15	2.22	0.84	-	-	-
Ngosong et al. (2020)	Temperate forest	14	16	1.14	0.2	-	-	-	-	Storage
Honvault et al. (2021)	Rhizosheath soils	8	8	1	0.98	0.98	0.67	0.34	1.02	Necromass
Butler et al. (2023)	Soils from West head, Australia	-	-	-	2.26	-	-	-	-	Storage*
Gorka et al. (2023a)	Agricultural soil	14	5	0.36	4.01	1.93	0.31	0.22	0.43	Storage*
	Beech forest	11	12	1.09	1.05	1.32	0.71	1.05	0.47	
	Grassland	15	5	0.33	3.68	2.31	0.81	0.52	1.1	
Gorka et al. (2023b)	Beech forest	13	13	1	0.97	0.76	0.82	1.29	0.53	Storage*
	Mean	**12.3**	**8.55**	**0.69**	**1.5**	**1.26**	**0.39**	**0.49**	**0.42**	
This publication	Beech forest	12	13	1.08	1.33	2.42	0.69	0.39	1.01	
	Pine forest	11	10	0.91	1.14	1.1	0.6	0.33	0.8	
	Grassland	11	10	0.91	1.4	2.74	0.77	0.91	0.69	
	Mean	**11.3**	**11**	**0.96**	**1.29**	**2.09**	**0.69**	**0.54**	**0.83**	

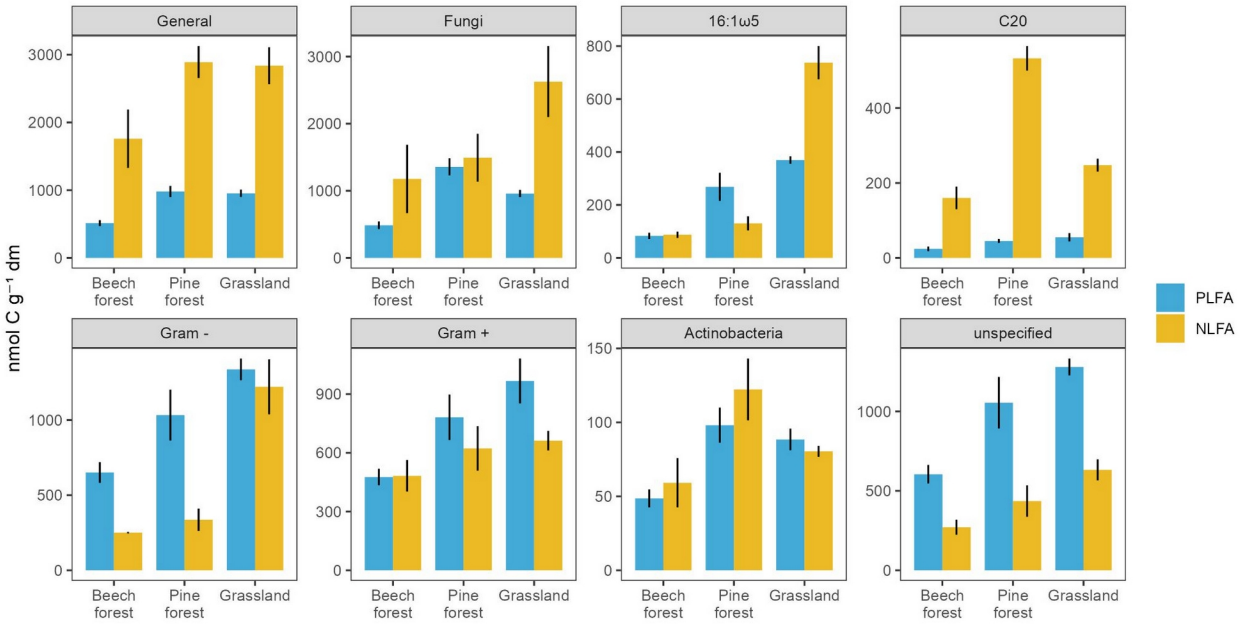

Figure 1 Abundances of PLFAs and NLFAs in three different soils. In all analysed soils, considerable amounts of bacteria-specific NLFAs were found. The fatty acid 16:1ω5 is depicted separately as it is specific to both arbuscular-mycorrhizal fungi and gram-negative bacteria. C20 denotes fatty acids with 20 carbon atoms. Data is presented as the sum of all fatty acids specific to each respective microbial group. Error bars represent the standard error. For a description of the sampling and extraction procedures, see *Materials and Methods* section 9.2.

Where do these bacterial NLFAs come from? In the literature, they have been interpreted in two different ways: (i) bacterial NLFAs indicate storage compounds, and/or (ii) they indicate bacterial necromass (Fig. 2). However, the majority of studies which measured bacterial NLFAs in soils used them as markers for necromass (Table 1). The underlying reasoning is that bacterial cells (presumably) do not contain TAGs, and when a cell dies, phospholipids are released and undergo rapid degradation (Bååth, 2003; Zhang et al., 2019). The initial step of this process involves the cleavage of the charged phosphate head, leading to the formation of a neutral diacylglycerol (DAG), which may then be stabilised in soil for an unknown amount of time. Using bacterial NLFAs as necromass biomarkers implicitly assumes that DAGs will—similar to TAGs—elute in the neutral lipid fraction of the FAME protocol, even though they are slightly more polar than TAGs due to the additional hydroxyl group. This assumption has never been verified to the best of our knowledge. Consequently, we tested the recovery of fatty acids derived from a DAG in the FAME extraction method. Our results show that both, TAG and DAG, co-elute in the neutral lipid fraction (Table 2; Gorka et al., 2023a), which confirms that potential remains of former phospholipids in the soil will be measured as

NLFAs in the FAME extraction method. Whether they are representative of bacterial necromass, however, also depends on how stable phospholipid-derived DAGs are in soils, a point which we discuss in section 4.2. In contrast, use of bacterial NLFAs as markers for carbon storage is supported by multiple observations of bacterial TAG storage in intracellular lipid droplets (Wältermann et al., 2005; Wältermann and Steinbüchel, 2005; Wältermann and Steinbüchel, 2006). These are usually limited to bacterial isolates, and are predominantly found in Actinobacteria (Barksdale and Kim, 1977; Arabolaza et al., 2008; Hernández and Alvarez, 2010; Cortes and de Carvalho, 2015; Eberly et al., 2013; Röttig et al., 2016). However, the prevalence of bacterial TAG production in soils is unknown. We provide a more detailed exploration of this in section 5.1.

Table 2 Recovery of fatty acids derived from a diacylglycerol (DAG), and the fatty acid and alcohol moieties of a wax ester (WE) in the three lipid fractions of neutral lipids (NL), glycolipids (GL) and phospholipids (PL). Pure lipid standards were fractionated on silica gel columns and derivatised via mild alkaline methanolysis. The recovery was calculated by comparison to unfractionated samples, and values are presented in % recovery. DAG, n=3; WE, n=4; n.d., not detected. See *Materials and Methods* section 9.4 for a detailed methodology.

	% Recovery		
Lipid fraction	**DAG-fatty acids**	**WE-fatty acid**	**WE-alcohol**
NL	99.3	78.9	69.6
GL	0.7	1.4	n.d.
PL	0.2	0.3	n.d.

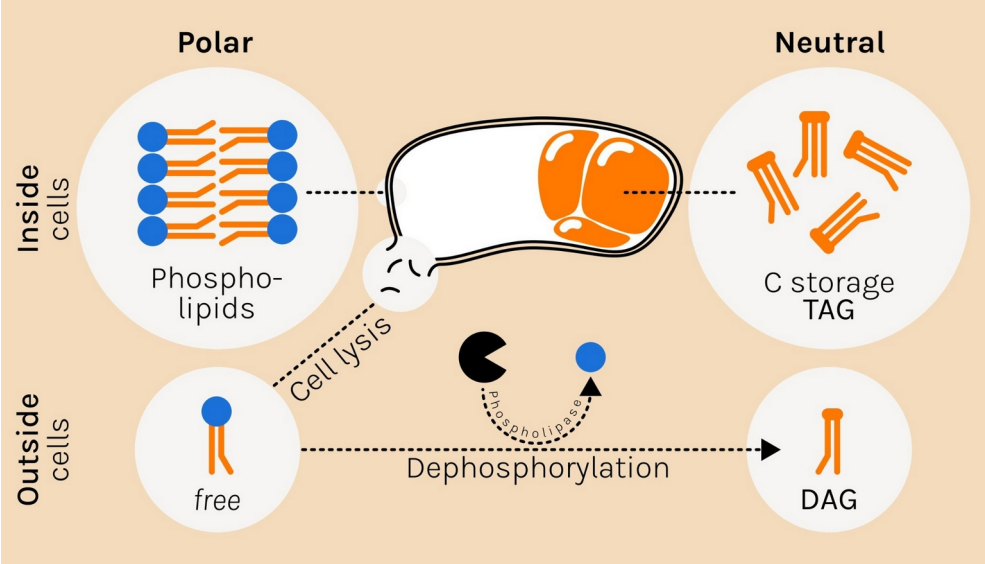

Figure 2 Soil bacterial NLFAs have been interpreted in two ways. (i) They are derived from membrane phospholipids, which are decomposed to DAGs by extracellular phosphatases after cell death, and thus indicative of bacterial necromass. (ii) They are derived from intracellular TAGs and are indicative of carbon storage.

