Biology and Fertility of Soils (2022) 58:835–842
https://doi.org/10.1007/s00374-022-01670-9

POSITION AND OPINION PAPERS

Fatty acid 16:1ω5 as a proxy for arbuscular mycorrhizal fungal biomass: current challenges and ways forward

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Received: 14 February 2022 / Revised: 14 September 2022 / Accepted: 27 September 2022 / Published online: 3 October 2022

Abstract
Fatty acid biomarkers have emerged as a useful tool to quantify biomass of various microbial groups. Here we focus on the frequent use of the fatty acid 16:1ω5 as a biomarker for arbuscular mycorrhizal (AM) fungi in soils. We highlight some issues with current applications of this method and use several examples from the literature to show that the phospholipid fatty acid (PLFA) 16:1ω5 can occur in high concentrations in soils where actively growing AM fungi are absent. Unless the study includes a control where the contribution of other microbes can be estimated, we advocate for the use of the neutral lipid fatty acid (NLFA) 16:1ω5. This biomarker has higher specificity, is more responsive to shifts in AM fungal biomass, and quantification can be conducted along with PLFA analysis without doubling analytical efforts. We conclude by contrasting various methods used to measure AM fungal biomass in soil and highlight future research needs to optimize fatty acid analyses.

Keywords PLFA 16:1ω5 · NLFA 16:1ω5 · Arbuscular mycorrhizal fungi · Biomass · Gram-negative bacteria

Background

Soil microbes are integral components of soil health, but how we measure and interpret microbial abundance and function is less clear (Fierer et al. 2021). Arbuscular mycorrhizal (AM) fungi are obligate root symbionts with most land plants and obtain carbon (C) in exchange for phosphorus and other putative services, such as increased drought tolerance and pathogen protection (Smith and Read 2008). Many methods are used to quantify AM fungal biomass in soils, including hyphal length and spore counts, DNA-based approaches, and fatty acid analysis, each with its pros and cons. For the fatty acid analysis, phospholipids are generally separated from neutral lipids to yield phospholipid fatty acids (PLFA), which are components of cell membranes, and neutral lipid fatty acids (NLFA) that are associated with storage (Fig. 1). The number of C atoms, number and positions of double bonds, and cyclic rings in fatty acid residues follow the phylogeny of organisms, and their analysis can therefore be used to estimate the biomass of various microbial groups (Tunlid and White 1990). More than 350 papers have been published using the fatty acid 16:1ω5 to quantify AM fungal biomass since this method was first developed in the early 1990s (Olsson and Lekberg 2022). Our concern is that the method is not always used accurately, which likely affects assessments of AM fungal biomass.

A recent review published in this journal describes current challenges and future use of phospholipid fatty acid (PLFA) analysis as an indicator for soil microbial biomass and the composition of microbial communities (Joergensen 2022). The review is timely and highlights important issues