Both interpretations of bacteria-specific NLFAs are theoretically plausible and not mutually exclusive. This problem is exemplified when comparing microbial pure culture and soil fatty acid profiles. In a previous publication, we extracted PLFA and NLFA profiles from pure cultures of bacteria and fungi (Gorka et al., 2023a). These profiles were primarily separated by taxa, regardless of whether the fatty acids came from phospholipids or neutral lipids (PERMANOVA $P < 0.001$, $R^2 = 0.52$; Fig. 3a, Table 3a). PLFAs and NLFAs were also separated, but with a much lower explanatory power ($P < 0.001$, $R^2 = 0.07$).. This not only shows that bacteria produce neutral lipids—likely TAGs—but also that microbes use the same fatty acids for phospholipids and neutral lipids and in similar proportional distribution in the two fractions. However, in the soil environment this interpretation is more complicated. In contrast to the pure culture profiles, the PLFA and NLFA profiles of soils were primarily separated by the lipid type (Fig. 3b), and PERMANOVA analysis showed similar contributions of the lipid source ($P < 0.001$, $R^2 = 0.33$) and soil type ($P < 0.001$, $R^2 = 0.30$; Table 3b). At a first glance this data could be interpreted as indicative of NLFA representing necromass rather than carbon storage since the soil fatty acid profiles do not reflect the pure culture profiles. However, from the comparison of soil PLFA and NLFA profiles one cannot conclusively infer the origin of NLFAs. If bacteria produce TAGs, we would expect different relative concentrations of NLFAs to PLFAs, as storage production depends on the overall physiological state of the microbial community, which is linked to environmental nutrient limitations (Bååth et al., 2003; Wältermann et al.,

2005). Moreover, there may be a preferential allocation of specific fatty acids into storage lipids. For example, this is the case in arbuscular mycorrhizal fungi which preferentially allocate the fatty acid 16:1ω5 into TAG (Olsson et al., 1995). On the other hand, if bacterial neutral lipids are derived from decomposed phospholipids (i.e. DAGs), the NLFA concentration would depend on the rate of phosphorylation, microbial turnover, and the retention of DAG, potentially leading to different concentrations of PLFAs and NLFAs. Thus, neither interpretation can be dismissed nor proved.

Table 3 Permutational multivariate analysis of variance (PERMANOVA) results of fatty acid profiles from **(a)** pure culture strains, and **(b)** soil profiles (see Fig. 3). 'Lipid type' refers to PLFA vs. NLFA. df, degrees of freedom; SS, sum of squares.

a		**df**	**SS**	**R^2**	**F**	**P**
	Lipid type	1	1.607	0.075	12.785	0.001
	Taxon	5	11.223	0.522	17.863	0.001
	Residual	69	8.67	0.403		
	Total	75	21.50	1		

b		**df**	**SS**	**R^2**	**F**	**P**
	Lipid type	1	0.838	0.330	23.165	0.001
	Soil	2	0.759	0.299	10.483	0.001
	Residual	26	0.941	0.371		
	Total	29	2.538	1		

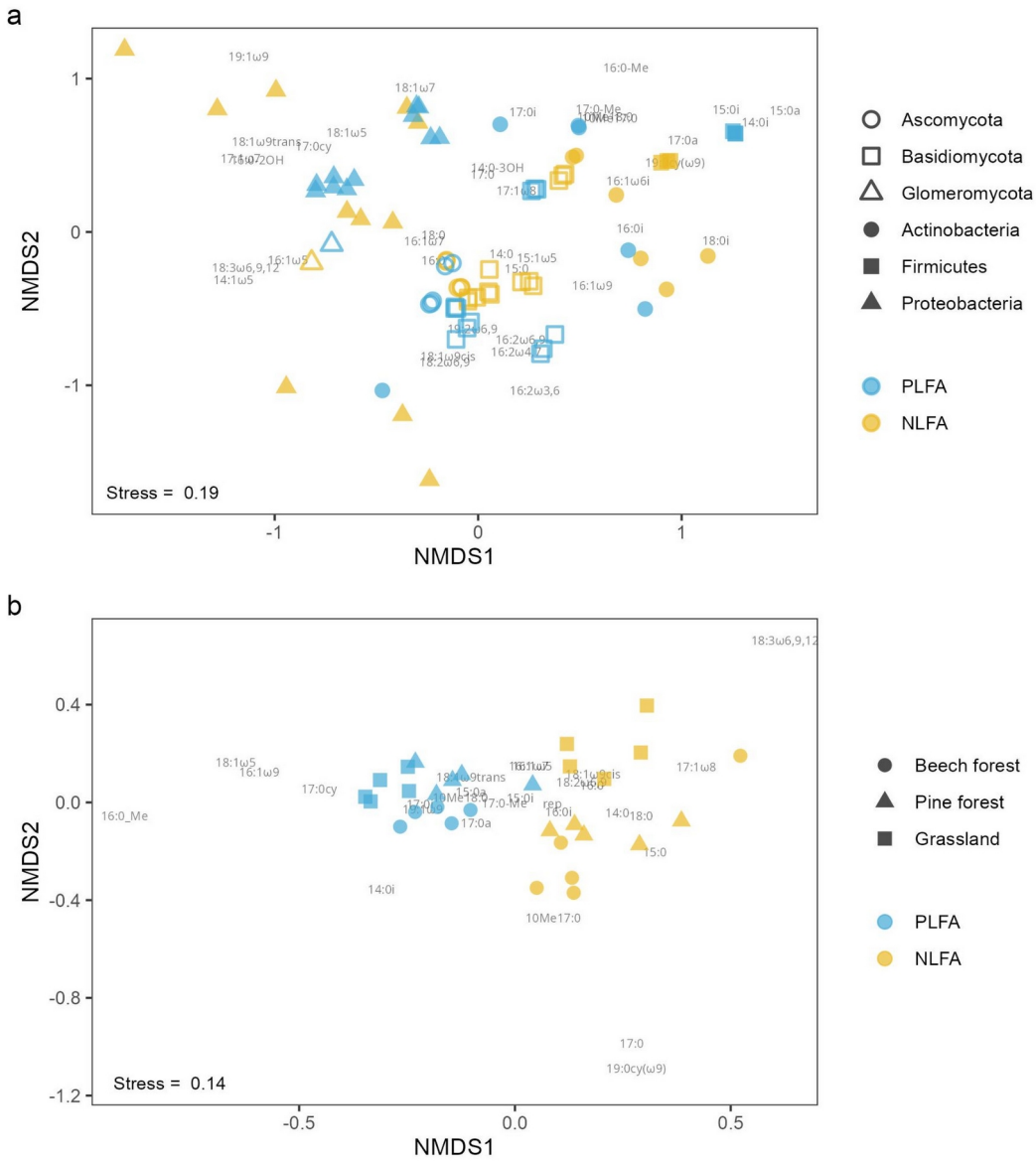

Figure 3 Non-metric multidimensional scaling (NMDS) plots of PLFA and NLFA profiles from **(a)** pure culture strains (data from Gorka et al., 2023a), and **(b)** three different soils (same data as Fig. 1). While pure culture profiles primarily cluster by phylum, soil profiles primarily cluster by lipid type (Table 3). Data provided in (a) mol%, and (b) nmol C g^{-1} dm. Individual points represent replicate samples.

4. Bacterial NLFAs as necromass markers

Soil microbial necromass is an important contributor to soil organic matter, accounting for approximately 50% of total soil organic carbon (Liang et al., 2017; Liang et al., 2019; Wang et al. 2021). Amino sugars, which are found in cell walls as chitin and peptidoglycan, are usually used to quantify microbial necromass

(Kästner et al., 2021; Salas et al., 2024). They allow estimates of fungal and bacterial necromass, but deeper taxonomic analysis is restricted as there are only four types of necromass-specific amino sugars, with only muramic acid being unique to bacterial peptidoglycan (Joergensen, 2018).

Apart from chemically recalcitrant cell wall fragments, dead cells leach a diverse set of cytoplasmic and membrane molecules (Camenzind et al., 2023). These are continually decomposed from polymers to monomers, and their stabilisation is intricately linked to the spatial heterogeneity and temporal variability of the soil matrix, which determine their accessibility to soil microbes (Lehmann and Kleber, 2015; Lehmann et al., 2020). Furthermore, the chemical nature of the decomposition products (e.g. polarity, ionisation) is itself a factor for stabilisation (von Lützow et al., 2006). Lipids may therefore form a significant part of necromass that is usually not measured.

Soil bacterial NLFAs may give valuable insight into membrane-lipid derived bacterial necromass at a certain stage of its decomposition. However, in order for NLFAs to be derived from decomposed phospholipids (DAGs), it is a prerequisite that (i) phospholipids derived from dead cells are decomposed extracellularly into DAGs, and (ii) these DAGs are stabilised in a recalcitrant manner (physico-chemically or spatially). In the following, we discuss current knowledge on these aspects.

4.1 Decomposition pathways of soil phospholipids

Soil phospholipids are rapidly degraded within hours to days (White et al., 1979; Janzen et al., 1994; Zhang et al., 2019). The interpretation that soil NLFAs are derived from degraded phospholipids (DAGs) assumes that the majority of phospholipids is hydrolysed by a particular class of phosphodiesterases. These enzymes belong to the phospholipase C family, which cleave between the glycerol and the phosphate group (Titball, 1993). Other proteins in the phospholipase superfamily cleave phospholipid molecules at various positions, which leads to the formation of distinct breakdown products other than DAGs (Fig. 4a; Fisher and Jain, 2009; Ramrakhiani and Chand, 2011; Barman et al., 2018). Phospholipases A_1 and A_2 hydrolyse the fatty acyl-ester bonds at the respective *sn*-1 and *sn*-2 positions of the glycerol moiety, producing free fatty acids and lysophospholipids (Köhler et al., 2006, Sitkiewicz et al., 2007). Phospholipase B cleaves both fatty acids, producing free fatty acids and the phosphorylated head group attached to glycerol-3-phosphate. Phospholipase D cleaves after the phosphate, producing a polar phosphatidic acid and an unphosphorylated head group. Continuing this pathway, phosphatidic acid can further be dephosphorylated to generate DAG

(Pyne et al., 2004; Carman and Han, 2006; Fisher and Jain, 2009). Whether extracellular DAGs are produced is therefore determined by the relative abundances of the various phospholipases in soil, where only phospholipase C, as well as downstream phosphorylation by phospholipase D, can yield DAGs from phospholipids (Fig. 4b).

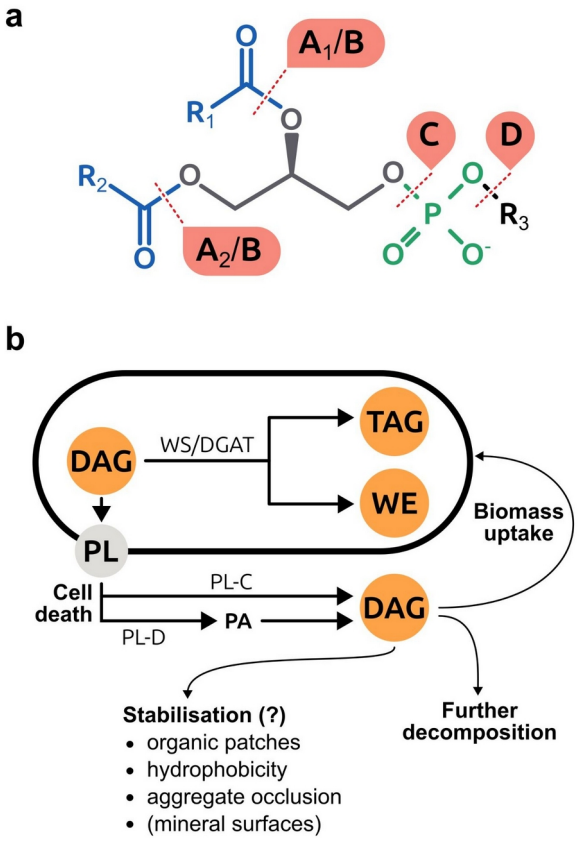

Figure 4 Pathways for degradation of phospholipids that lead to DAG formation. **(a)** The letters in red boxes A_1, A_2, B, C, and D indicate the sites of hydrolytic cleavage on a phospholipid by the respective phospholipase families. **(b)** Synthesis and decomposition pathways that lead to intracellular and extracellular neutral lipids, which are possible sources of soil bacterial NLFAs (marked in orange). TAG, triacylglycerols; WE, wax esters; DAG, diacylglycerols; PL, phospholipid; WS/DGAT, wax ester synthase/diacylglycerol acyltransferase; PL-C and -D, phospholipase C and D; PA, phosphatidic acid.

All phospholipase classes can be secreted by bacteria (Stinson and Hayden, 1979; Flieger et al., 2000; Hagishita et al., 2000; Farn et al., 2001; Sugiyama et al., 2002; Simkhada et al., 2007; Sugimori et al., 2012), and phosphodiesterases have been suggested to be widespread among soil bacteria (Kuroshima and Hayano, 1982;

Lidbury et al., 2017). However research is lacking on their relative contributions to soil phospholipid turnover. Only in a recent study, Wasner et al. (2023) found direct uptake of phospholipid-derived, organic phosphorus in an ^{33}P radio-isotope incubation experiment. Since transporters have only been identified for glycerol-3-phosphate, the authors speculate that a portion of phospholipid breakdown occurs through a pathway that produces glycerol-3-phosphate as an end product. This pathway is likely to involve phospholipase D, as well as phospholipase A or B, which suggests that phospholipids are consecutively degraded by several phospholipase classes.

DAGs can further be hydrolysed by DAG- and monoacylglycerol lipases into glycerol and free fatty acids (Rengachari et al., 2012; Yuan et al., 2016; Li et al., 2020). In a previous experiment, we found that a freeze-thawing event, which likely killed a large part of the soil microbial community, decreased both fungal and bacterial NLFAs within days (Gorka et al., 2023b). This either suggests phospholipase pathways which did not yield DAGs, or further decomposition of phospholipid-derived DAGs (or, alternatively, that bacterial NLFAs are derived from TAGs used for storing carbon in living biomass which degraded after cell death). Although DAGs can theoretically be produced during phospholipid degradation, they are a potentially intermediate compound in only two of several pathways during phospholipid degradation. A more systematic investigation of soil phospholipid degradation products is needed to assess their contribution to soil bacterial NLFA pools.

4.2 Stability of neutral lipids in soils

It is unclear how long extracellular neutral lipids persist, but there are indications that they may be stabilised. Rethemeyer et al. (2004) found ^{14}C abundances in PLFAs close to atmospheric values, while neutral lipid extracts were depleted in ^{14}C, indicating contributions of old carbon (and hence recalcitrance or slower turnover) to the neutral lipid pool. Soliman and Radwan (1981) and Hita et al. (1996) recovered significant amounts (up to 60%) of TAG-18:0 weeks after its addition to soils. However, Hita et al. (1996) found significant increases in free fatty acids after TAG addition, indicating TAG decomposition. We also note that the measured TAGs may have been extracted from microbial biomass as the authors could not differentiate between added and newly produced TAGs.

Parts of stable microbial necromass can persist as lipids (Kallenbach et al., 2016; Hall et al., 2020), possibly by interaction with mineral surfaces (Olivelli et al., 2020; Neurath et al., 2021). Indeed, lipids derived from bacterial extracellular polymeric substances (EPS) have been found to attach to goethite heterogeneously

in lipid-rich spots (Liu et al., 2013). While lipids are only minor components of EPS, goethite-adsorbed EPS consisted of >25% lipids, suggesting preferential adsorption of lipids. Nonetheless, since DAGs are only slightly polar, their stabilisation via organo-mineral interactions is hypothetically minor due to weak adsorption. The -CH and -OH groups of DAGs are electrostatically neutral in typical soil pH ranges, which makes them less mobile in aqueous solutions and less likely to bond to mineral surfaces or metal cations (Kleber et al., 2015). Organic matter binds to minerals in clusters, indicating that new necromass compounds preferentially associate with existing organic matter attached to mineral sites (Vogel et al., 2014). It has thus been suggested that organo-organo interactions might be another form of necromass stabilisation (Buckeridge et al., 2020; Buckeridge et al., 2022).

The hydrophobic character of lipids plays a role in protection from mineralisation (Kästner et al., 2021). Hydrophobicity has been positively linked to carbon sequestration as it reduces surface wettability and thus accessibility of organic matter to microorganisms (Piccolo et al., 1999; von Lützow et al., 2006; Song et al., 2013). Hydrophobicity also increases aggregate stability, which stabilises organic matter spatially via occlusion or clogging of intra-aggregate pores (von Lützow et al., 2006; Hafida et al., 2007; Totsche et al., 2018). Furthermore, mineralisation of reduced organic compounds like lipids is highly limited in anaerobic conditions, which can result in their preservation (Keiluweit et al., 2017). This suggests that neutral lipids may accumulate particularly in water-inaccessible pores as organic patches, and anaerobic microsites within aggregates.

Phospholipid-derived DAGs are only measured in the NLFA fraction if sufficient amounts are stabilised in soil. Neutral lipids, including DAGs, may be stabilised through mechanisms such as mineral interactions, hydrophobicity, and protection in microsites. However, TAGs leached from dead (particularly fungal) cells could also persist under these conditions. This implies that the presence of fungal NLFAs in soil could reflect both microbial carbon storage and necromass, which is in stark contrast to their interpretation as solely carbon storage markers.

5. Bacterial NLFA as carbon storage markers

Production of carbon storage compounds is common in microorganisms (Lee, 1996; Elbein et al., 2003; Rontani, 2010; Wilson et al., 2010; Dalpé et al., 2012; Murphy, 2012; Cifuente et al., 2019; Kumar et al., 2020). They can be readily mobilised to meet energy or carbon demands under limiting conditions. Two strategies for storage can be differentiated: (i) reserve storage, where resources are continuously allocated into storage compounds at the cost of other metabolic

demands, and (ii) surplus storage, where only abundant resources are allocated into storage compounds (Mason-Jones et al., 2021). While reserve storage comes with trade-offs against other physiological demands, it can be a valuable investment into future survival and reproduction. Surplus storage allows greater stoichiometric buffering and use of carbon resources, which can be particularly advantageous in environments of fluctuating resources (Manzoni et al., 2021).

Bacteria can use a wide spectrum of compounds to store carbon and energy: PHAs, glycogen, trehalose, wax esters, and TAGs are all used for carbon storage. PHAs and wax esters are unique storage compounds in bacteria and have been found in several phyla, predominantly in Proteobacteria (PHAs) and Actinobacteria (wax esters; Dalal et al., 2010; Murphy, 2012; Sehgal and Gupta, 2020). Other carbon storage compounds like glycogen, trehalose, and TAGs also occur in fungi and are not unique to bacteria (Preiss, 1984; Genet et al., 2000; Rangel-Castro et al., 2002; Elbein et al., 2003; Wilson et al., 2010; Mason-Jones et al., 2021). In contrast to glycogen and trehalose, TAGs are hydrophobic which allows compact storage in lipid droplets with higher energy density. Although TAGs have been observed in several bacterial species (particularly Actinobacteria, see section 5.1), their abundance in soil bacterial communities is still unclear. Below we discuss the potential across known bacterial species for TAG production as well as experimental evidence.

5.1 Use of triacylglycerols (and wax esters) for carbon storage in soil bacteria

Although other forms of carbon storage like PHAs (Mason-Jones et al., 2019; Kumar et al., 2020) or glycogen (Sekar et al., 2020; Wang et al., 2020a) are assumed to occur more prevalently, TAGs have been observed in several bacterial species—particularly Actinobacteria (Alvarez and Steinbüchel, 2002). TAGs are stored in intracellular lipid droplets (Packter and Olukoshi, 1995; Alvarez et al, 1996), and their hydrophobic nature allows the accumulation of large intracellular reservoirs without affecting the osmolarity of the cytoplasm (Alvarez and Steinbüchel, 2002). Accumulation of TAGs in bacterial cultures can be stimulated if nitrogen availability is limiting growth and carbon is available in excess, and occurs predominantly during the stationary growth or sporulation phase (Hoskisson et al., 2001; Alvarez and Steinbüchel, 2002; Wältermann and Steinbüchel, 2006). Some bacteria are able to accumulate considerable amounts of TAGs when grown on carbon-rich media. For instance, *Micromonospora echinospora* was found to accumulate up to 8% TAGs of their cellular dry weight (Hoskisson et al., 2001), *Rhodococcus opacus* strain PD630 up to 76% TAGs (Wältermann et al., 2000), or 87% fatty acids (the majority of which were TAG-derived; Alvarez et al., 1996),

and *Gordonia* sp. 29-96% total lipids on various growth media with significant amounts of TAGs (5-58 mg L^{-1} medium; Gouda et al., 2008).

Organisms that accumulate TAGs for carbon storage typically use specific DAG acyltransferases (DGAT) to catalyse the final addition of the third fatty acid moiety to the hydroxyl group on the DAG glycerol backbone. Contrary to eukaryotes, which use acyltransferases from two diverse DGAT families (Liu et al., 2012), bacteria preferentially utilise a specialised wax ester synthase/diacylglycerol acyltransferase (WS/DGAT, Fig. 4b; Kalscheuer and Steinbüchel, 2003; Röttig and Steinbüchel, 2013; Alvarez, 2016). As the name indicates, WS/DGAT can accept DAGs for TAG synthesis (i.e., DAG plus fatty acyl-coenzyme A, resulting in a TAG), as well as fatty alcohols to produce wax esters (i.e., fatty alcohol plus fatty acyl-coenzyme A, resulting in a wax ester).