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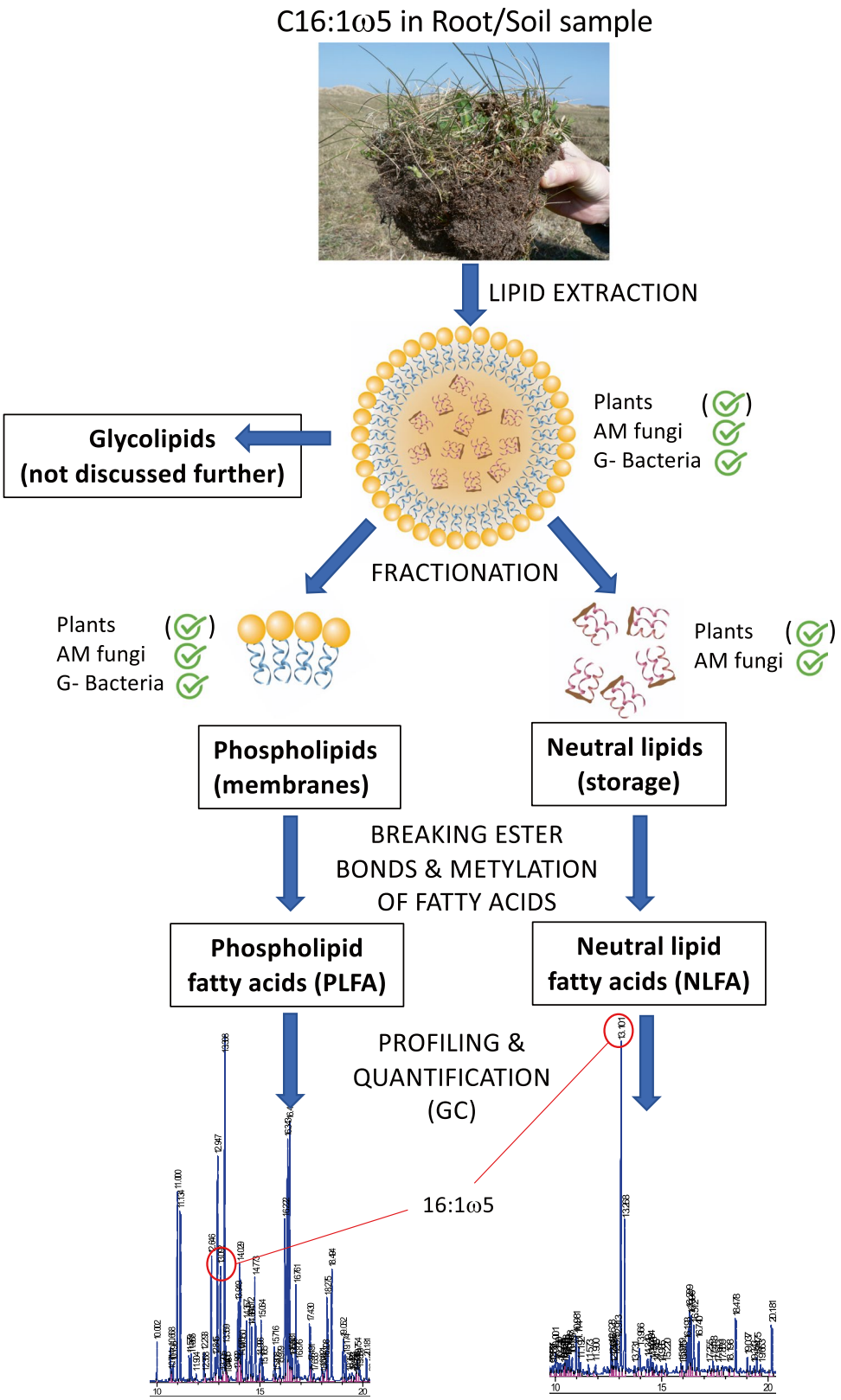

Fig. 1 Extraction and quantification pathways using gas chromatography (GC) of neutral lipids and phospholipids, as well as organisms known to contain 16:1ω5 phospholipid and neutral lipid fatty acids, respectively

associated with the method, but we take issue with one argument put forward, which is that PLFA 16:1ω5 should be used to quantify the biomass of AM fungi. This has been advised against previously because PLFA 16:1ω5 is also found in Gram negative (G −) or diderm bacteria, which makes it a less suitable marker for AM fungi (Nichols et al 1986;

Frostegård et al. 2011). The recent review (Joergensen 2022) argues that this is not problematic based on two assumptions: (1) PLFA 16:1ω5 is found only in minute amounts in G − bacteria and (2) this group of bacteria contribute at most 10% to the total microbial biomass. As such, any contribution by bacteria to the PLFA 16:1ω5 ought to be minimal.

Here we question those two assumptions and use examples from the literature to illustrate that PLFA 16:1ω5 may be a poor biomarker for actively growing AM fungi. This is important because more than half of all the papers published to date have used PLFA 16:1ω5 to quantify AM fungal biomass, predominately in soils and lacking proper controls where the non-AM fungal contribution can be estimated (Olsson and Lekberg 2022). We then contrast the performance of PLFA 16:1ω5 with NLFA 16:1ω5, compare fatty acid analysis with other methods to quantify AM fungal biomass, and conclude by outlining current challenges and future approaches. Our objective with this Position paper is to provide a sound basis for choosing methods to quantify the biomass of this important group of symbionts as accurately as possible.