Wax esters represent a potentially additional source of NLFAs apart from TAGs, as they consist of a fatty alcohol and a fatty acid component (Alvarez, 2016). We tested the recovery of fatty acids derived from a wax ester in the FAME extraction method. We found that wax esters co-elute in the neutral lipid fraction with relatively high recovery (ca. 80%), and the fatty acid moiety is methylated to a FAME in the derivatisation step (Table 2). Wax esters will therefore be measured as NLFAs in the FAME extraction method. We also found that the alcohol derived from a pure wax ester standard can be identified as a distinct peak in NLFA chromatograms (Fig. S1) and has reasonable recovery (ca. 70%; Table 2). Thus, it should be possible to estimate the proportion of wax esters in the total soil neutral lipid pool by comparing the total concentrations of alcohols in the neutral lipid fraction (assuming that all of them are derived from wax esters) to NLFAs. We did not, however, detect any alcohols in soil NLFA extracts (in data presented in this publication, as well as past extractions), which suggests that wax esters are not widely used for storage by soil microbes.

To estimate the potential of soil bacteria to produce TAGs (and potentially wax esters), we employed the Joint Genome Institute's (JGI) *Integrated Microbial Genomes with Microbiome Samples Expert Review* (IMG/M-ER; Chen et al., 2023; https://img.jgi.doe.gov/) database of bacterial isolate genomes to search for protein families from the Pfam database (Paysan-Lafosse et al., 2023; https://www.ebi.ac.uk/interpro/). We specifically searched for WS/DGAT (C-terminal domain; pfam06974) and DAGAT (pfam03982) as marker enzymes for potential TAG production. The search was restricted to all bacteria that occur in the specified ecosystem type 'soil'. Since datasets of bacterial isolates cannot fully portray actual soil bacterial communities, we additionally tested how representative

the search in the JGI dataset is by estimating the occurrence of the storage compound PHA which is considered common in bacteria. For this, we searched for the PHA synthesis regulator PhaR via the PHB/PHA accumulation regulator DNA-binding domain (pfam07879; Maehara et al., 2002) as a proxy for PHA–producing bacteria. The search resulted in a total dataset of 2926 unique bacterial isolates, of which 918 were assigned to WS/DGAT, 74 to DAGAT, and 790 to PhaR.

Based on this dataset, the majority of soil Actinobacteria (66%) and a significant portion of Proteobacteria (19.2%) encode for WS/DGAT (Fig. 5, Table 4). Actinobacteria showed WS/DGAT occurrences in the class Actinomycetia (66%, n=1065), but also in Thermoleophilia (100%, n=7) and the singular species of Rubrobacteria in the dataset (Table S2). Indeed, TAG production has been observed in Actinobacteria previously in the genera *Dietzia* (Alvarez, 2003), *Gordonia* (Alvarez, 2003; Eberly et al., 2013), *Micromonospora* (Hoskisson et al., 2001), *Mycobacterium* (Akao and Kusaka, 1976; Maurya et al., 2019), *Nocardia* (Alvarez, 2003; Röttig et al., 2016), *Rhodococcus* (Alvarez, 1996; Alvarez et al., 2000; Wältermann et al., 2000, Hernández and Alvarez, 2010; Cortes and de Carvalho, 2015; Röttig et al., 2016), and *Streptomyces* (Arabolaza et al., 2008; Röttig et al., 2016). All these genera were also present in the JGI dataset and encoded for WS/DGAT.

We also found substantial occurrences of WS/DGAT in Proteobacteria in the classes Deltaproteobacteria (73%, n=90), Gammaproteobacteria (16%, n=333), Betaproteobacteria (17%, n=287), and Alphaproteobacteria (10%, n=335). TAG production was observed previously in the Gammaproteobacteria *Acinetobacter* (Scott and Finnerty, 1976) and *Alcanivorax* (Kalscheuer et al., 2007); both genera also encoded WS/DGAT in the JGI dataset. Occurrences of WS/DGAT were also found in Acidobacteria in the class Acidobacteriia (7.1%, n=28), but were low to non-existent in Firmicutes (0.3% in Bacilli, n=368; 2.3% in Clostridia, n=43). Although the DAGAT family is usually associated with eukaryotes, we found occurrences in Cyanobacteria (87%, n=15) and Deltaproteobacteria (49%, n=90), and in a few species of Actinomycetia (1.2%, n=1065) and Gammaproteobacteria (1%, n=333; Table S2).

The highest occurrences of PhaR were found in Proteobacteria (73%), and fewer in Acidobacteria (16%) and phyla with low coverage in the JGI dataset (n < 20, denoted as ‘Other’; 11.5%). All other bacterial groups showed only few occurrences of PhaR (< 1%; Fig. 5, Table 4). This is consistent with available literature on PHA producing bacterial isolates (Wang and Bakken, 1998; Kadouri et al., 2005; Dalal et al., 2010; Sehgal and Gupta, 2020).

Table 4 Occurrence of specific genes for wax ester synthase/diacylglycerol acyltransferase (WS/DGAT), diacylglycerol acyltransferase (DAGAT), and the polyhydroxyalkanoate synthesis regulator PhaR in phyla of soil bacterial isolates (Fig. 6). Both WS/DGAT and DAGAT catalyse the last step in TAG synthesis. 'Other' denotes multiple phyla with low species coverage (n < 20).

Phylum	**n**	**WS/DGAT(%)**	**DAGAT (%)**	**PhaR (%)**
Actinobacteria (G+)	1073	66.4	1.2	0.8
Proteobacteria (G-)	1054	19.2	4.5	72.9
Firmicutes (G+)	434	0.5	0.2	-
Bacteroidota (G-)	273	-	-	0.4
'Other'	61	-	21.3	11.5
Acidobacteria (G-)	31	6.5	-	16.1

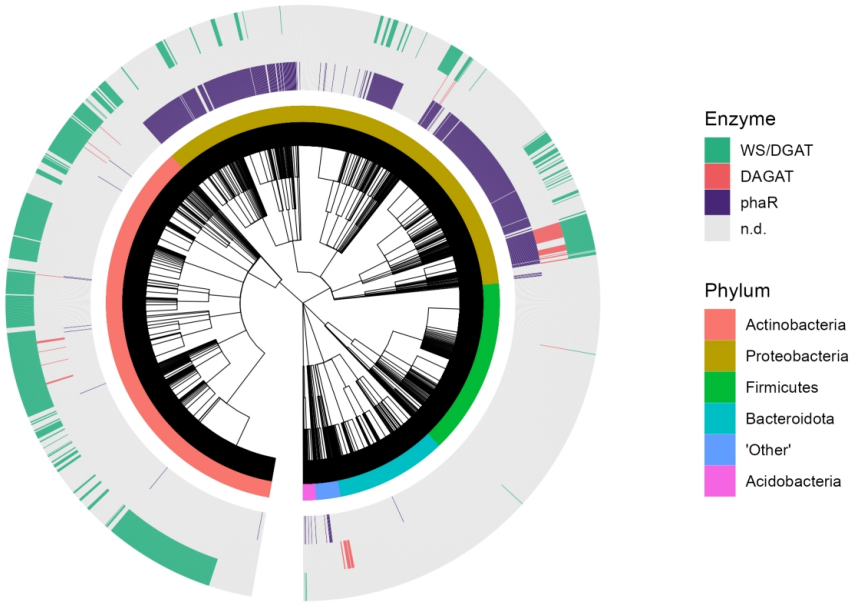

Figure 5 Occurrence of wax ester synthase/diacylglycerol acyltransferase (WS/DGAT), diacylglycerol acyltransferase (DAGAT), and the polyhydroxyalkanoate synthesis regulator PhaR in soil bacterial isolates based on the IMG/M-ER database. Both WS/DGAT and DAGAT catalyse the last step in TAG synthesis. 'Other' denotes multiple phyla with species coverage (n < 20). The cladogram shows hierarchical relationships of soil bacteria. For a detailed description of the methodology, see *Materials and Methods* section 9.5.

This search of soil bacterial isolate genomes certainly comes with biases (overrepresentation of culturable species, annotation reliance on known protein families), but it nonetheless provides insight on the potential use of lipids in bacterial carbon storage. We identified WS/DGAT in classes of both gram-positive bacteria (Actinobacteria) and gram-negative bacteria (Proteobacteria, Acidobacteria), and found that certain bacterial classes lacked WS/DGAT in most of their species (Firmicutes, Bacteroidota; Fig. 5, Table 4). Intriguingly, we found similar occurrences of WS/DGAT compared to phaR (31% and 27% of all analysed genomes respectively), suggesting that bacterial TAG production may be as common as PHA production. Overall, these results indicate that the potential for production of TAGs may be more widespread in soil bacteria than previously assumed.

5.2 Abundant labile carbon is readily incorporated into both soil PLFAs and NLFAs

We experimentally tested whether bacteria utilise neutral lipids for carbon storage by creating conditions of excess abundance of a labile carbon source. Specifically, we added ^{13}C-labelled glucose to sieved beech forest soil (4 mg glucose g^{-1} soil). We then incubated the soil at a constant temperature of 20 °C, and harvested it after 4 hours, 1 day, 4 days, and 6 days. The use of the isotopic label allowed us to trace glucose-derived ^{13}C into soil PLFAs and NLFAs. We expected the excess amount of labile carbon to trigger a strong response in microbial activity, where surplus carbon is distributed into storage compounds. Our hypothesis was that if bacterial NLFAs are necromass-markers derived from degraded phospholipids, they would show a much lower relative enrichment in ^{13}C than their PLFA counterparts, as their majority would not originate from newly formed, ^{13}C-labelled biomass. In contrast, if bacterial NLFAs are TAG-derived, bacterial NLFAs would show a similar or higher relative enrichment in ^{13}C than their PLFA counterparts as glucose-^{13}C would be allocated into newly formed storage compounds.

We found an immediate enrichment of ^{13}C in fungi- and bacteria-specific PLFAs and NLFAs already four hours after glucose addition (Mann-Whitney test, $P < 0.05$; Fig. 6a). The ^{13}C-enrichment increased for four days, and remained constant from day four to six. Fungal and gram-negative bacterial NLFAs showed higher relative ^{13}C-enrichment compared to PLFAs throughout the experimental duration (t-test, $P < 0.05$). Conversely, gram-positive bacterial NLFAs were similarly enriched in ^{13}C as PLFAs, except on day six. Fungal NLFAs showed consistently higher enrichment compared to bacterial NLFAs after the first day (t-test, $P < 0.1$). Most individual fatty acids showed higher ^{13}C enrichment in NLFAs than PLFAs

on day six (Fig. 6b). Of these, all fatty acids specific to general microbial biomass and fungi, two out of five gram-negative fatty acids (16:1ω7 and 17:0cy), and three out of six gram-positive fatty acids (16:0i, 17:0a, 17:0i) were more ^{13}C-enriched in NLFAs than PLFAs (t-test, $P < 0.05$). None of the fatty acids were more ^{13}C-enriched in PLFAs than NLFAs.

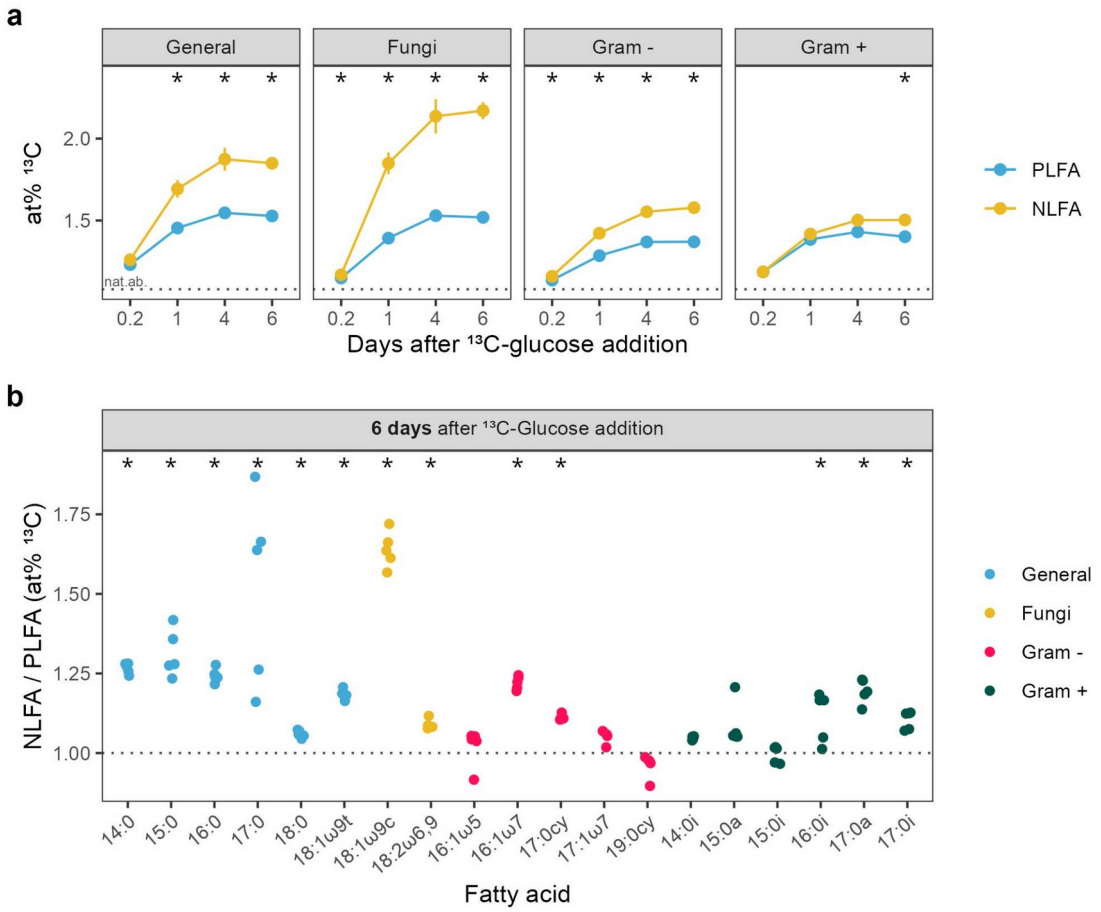

Figure 6 Incorporation of ^{13}C into soil PLFAs and NLFAs after addition of ^{13}C-glucose. **(a)** Time course of relative ^{13}C incorporation into microbial PLFAs and NLFAs. Data is presented as the sum of all fatty acids specific to each respective microbial group. NLFAs tended to be more enriched than PLFAs. The dotted line represents average natural abundance ^{13}C from control samples. Points represent means, and error bars represent the standard error. **(b)** NLFA/PLFA ratio of relative ^{13}C incorporation into individual fatty acids, six days after the addition of ^{13}C-glucose. Individual points represent replicate values. In both figures, asterisks indicate significant differences between PLFAs and NLFAs (t-test, $p < 0.05$). All fatty acids (PLFAs and NLFAs) in all time points were significantly enriched in ^{13}C relative to unlabelled control values (Mann-Whitney test, $p < 0.05$). For a detailed description of the experimental setup and extraction procedure, see *Materials and Methods* section 9.3.

The rapid incorporation of added glucose-carbon into bacterial NLFAs, as well as the similar relative enrichment patterns of fungal and bacterial NLFAs, indicates bacterial accumulation of excess carbon in TAGs. This is similar to the findings of Rinnan et al. (2013) and Mason-Jones et al. (2023), who found allocation of labile-substrate-derived ^{13}C into soil microbial NLFAs (without differentiating bacterial or fungal origin) within days after substrate addition. Previously we also observed that glucose addition to the same soil increased both fungal and bacterial NLFAs (Gorka et al., 2023b). Similarly, Koyama et al. (2018) found increased fungal and bacterial NLFAs, but not PLFAs, under elevated CO_2 in plots with vegetation cover, suggesting that higher root exudation of labile carbon compounds increased microbial carbon storage. Thus, accumulating evidence from our research and others indicates that bacteria can accumulate TAGs in their biomass in response to certain environmental stimuli.

6. Contribution of intracellular DAGs to the NLFA pool

Intracellular DAGs, the anabolic precursors of both phospholipids and TAGs in cellular lipid synthesis, are a potentially third source of soil NLFAs. They can be formed through *de novo* synthesis via esterification of monoacylglycerol by acyltransferases, or turnover of TAGs, phospholipids, or phosphatidic acid (the intermediate compound between DAGs and phospholipids; Eichmann and Lass, 2015; Alvarez, 2016). The balance between these DAG formation pathways and further downstream DAG transformations (to membrane lipids or TAGs) determines the pool size of intracellular DAGs. Although DAGs are intermediate compounds in lipid synthesis, they can constitute a substantial fraction of intracellular neutral lipids. Indeed, DAGs were found to occur in similar amounts (2–5 mol% of the total lipidome) as TAGs (2–8 mol%) in yeast (Ejsing et al., 2009; Klose et al., 2012). Since DAGs are also precursors for phospholipids, non-TAG-producing bacteria can still contribute to the soil neutral lipid pool. In these bacteria, fatty acid profiles from DAGs likely reflect PLFA profiles. Thus, even though data is lacking for bacteria, the potential presence of sizable intracellular DAG pools may explain the comparable diversity of soil PLFA and NLFA profiles.

7. How to interpret bacterial NLFAs?

7.1 Bacterial NLFAs remain enigmatic—but data point towards storage compounds

We found several arguments which indicate that bacterial NLFAs are derived from storage compounds: (i) the literature provides several observations of bacterial TAGs, (ii) excess labile carbon is rapidly allocated into both soil bacterial NLFAs (similar to fungal NLFAs), suggesting usage of TAGs for surplus storage, and (iii)

a wide range of soil Actinobacteria and Proteobacteria are able to produce WS/DGAT enzymes catalysing the last step in TAG synthesis.

Nevertheless, we agree with the notion that bacterial NLFAs may be partly derived from sources other than storage compounds. Turnover of phospholipids from dead cells can result in neutral DAGs, which, as we show, is also recovered in the NLFA fraction (Table 2). A potential third source of NLFAs are intracellular DAGs, which are ubiquitous precursor compounds in phospholipid synthesis.

7.2 Suggestions for further experiments

Understanding whether NLFAs originate from carbon storage or necromass compounds requires identifying their source, either from TAGs or DAGs. The downside of the FAME extraction method is that the original lipid molecule containing the measured fatty acid can only be classified by its polarity, as structural information is lost once the fatty acid is cleaved. Shotgun lipidomics of bacterial pure cultures, where mass spectra of intact lipids are measured without prior chromatographic separation, can overcome this limitation (Ejsing et al., 2009; Klose et al., 2012; Wang et al., 2016). Furthermore, liquid chromatography (LC)-based approaches like ultra-high-pressure LC coupled with high-resolution mass spectrometry (UHPLC-HRMS) not only allow identification of intact lipid species (Bale et al., 2021; Couvillion et al., 2023)—and thus fingerprint TAGs and DAGs —but potentially enable absolute quantifications. Such lipidomics approaches could give valuable insight in the distribution of TAGs and DAGs not only in bacterial pure cultures, but also soil environments.

Experiments utilising stable isotopes could also elucidate the origin of bacterial NLFAs. For instance, addition of ^{13}C-labelled TAGs and/or phospholipids and subsequent measurement of ^{13}C incorporation in PLFAs, NLFAs, and respired CO_2 could serve as a more comprehensive approach on the dynamics of retention, turnover, and microbial incorporation of lipids in soils. Experiments utilising stable isotopes which do not interfere with microbial metabolism (^{2}H or ^{18}O), could also give valuable insights into the origin of bacterial NLFAs. Canarini et al. (2023) reported that ^{2}H in water vapour, which was in equilibrium with soil water, was detected in microbial PLFAs and NLFAs within 48 hours. Under a similar setup with higher temporal resolutions, simultaneous ^{2}H enrichment in PLFAs and NLFAs would suggest NLFAs as storage markers, whereas a time-lag in ^{2}H enrichment would indicate NLFAs to be derived from necromass as community turnover is required for the formation of phospholipid-derived NLFAs.