PLFA 16:1ω5 in sources other than actively growing AM fungi

While several G − bacteria contain no detectable or only trace amounts of PLFA 16:1ω5 (Zelles 1997), this fatty acid can dominate in other isolates, representing up to 50% of all PLFA or 30% of the total fatty acids in their cells (Walker 1969; Livermore et al. 1969; Joung et al. 2015; Islam et al. 2020; Chen et al. 2020). Indeed, PLFA 16:1ω5 is considered a biomarker for some G − bacteria (Findlay et al. 1990; Findlay and Dobbs 1993). If the biomass of G − bacteria is small relative to AM fungi, this may not be a problem, but what support do we have for this assertion? One way to address this question is to look at environments where AM fungi are expected to be absent or in very low abundance, and here results vary. For example, PLFA 16:1ω5 was in low abundance or apparently absent in lake sediments and compost (Findlay and Dobbs 1993; Cooper et al. 2002), but it was the fourth most abundant fatty acid based on mol % in wood fiber collected from other lake sediments (Regnell et al. 1996). Also, the PLFA 16:1ω5 responded more than any other fatty acid to lime additions in coniferous forests and spruce plantations that contained very few AM hosts (Frostegård et al. 1993; Cruz-Paredes et al. 2017). Another way to address this is to consider results from microcosm experiments where soils are incubated without plants. Here, some experiments have found that PLFA 16:1ω5 was among the markers responding most to the addition of leaf litter (e.g., Benito-Carnero et al. 2021), or to treatments that increase soil pH (Cruz-Paredes et al. 2017). Because AM fungi are obligate biotrophs they may survive for some time without hosts, but their biomass will not increase without carbon supplied by a living host plants (with some notable exceptions in highly sophisticated in vitro systems; e.g., Tanaka et al. 2022). Thus, AM fungi cannot be the source of the increasing PLFA 16:1ω5 concentrations in these types of experiments. Likewise, despite assertions that plants do not produce PLFA 16:1ω5 (e.g., Brands et al. 2020), some species within the family Proteaceae are metabolically capable of synthesizing this fatty acid (Vickery 1971). A public database tool (https://plantfadb.org/tree; Ohlrogge et al. 2018) based on the Seed Oil Fatty Acid (SOFA) database (Matthäus 2012) reports 93 occurrences of 11-Z-hexadecenoic acid (i.e., 16:1ω5) in 60 out of 8000 plant species, albeit in seeds or leaves, not roots. Combined, these studies illustrate that PLFA 16:1ω5 can be quite abundant and show treatment effects that cannot be explained by shifts in AM fungal biomass.

What is the support for AM fungal biomass greatly exceeding G − bacterial biomass in AM-dominated systems? Clearly such data are very difficult to obtain and will be rough estimates at best. In a Danish linseed field, AM fungal biomass was estimated to be ~ 0.3–4 times that of total bacterial biomass (Olsson et al. 1999), and in arable soils in Germany, the value ranged from 1.3 to 1.6 (Faust et al. 2017) when applying a conversion factor of 322 nmol mg^{-1} dry biomass for bacteria and 19 nmol mg^{-1} for AM fungal biomass (Olsson et al. 1999). These conversion factors incidentally also illustrate that bacteria, with a higher surface area per volume, contain an order of magnitude more PLFAs per g biomass than AM fungi (Joergensen and Wichern 2008). Stable isotope probing (SIP) combined with fatty acid analysis offers another way to assess whether AM biomass exceeds G − bacterial biomass by tracing the relative proportion of ^{13}C in PLFA 16:1ω5 originating from living plants (indicative of AM fungi) and dead organic matter (indicative of bacteria, assuming that AM fungi are fully biotrophic and acquire all their carbon from plants under most situations). The study by Elfstrand et al. (2008) showed more ^{13}C in PLFA 16:1ω5 originated from green manure than from actively growing leek plants in an agricultural setting, and that G − bacteria dominated irrespective of C-source. Likewise, Ven et al. (2020) used host plant and soil with different $\delta^{13}C$ signatures (C_4 plant grown in soil collected underneath C_3 vegetation) and found that the $\delta^{13}C$ signal in the PLFA 16:1ω5 extracted from sand-filled mesh bags was 30–50% higher and more similar to plant root than the $\delta^{13}C$ signal extracted from the soil, which was more similar to soil organic matter. This probably signifies a much greater AM fungal contribution to PLFA 16:1ω5 in the mesh bags (where the only source of C for bacteria would be AM fungal exudates or microbial necromass), and a much greater bacterial contribution to PLFA 16:1ω5 in soils.