8. Conclusions

Full NLFA profiles are sensitive markers for soil carbon dynamics, but their enigmatic origin makes it difficult to define their functional role. Both suggested origins—storage compounds and necromass—potentially contribute to the final NLFA pool, as both TAGs and phospholipid-derived DAGs can elute in the neutral fraction in the FAME method. To date, soil studies have used bacterial NLFA mainly as necromass biomarkers. However, accumulating evidence from our research and others favours bacterial NLFA to be derived mostly from storage compounds (i.e., TAGs). We suggest further experimental avenues to potentially quantify the contribution of each pathway to NLFA formation, such as coupling NLFA with stable isotopes or lipidomics approaches. Given the important role that NLFAs can have for soil biogeochemistry and microbial ecology, we hope that this perspective can foster new experimental avenues to quantify their source contribution as well as help researchers in guiding their interpretation of the function role of NLFAs in soil.

9. Materials and methods

9.1 Full NLFA profile literature search

A literature search was conducted on Web of Science using the search string *(NLFA* AND PLFA*) OR ("phospholipid fatty acid*" AND "neutral lipid fatty acid*") OR ("lipid biomarker*" AND soil AND microbial)*. This resulted in a total list of 248 publications. Our selection criteria entailed that the publication has to show full PLFA and NLFA profiles (or grouped abundance data), and analyse soils. This yielded a filtered list of 15 publications. Plot data was extracted using PlotDigitizer (https://plotdigitizer.com/app). If the study used experimental treatments, only control values were extracted.

9.2 Extractions of soil and microbial pure culture PLFAs and NLFAs

Three soil types were harvested for the soil lipid extractions: a beech forest soil from Klausen-Leopoldsdorf, and a spruce forest as well as a grassland soil from Ausserneuwald. All sites were located in Lower Austria, Austria. After removing aboveground biomass with a knife, the upper 5 cm of the soil were collected with soil cores (10 cm diameter) in replicates of five (n=5). Soils were homogenised by sieving (mesh size = 2 mm), and subsequently frozen, lyophilised, and stored at -20 °C until extraction.

Microbial pure culture strains were extracted as described in Gorka et al. (2023a; see also for complete descriptions of the microbial strains). These were gram-positive bacterial strains of Actinobacteria (*Solirubrobacter soli* DSM 22325, *Streptosporangium roseum* DSM 43021) and Firmicutes (*Paenibacillus*

alginolyticus DSM 5050), gram-negative bacterial strains of Proteobacteria (*Labilithrix luteola* DSM 27648, *Chelativorans multitrophicus* DSM 6780, *Paraburkholderia xenovorans* DSM 17367), and fungal strains of arbuscular-mycorrhizal Glomeromycota (*Rhizophagus irregularis*), Ascomycetes (*Periconia macrospinosa* DSM 62880, *Trichoderma virens* DSM 3500), and Basidiomycetes (*Armillaria gallica* DSM 3732, *Psilocybe cyanescens* DSM 4999, *Lactarius subdulcis*, *Lactarius quietus*). All microbial pure cultures were lyophilised, and stored at -20 °C until extraction.

Total lipids were obtained with an adjusted FAME extraction method (Bligh and Dyer, 1959; Frostegård et al., 1991; Buyer and Sasser, 2012). In short, lyophylised samples were extracted with a monophasic solution of chloroform, methanol and citrate buffer (v:v:v = 1:2:0.8). A two-phase solution was reached by changing the solvent volume ratios via addition of chloroform and citrate buffer, allowing the collection of the lower phase containing the total lipids. These were subsequently fractionated via high-throughput silicic acid chromatography (Buyer and Sasser, 2012) with solvent of increasing polarity, yielding neutral lipids (chloroform and ethanol, v:v = 98:2; Drijber and Jeske, 2019), glycolipids (acetone), and phospholipids (chloroform, methanol and water, v:v:v = 5:5:1; Buyer and Sasser, 2012). Phospho- and neutral lipids were derivatised to FAME via mild alkaline methanolysis. The resulting PLFAs and NLFAs were injected on a gas chromatograph (7890B, Agilent Technologies) connected to a time of flight mass spectrometer (Tof-MS; Pegasus BT, LECO). Quantification was achieved via the internal standard methyl nonadecanoate. The exact specifications of the laboratory workflow are described in Gorka et al. (2023a).

Fatty acids below 0.5% of the total fatty acid abundance of each replicate sample were removed from further calculations. Taxonomic groups were calculated via the sum of the following fatty acids: Fatty acids were assigned as follows: General microbial biomass (14:0, 15:0, 16:0, 17:0, 18:0, 18:2ω9t), fungi (18:1ω9c, 18:2ω6,9, 18:3ω6,9,12), fatty acids with 20 carbon atoms (20:0, 20:1ω9, 20:2ω6,9, 20:3ω6,9,12, 20:4ω6,9,12,15, 20:5ω3,6,9,12,15), gram-negative bacteria (14:0-2OH, 14:1ω5, 16:0-2OH, 16:1ω7, 16:1ω9, 17:0cy, 17:1ω7, 17:1ω8, 18:1ω5, 19:0cy, 19:1ω9), gram-positive bacteria (14:0i, 15:0a, 15:0i, 16:0i, 17:0a, 17:0i, 18:0i), Actinobacteria (10Me-17:0, 10Me-18:0), and unspecified microbial biomass (15:1, 16:0-Me, 16:2ω6,9, 17:0-me). The fatty acid 16:1ω5, which is specific to both arbuscular-mycorrhizal fungi and gram-negative bacteria depending on the fatty acid type and ecosystem, was kept separate (Lekberg et al., 2022; Olsson and Lekberg, 2022).

9.3 ^{13}C-labelled glucose addition experiment

The experimental setup was identical to Gorka et al. (2023b). In short, sieved (2 mm mesh size) beech forest soil (top 5 cm) was transferred to glass jars with a lid which allowed gas exchange. After one week acclimation time at 20 °C (the temperature was kept constant throughout the experimental duration), ^{13}C-labelled glucose (99 at% ^{13}C) was added (4 mg glucose g^{-1} soil) and homogenised via rigorous shaking, while control soils did not receive any treatment. Soil was subsequently harvested 4 hours, 1 day, 4 days, and 6 days after glucose addition (n=5). PLFAs and NLFAs were extracted as described in section 9.2; only for lipid fractionation, the pure solvents chloroform (neutral lipids), acetone (discarded), and methanol (phospholipids) were used. PLFAs and NLFAs were quantified on a gas chromatograph (Trace GC Ultra, Thermo Scientific) coupled to a mass spectrometer (ISQ, Thermo Scientific), and their isotopic ^{13}C/^{12}C ratios were measured on a gas chromatograph (Trace GC-Ultra, Thermo Fisher Scientific) coupled to an isotope ratio mass spectrometer (Finnigan Delta-V Advantage, Thermo Fisher Scientific) via a GC IsoLink (Thermo Fisher Scientific). For a complete description of the experimental setup see Gorka et al. (2023b). Fatty acids were assigned as follows: general microbial biomass (14:0, 15:0, 16:0, 17:0, 18:0, 18:1ω9t), fungi (18:1ω9c, 18:2ω6,9), gram-negative bacteria (16:1ω5, 16:1ω7, 17:0cy, 17:1ω7, 19:0cy), and gram-positive bacteria (15:0a, 15:0i, 16:0i, 17:0a, 17:0i).

9.4 Recovery of a pure DAG and wax ester

We used a diacylglycerol (1,2-dioleoyl-sn-glycerol; Avanti Polar Lipids) and wax ester (oleyl oleate; Sigma-Aldrich) with known attached fatty acids to test their recovery after lipid fractionation. Both compounds were subjected to lipid fractionation and derivatisation (mild alkaline methanolysis), and subsequently measured on a GC-Tof-MS system (as described in section 9.2). Recovery was calculated relative to replicates of the same compounds which did not undergo lipid fractionation.

9.5 DAGAT and WS/DGAT gene search

We searched the Joint Genome Institute's (JGI) *Integrated Microbial Genomes with Microbiome Samples Expert Review* (IMG/M ER, tool 'Function Search'; https://img.jgi.doe.gov/) for WS/DGAT ('WS/DGAT C-terminal domain', pfam06974), diacylglycerol acyltransferase ('DAGAT', pfam03982), and additionally for the PHA synthesis regulator PhaR ('PHB/PHA accumulation regulator DNA-binding domain', pfam07879; Maehara et al., 2002). The search was restricted to all JGI-sequenced bacterial isolates that occur in the specified ecosystem type 'soil'. Species were classified based on their NCBI taxon

identification numbers. The resulting dataset consisted of 2926 unique entries, of which 918 showed WS/DGAT, 74 DAGAT, and 790 PhaR. Phyla with very low species coverage (n < 20) were collectively denoted as 'Other' (i.e., Aquificae, Armatimonadetes, Candidatus Microgenomates, Cyanobacteria, Chloroflexi, Deinococcus-Thermus, Fusobacteria, Nitrospirae, Planctomycetota, Spirochaetes, Synergistetes, Verrucomicrobia).

9.6 Data analysis

All data were analysed with R (v4.3.1), with preliminary data transformations and calculations in LibreOffice Calc (v7.6.4.1). Statistical significance between groups were tested with the t-test, or if its assumptions were violated (Shapiro-Wilk's test for normality and Levene's test for homogeneity of variance), with the Mann-Whitney test. Plots were created with the package *ggplot2* (Wickham, 2016).

The cladogram was constructed using hierarchical taxonomic categories (domain, phylum, class, order, family, genus, species) via the function *as.phylo* from the package *ape* (v5.7-1; Paradis et al., 2004), and plotted with the package *ggtree* (v3.10.0; Wang et al., 2020b; Xu et al., 2021; Xu et al., 2022).