Another indication that this was the case was a significant correlation between PLFA 16:1ω5 and AM hyphal density in the mesh bags, but not in the soil. Thus, the assumption that AM fungal biomass dominates in AM systems, which justifies the use of PLFA 16:1ω5 as an indicator specific for this group with negligible interferences from other microbial groups, is not supported under all situations. As such, PLFA 16:1ω5 should only be used when the contribution from sources other than AM fungi can be estimated.

Comparisons between PLFA and NLFA 16:1ω5

Given the issues raised above, we argue that NLFA 16:1ω5 is a more specific biochemical signature compound to use in AM studies because other dominant fungi (ascomycetes and basidiomycetes) do not produce it in significant amounts (Müller et al. 1994) and the common storage material for prokaryotes is polyhydroxyalkanoic acids, which do not contain 16:1ω5 or other long-chain fatty acids (Wältermann and Steinbüchel 2005). Some actinomycetes can store triacylglycerols or wax esters (Wältermann and Steinbüchel 2005) but there is no evidence that NLFA 16:1ω5 is produced by these organisms. Thus, there is no reason to believe that the 16:1ω5 fatty acid detected in the neutral fraction of lipid extracts has prokaryote origin. Despite this, NLFA analyses are less common than PLFA analyses for quantifying AM fungi (Olsson and Lekberg 2022), possibly because of the doubling of sample numbers postfractionation (Fig. 1) and/or lack of awareness among researchers regarding issues associated with PLFA.

Some studies that have analyzed both 16:1ω5 PLFA and NLFA show similar responses to treatments (Faust et al. 2017), but others do not. In a field experiment where a non-AM host (amaranth) replaced an AM-host plant (wheat), the NLFA 16:1ω5 in soils decreased to almost zero whereas the PLFA 16:1ω5 increased (Ngosong et al. 2012). Given that amaranth does not supply C to AM fungi, this increase in PLFA 16:1ω5 cannot reflect greater AM fungal biomass. More likely, the increase in PLFA 16:1ω5 in Ngosong et al.'s study was due to G − bacteria. This is supported by Elfstrand et al. (2008) where most of the ^{13}C in PLFA 16:1ω5 came from green manure, whereas most of the ^{13}C in NLFA 16:1ω5 came from plants. Moreover, soil NLFA, but not PLFA 16:1ω5, correlated well with AM colonization of bait plants in old field successions (Hedlund 2002), and with inoculum potential in bulk soil collected around plants that varied in host quality (Vestberg et al. 2012).

To quantitatively compare PLFA and NLFA 16:1ω5 in soils where actively growing AM fungi were present or not, we selected all publications from a recent literature review (Olsson and Lekberg, 2022) where both fatty acids were reported. We also included values extracted from roots to assess if patterns found in soils were similar in roots. We found seven papers in total. In each publication, a microbial inoculum lacking AM fungi was added to all treatments, and AM fungal inoculations resulted in substantial AM colonization of host plants whereas control plants were non-mycorrhizal (Table S1). This is not a perfect comparison if other microbial groups respond to the absence of AM fungi, which can occur sometimes (Gryndler et al. 2018) but not always (Olsson et al. 1996). However, it allows for some rough comparisons of the two fatty acids when viable AM fungi are present or not. This analysis showed that PLFA 16:1ω5 did not differ substantially between soil without AM fungi (0.80 nmol g soil^{-1} ± 0.23) and soil with AM fungi (1.3 nmol g soil^{-1} ± 0.74, means ± SD), and that actively growing AM fungi contributed on average 29% to the PLFA 16:1ω5 (Fig. 2). That is, roughly 70% of this fatty acid originated from sources other than actively growing AM fungal biomass, such as G − bacteria and dead AM fungi that had not yet decomposed. In contrast, AM fungi accounted for on average 92% of all NLFA 16:1ω5 in soil (Fig. 2; Table S1). Taken together, we argue that NLFA 16:1ω5 is not only more specific but also more responsive to changes in AM fungal biomass than PLFA. This is illustrated by the fact that the difference between mycorrhizal and non-mycorrhizal soils was 1.6-fold for PLFA 16:1ω5 and 22-fold for NLFA 16:1ω5.

The contribution by AM fungi to PLFA 16:1ω5 in roots, however, was greater and more similar to the contribution to NLFA 16:1ω5 (94% vs. 98%, respectively; Fig. 2; Table S1). This greater contribution by AM fungi to PLFA

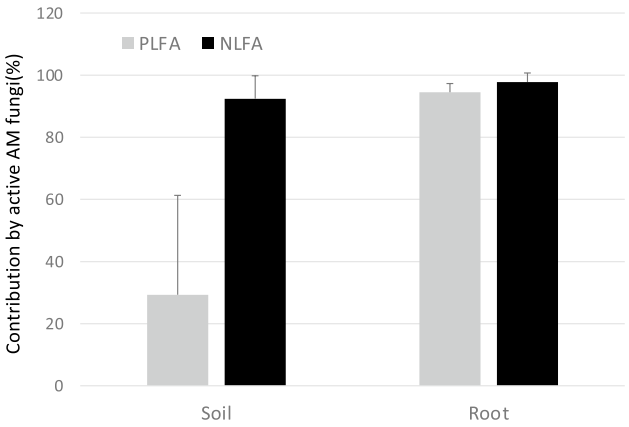

Fig. 2 Contribution by actively growing arbuscular mycorrhizal (AM) fungi to 16:1ω5 phospholipid fatty acids (PLFA) and neutral lipid fatty acids (NLFA) extracted from soil ($n = 5$ papers) or roots (6 papers) in studies that quantified both fatty acids were conducted in sterilized soil inoculated with AM fungi as well as a microbial wash. While variable among studies, it highlights that the contribution to PLFA [$PLFA_{AM} - PLFA_{NM}$)/$PLFA_{AM}$] is much smaller than NLFA in soil, but not in roots. Data used to generate this figure is in Table S1, mean ± SD

16:1ω5 in roots relative to soil is presumably because the biomass of AM fungi can constitute several percent of root biovolume (Toth et al. 1991), i.e., substantially more than in soil, whereas the abundance of bacteria is orders of magnitude lower in roots than in the rhizosphere (Bulgarelli et al. 2013; Liu et al. 2017). Based on this, PLFA 16:1ω5 could probably be used to quantify AM fungal abundance in roots, but not soil, unless accompanied by a proper control where AM fungi are absent.

There are a few situations where care should also be taken with NLFA 16:1ω5. Like PLFA, it has been found in small amounts in plants (Table S1), although AM roots contained on average 50 times more NLFA 16:1ω5 than non-mycorrhizal roots (Table S1). This suggests that most NLFA 16:1ω5 found in roots likely comes from AM fungi rather than plants (or plant endophytes). The exception may be when dried plant matter is used to stimulate hyphal colonization into ingrowth bags (Hammer et al. 2011), especially in situations where hyphal abundance is low. In such cases, including mesh bags impenetrable by AM fungal hyphae as controls is a good idea. Also, NLFA 16:1ω5 measures AM fungal biomass associated with storage structures (spores and vesicles primarily) where persistence in soil is little known (discussed more below). High background values due to slow turn-over or non-specificity do not seem to be a problem, however, because we observed rapid responses in NLFA when C allocation to AM fungi changed (see previous references) and high ratios in both soil and roots relative to non-mycorrhizal controls (Table S1). Finally, NLFA should never be used to assess AM fungal biomass when the ratio NLFA/PLFA for 16:1ω5 is < 1 as it indicates that NLFA values primarily derive from sources other than AM fungi (Olsson 1999). A common misunderstanding from Olsson (1999) is that PLFA (not NLFA) 16:1ω5 primarily measures AM fungal biomass when ratios > 1, which may not be the case.