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Supplementary Figure and Tables

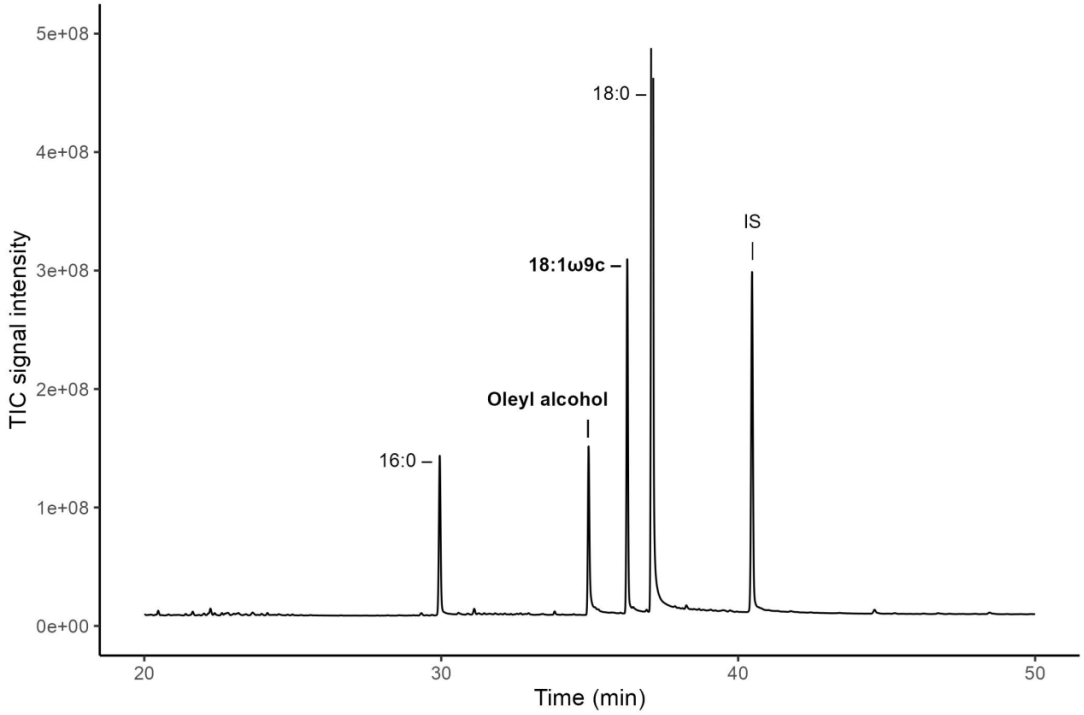

Figure S1 Chromatogram of a methylated pure wax ester (oleyl oleate; NLFA fraction). Both moieties of the wax ester, i.e. the alcohol (oleyl alcohol) and methylated fatty acid (18:1ω9c) are measured in the same GC chromatogram with a standard temperature program for FAME measurements (Gorka et al., 2023a). Their recovery values are listed in Table 2. The fatty acids 16:0 and 18:0 are common contaminants in the neutral lipid fraction stemming from the plastic walls of silicic acid columns. IS, internal standard (FAME 19:0).

Table S1 Individual PLFA and NLFA concentrations in nmol C g^{-1} dm, and their respective NLFA/PLFA ratios, of the three analysed soils (see Fig. 1). NLFA/PLFA ratios above or equal 1 are marked in bold.

Specificity	FA	Beech forest			Pine forest			Grassland		
		PLFA	NLFA	NLFA/PLFA	PLFA	NLFA	NLFA/PLFA	PLFA	NLFA	NLFA/PLFA
General	14:0	18.5	76.8	**4.1**	39.9	176.8	**4.4**	35.1	91.8	**2.6**
	15:0	17.7	36.9	**2.1**	29.9	46.2	**1.5**	-	46.2	-
	16:0	355.2	873.8	**2.5**	691.7	933.1	**1.3**	690.4	1718.2	**2.5**
	17:0	-	23.1	-	-	-	-	-	-	-
	18:0	136.6	770.7	**5.6**	224.7	1734.1	**7.7**	228.5	999.2	**4.4**
	18:1ω9trans	485.2	167.7	0.3	869.1	253.8	0.3	998.8	530.5	0.5
Fungi	18:1ω9cis	322.9	852.6	**2.6**	843.4	901.1	**1.1**	692.2	1913.1	**2.8**
	18:2ω6,9	164.0	324.6	**2.0**	514.8	591.4	**1.1**	266.8	606.7	**2.3**
	18:3ω6,9,12	-	-	-	-	-	-	-	107.1	-
16:1ω5	16:1ω5	83.3	87.4	**1.0**	268.4	130.3	0.5	369.7	737.1	**2.0**
C20	20:0	-	153.1	-	-	509.1	-	-	152.9	-
	20:4ω6,9,12,15cis	20.6	17.7	0.9	45.3	39.2	0.9	47.0	62.8	**1.3**
	20:5ω3,6,9,12,15	18.1	-	-	-	-	-	40.2	53.0	**1.3**

Table S1 (*continued*)

Specificity	FA	Beech forest			Pine forest			Grassland		
		PLFA	**NLFA**	**NLFA/PLFA**	**PLFA**	**NLFA**	**NLFA/PLFA**	**PLFA**	**NLFA**	**NLFA/PLFA**
Gram -	16:1ω7	200.6	172.3	0.9	468.9	178.5	0.4	534.7	1129.2	**2.1**
	16:1ω9	21.1	-	-	60.0	-	-	-	-	-
	17:0cy	76.5	39.1	0.5	135.1	65.7	0.5	194.4	35.8	0.2
	17:1ω8	-	26.0	-	-	-	-	-	68.4	-
	18:1ω5	22.9	-	-	111.8	-	-	100.4	-	-
	19:0cy(ω9)	-	33.5	-	-	-	-	-	-	-
	19:1ω9	433.7	52.0	0.1	505.1	131.2	0.3	507.5	54.3	0.1
Gram +	14:0i	-	24.6	-	-	-	-	46.9	-	-
	15:0a	117.2	68.1	0.6	188.9	96.0	0.5	340.3	140.4	0.4
	15:0i	217.8	171.2	0.8	358.3	232.2	0.6	400.1	294.8	0.7
	16:0i	75.2	162.3	**2.2**	110.3	181.9	**1.6**	107.7	142.8	**1.3**
	17:0a	31.2	41.7	**1.3**	76.6	93.1	**1.2**	82.5	58.3	0.7
	17:0i	35.0	34.7	**1.0**	59.3	47.3	0.8	78.8	61.6	0.8

Table S1 (*continued*)

Specificity	FA	Beech forest			Pine forest			Grassland		
		PLFA	NLFA	NLFA/PLFA	PLFA	NLFA	NLFA/PLFA	PLFA	NLFA	NLFA/PLFA
Actino	10Me17:0	16.6	31.2	**1.9**	-	50.9	-	-	-	-
	10Me18:0	32.1	56.0	**1.7**	98.1	71.3	0.7	88.5	80.4	0.9
unspecified	16:0_Me	19.5	-	-	-	-	-	59.6	-	-
	17:0_Me	115.5	103.3	0.9	185.6	182.0	**1.0**	257.3	101.6	0.4

Table S2 Occurrence of diacylglycerol acyltransferase (DAGAT) and wax ester synthase/diacylglycerol O-acyltransferase (WS/DGAT) specific genes in bacterial classes of soil isolate species. Both enzymes catalyse the last step in TAG synthesis. Asterisks indicate phylum assignment to 'Other' in the main figure and table.

Phylum	Class	n	DAGAT (%)	WS/DGAT (%)
Acidobacteria	Acidobacteriia	28	-	7.1
	Blastocatellia	1	-	-
	Holophagae	1	-	-
	Vicinamibacteria	1	-	-
Actinobacteria	Actinomycetia	1065	1.2	66.1
	Rubrobacteria	1	-	100
	Thermoleophilia	7	-	100
Aquificae*	Aquificae	2	-	-
Armatimonadetes	Chthonomonadetes	1	-	-
Bacteroidota	Bacteroidia	5	-	-
	Chitinophagia	49	-	-
	Cytophagia	81	-	-
	Flavobacteriia	78	-	-
	Saprospiria	1	-	-
	Sphingobacteriia	59	-	-
Candidatus Microgenomates*	unclassified	1	-	-
Chloroflexi*	Ktedonobacteria	3	-	-
	unclassified	2	-	-
Cyanobacteria*	unclassified	15	86.7	-
Deinococcus-Thermus*	Deinococci	15	-	-
Firmicutes	Bacilli	386	0.3	0.3
	Clostridia	43	-	2.3
	Negativicutes	2	-	-
	Tissierellia	2	-	-
	unclassified	1	-	-

Table S2 *(continued)*

Phylum	Class	n	DAGAT (%)	WS/DGAT (%)
Fusobacteria*	Fusobacteriia	1	-	-
Nitrospirae*	Thermodesulfovibrionia	1	-	-
Planctomycetota*	Planctomycetia	4	-	-
Proteobacteria	Acidithiobacillia	3	-	-
	Alphaproteobacteria	335	-	9.9
	Betaproteobacteria	287	-	17.1
	Deltaproteobacteria	90	48.9	73.3
	Epsilonproteobacteria	3	-	-
	Gammaproteobacteria	333	0.9	16.2
	Oligoflexia	3	-	-
Spirochaetes*	Spirochaetia	7	-	-
Synergistetes*	Synergistia	1	-	-
Verrucomicrobia*	Methylacidiphilae	1	-	-
	Opitutae	1	-	-
	Spartobacteria	1	-	-
	Verrucomicrobiae	5	-	-

Chapter 5

Summary and synthesis

Soil is a highly heterogeneous environment that continuously exposes microbial communities to fluctuating changes in their environment. To understand how soil microbial communities respond to such dynamic conditions, it is important to have biological marker compounds which accurately reflect both the structure and function of the communities. In this thesis, I used soil lipid-derived fatty acids to trace microbial community development after sudden changes in their physico-chemical environment, which gives insight into the resilience of soil microbial communities towards commonly experienced perturbations. Furthermore, I extended the existing PLFA extraction protocol to analyse neutral lipids and glycolipids in addition to phospholipids, which are potentially sensitive markers for microbial community functions. Finally, I discuss whether bacterial NLFAs extracted from soil are markers for carbon storage or bacterial necromass.

In **Chapter 2,** I simulated strong single-pulse perturbations of a temperate soil microbial community. These perturbations (glucose addition at 4 mg g^{-1} soil, freeze-thawing overnight at -20°C) mimicked common pulse events in natural soils, hence a high resilience of the microbial community towards them was expected. Microbial community dynamics were traced via phospholipid and neutral lipid fatty acid (PLFA, NLFA) analysis, as well as carbon and nitrogen stoichiometry in microbial biomass and dissolved pools shortly after treatment application (0.4–6 days), and at long-term timepoints (84 and 160 days).

There was a restructuring of the microbial communities and fluctuations in carbon and nitrogen stoichiometry over the experimental duration, regardless of the applied perturbations. Microbial communities showed high initial variation between sampling replicates, an effect which wore off in the long term. This indicates a transient change of the overall soil system. Moreover, the overall microbial community composition significantly changed (PERMANOVA, $P < 0.001$, $R^2 = 0.48$), and notably dissolved nitrogen increased slowly over time. Although it was expected that such a controlled system would bear an effect, this not only suggests that the trajectory of the microbial community initially changed in a stochastic (rather than deterministic) way due to stress caused by the incubation or soil sieving, it also indicates a transient change of the overall soil system. Resilience is sometimes measured only in reference to an initial state, but in this experiment, the reference state was intentionally exposed to the same experimental stressors, except for the perturbation treatments, at each sampling timepoint (Orwin and Wardle, 2004; Allison and Martiny, 2008). This allowed concurrent measurement of soil microbial response against an overall background of a non-steady state.

Both glucose addition and freeze-thawing significantly affected the microbial community composition based on PLFAs within the first week after treatment application (PERMANOVA, $P < 0.001$, $R^2 = 0.36$). Furthermore, legacy effects of both perturbations were still apparent up to 160 days after the perturbations were applied (PERMANOVA, $P < 0.05$, $R^2 = 0.14$), with PLFAs visibly separated in the multivariate correspondence analysis biplot (two-sample Mann-Whitney test on scores on first CA axis, $P < 0.05$). Fungi- and bacteria-specific PLFAs and NLFAs also increased (glucose-addition) and decreased (freeze-thawing) in the short term, and stayed increased after glucose addition up to 160 days. Carbon:nitrogen ratios in soil microbial biomass and dissolved pools were consistently different from control soils throughout the experiment.

These results demonstrate that single pulse perturbations can have long-lasting effects on soil microbial community structure and function. This is in contrast to our initial hypothesis, which expected high resilience of the soil microbes to environmental changes that are arguably common in the analysed soils. I speculate that feedback mechanisms between the microbial community and its soil environment could have maintained the altered state.

In **Chapter 3**, I expanded the high-throughput PLFA method to additionally yield neutral and glycolipids (Buyer and Sasser, 2012). For this, I first tested the recoveries of the three lipid fractions on silica solid phase extraction with pure lipid standards. While the PLFA method had been tested for recoveries of specific lipid fractions previously, it was never comprehensively assessed with all three lipid classes (Heinzelmann et al., 2014; Drijber and Jeske, 2019; Warren, 2019). Full fractionation of neutral, glyco-, and phospholipids was achieved by adding 2% ethanol to chloroform, while the classically used pure chloroform resulted in a significant loss of neutral TAGs and sterols into the glycolipid fraction (Drijber and Jeske, 2019). The betaine lipid diacylglyceryl trimethylhomoserine (DGTS) was mainly recovered in the phospholipid fraction (80%), but 10% were recovered in the neutral and glycolipid fraction respectively (Warren, 2019).

The second approach in this project was to assess whether the taxonomic specificities of marker fatty acids are retained across lipid fractions. Lipids were extracted from representative soil microbial pure cultures strains. Non-metric multidimensional scaling (NMDS) analysis of the pure culture fatty acid profiles revealed that NLFA, GLFA, and PLFA primarily cluster by taxonomy (PERMANOVA, $P < 0.001$, $R^2 = 0.74$). Specific fatty acids that are used as taxonomic markers corresponded with previous publications (e.g. Zelles, 1997; Willers et al., 2015). For instance, iso- and anteiso-branched fatty acids were found

in gram-positive bacteria, 10-methyl branched fatty acids in Actinobacteria, monounsaturated fatty acids in gram-negative bacteria, the fungal marker 18:2ω6,9 in Ascomycetes and Basidiomycetes, and 16:1ω5 in the arbuscular-mycorrhizal fungus. Interestingly, we found the iso-branched fatty acid i16:0 to be abundant in Actinobacteria, which contrasts the recent proposal by Joergensen (2022) to apply iso- and anteiso-branched fatty acids only to Firmicutes. Moreover, 18:1ω9t was found in both bacteria and fungi, suggesting it to be an unspecific microbial marker.

Thirdly, I analysed complete PLFA, NLFA, and GLFA profiles of soil extracts. The majority of fatty acids were recovered in the PLFA and NLFA fractions (together ca. 88%), although significant amounts were still recovered in the GLFA fraction. Multivariate NMDS analysis contrasted with the pure culture data, as the fatty acid profiles were primarily separated by the lipid class (PERMANOVA, $P < 0.0001$, $R^2 = 0.47$), albeit soils were also well separated (PERMANOVA, $P < 0.0001$, $R^2 = 0.21$). While the origin of bacterial NLFAs (either carbon storage or necromass markers, which I discuss in depth in Chapter 4), and functions of microbial GLFAs, are not fully understood, their strong separation between soils suggests them to be powerful markers for changes in microbial community physiology.

The fourth effort in this project was to integrate sterol analysis into the PLFA method. Pure sterols eluted in the neutral lipid fraction with high recovery upon addition of 2% ethanol to chloroform. Gas chromatographic measurement of underivatised ergosterol gave excellent standard row linearity ($R^2 = 0.99$), and it was possible to integrate fatty acid methyl ester (FAME) and sterol elution within single chromatographic runs. This was achieved by running isothermally at 300°C after all FAMEs had eluted. Fungal pure culture and sporocarp extracts yielded significant amounts of sterols, particularly ergosterol. However, soil extracts only yielded miniscule amounts of sterols. Efforts to improve the ergosterol yield from soil by increasing the extractant:soil ratio vastly underperformed compared to a standard ergosterol extraction protocol with pure ethanol (Djajakirana et al., 1996). This suggests that the PLFA method is not suitable for sterol measurements in soils. Nonetheless, the same method can be applied to biological tissues, and the developed gas chromatographic measurement of underivatised sterols can still be applied to soil extracts from alternative extraction methods.

Chapter 4 aims to elucidate whether soil bacteria-specific NLFAs derived from recently degraded phospholipids (suggesting necromass) or TAGs (suggesting carbon storage). Both interpretations have been used in the literature (e.g. Bååth,

2003; Mason-Jones et al., 2021). Although neutral lipids can theoretically stabilise via organo-organo interactions or intra-aggregate micropores due to their hydrophobic character, only two of several phospholipid degradation pathways can lead to neutral lipid formation (Fisher and Jain, 2009; Kästner et al., 2021; Buckeridge et al., 2020). Phospholipases can cleave phospholipids at various positions, and only phospholipase C and D pathways, which cleave between the glycerol and the phosphate group (PL-C) and after the phosphate group (PL-D) can potentially yield neutral diacylglycerols (DAGs; Titball, 1993; Flores-Díaz et al., 2016). Furthermore, these are only initial steps in phospholipid degradation as DAG can be further degraded to glycerol and free fatty acids (Yuan et al., 2016). Thus, phospholipid-derived DAGs likely reflect only an intermediate step during phospholipid degradation.

Instead, TAG production is often observed in bacterial pure culture studies, particularly Actinobacteria (Alvarez and Steinbüchel, 2002; Wältermann and Steinbüchel, 2006). These use a specialised wax ester synthase/diacylglycerol acyltransferase (WS/DGAT) for TAG production (Alvarez, 2016). Utilising the Joint Genome Institute's (JGI) database of bacterial isolate genomes, I found that a significant portion (31%) of soil bacterial isolates encode genes for WS/DGAT. Intriguingly, polyhydroxyalkanoate (which is a common bacterial storage compound) showed similar coverage across bacterial isolates (27%, based on the synthesis regulator PhaR). This indicates that bacterial TAG production is more widespread in soil bacteria than previously assumed.

Furthermore, I experimentally tested the fate of an added glucose-^{13}C into soil microbial NLFAs and PLFAs. The addition of glucose created conditions of access carbon for storage compound production. Glucose-^{13}C was rapidly allocated into fungi- and bacteria-specific PLFAs and NLFAs already four hours after glucose addition (Mann-Whitney test, $P < 0.05$). Relative ^{13}C enrichment increased within six days, and NLFAs specific to gram-positive as well as gram-negative bacteria were even more enriched than the corresponding PLFAs (t-test, $P < 0.05$).

These findings strongly favour bacterial NLFA to be derived mostly from storage compounds. Nonetheless, degraded phospholipids potentially may contribute to the final NLFA pool, and further studies are needed to test whether this contribution is significant. Modern lipidomics approaches which facilitate the analysis of intact lipid species could further elucidate the origin of NLFAs extracted from soil (Bale et al., 2021; Couvillion et al., 2023).

Overall, this thesis contributes to understanding the resilience and functional responses of soil microbial communities to environmental perturbations, as well as refining lipid analysis methodologies to better characterise these communities. Through the combination of experimental perturbations and method development in lipid profiling, this thesis offers new insights into the long-term effects of environmental changes on microbial community structure, and the applicability of fatty acid profiling to trace microbial community functions. Analysing soil microbial responses to single-pulse perturbations revealed that short environmental changes can induce long-lasting alterations in microbial community composition and function. This finding challenges the assumption of high microbial resilience in soils that regularly experience such perturbations and suggest that microbial feedback mechanisms could contribute to the persistence of these changes over time. The expansion of the traditional PLFA method by incorporating neutral and glycolipid fractions offers a more comprehensive view of microbial community dynamics. NLFAs and GLFAs can serve as sensitive markers for physiological traits like carbon storage or stress response. My findings also indicate that bacterial NLFAs are markers for carbon storage rather than necromass. Thus, analysing complete NLFA profiles (covering both fungi and bacteria) can serve as a sensitive approach to quantify microbial carbon storage. Combining NLFA and GLFA measurements with PLFAs adds minimal effort to the extraction process while potentially providing valuable insights into soil microbial physiology. Their consistent taxonomic specificity across lipid fractions, and different functional meaning, underscores the potential of these expanded profiles as powerful tools for soil microbial ecology.

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