When estimates of bacterial background for 16:1ω5 PLFA can be obtained (e.g., by including mesh bags and non-mycorrhizal treatments), using this fatty acid to quantify AM fungi allows for direct comparisons with other microbial groups also quantified by PLFA profiling. There are also advantages of using both PLFA and NLFA to quantify AM fungi as they can provide insights into (i) AM fungal energy status (Lekberg et al. 2013), (ii) relative allocation to growth vs. storage (van Aarle and Olsson 2003), and (iii) shifts in AM fungi and G − bacteria across environmental gradients, including changes in host abundance, soil organic matter, and pH (Högberg et al. 2006; Welc et al. 2012). If estimates of AM fungal biomass in soils are of interest and bacterial contributions cannot be estimated, however, we argue that NLFA 16:1ω5, *not* PLFA 16:1ω5, should be used because of its higher specificity.

AM fungal biomass measurements — comparisons with other methods and way forward

It is important to remember that all methods are estimates at best and can be biased to various extents. For example, most measurements of AM fungal abundance in roots have been based on microscopic assessment of presence/absence in root intercepts (McGonigle et al. 1990). This method does not differentiate between viable and non-viable AM fungi and does not consider the proportion of each intercept filled (Trouvelot et al. 1986), which may result in poor correlations with AM fungal biomass. Likewise, molecular approaches targeting DNA or RNA, such as qPCR, may overestimate the abundance of particular taxa that sporulate readily or contain high concentration of DNA per unit weight of biomass and/or high copy number of marker genes per genome (Thonar et al. 2012). Internal transcribed spacer region (ITS) sequences have also been used to assess relative abundance of different fungal clades including AM fungi, but amplification of Glomeromycotan fungi can sometimes be low relative to other fungal taxa, especially in soil (Lekberg et al. 2018; Fu et al. 2022). Such methods are also sensitive to the choice of primers and amplification (Suzuki et al. 2020). A study involving consecutive harvests and characterizations of both intraradical and extraradical AM fungal biomass using both PLFA and NLFA analyses, microscopy and molecular approaches would allow for assessments of congruence. We are not aware of many such studies to date, although some exists (e.g., Vestberg et al. 2012; Voříšková et al. 2017), which highlights an important knowledge gap.

One method that holds great promise is SIP where ^{13}C incorporation into specific fatty acids or RNA is measured. This technique has been used to trace recently assimilated C from plants to AM fungi and other soil biota groups, including individual microbial taxa (Olsson and Johnson 2005; Drigo et al. 2010; Kiers et al. 2011; Kaiser et al. 2015; Forczek et al. 2022). The SIP method can be coupled with radioactive isotopes and molecular approaches targeting functional genes such as nutrient/carbon transporters (Watts-Williams et al. 2015; Sawers et al. 2017) or measurements of enzyme activity (e.g., phosphatase; Van Aarle et al. 2001) to calculate cost–benefit ratios in the AM symbiosis, or nanoscale secondary-ion mass spectrometry (NanoSIMS) to visualize C flow through AM fungal structures in roots (Kaiser et al. 2015).

Interpretation of fatty acid analyses could be improved by increased attention to a few areas. Opinions differ regarding persistence after cell death, with some arguing that fatty acid analysis are not suitable for short-term

experiment (Joergensen 2022), while others claim rapid degradation upon cell death (Tunlid and White 1992; Herzberger et al. 2014). This may be particularly relevant for AM fungi because, depending on environmental conditions, they can survive on stored carbon for an extended period of time (Lekberg and Koide 2008). How this may influence NLFA and PLFA 16:1ω5 values and correspond to AM fungal viability is little known but could be assessed by sampling stored soil over time for parallel fatty acid analyses and infectivity assays. Future experiments should also quantify the possible contribution of extracellular lipids stabilized on soil minerals and/or microbial necromass in soil to both NLFA and PLFA fractions after cell death (Zelles 1999; Joergensen 2022). One approach would be to isotopically (^{13}C) label AM fungal mycelium and then track microbial decomposition by measuring ^{13}C incorporation into metabolites (fatty acids, proteins, and nucleic acid) within specific microbial groups.

Other areas that would benefit from more information are these analyses' degree of specificity and context dependency. For example, we currently do not know if fungi that can co-occur with AM fungi, such as arbuscule-forming fine root endophytes from the Mycoromycotina clade (Hoysted et al. 2019) produce the fatty acid 16:1ω5. Also, the presence and absence of certain endobacteria seem to influence fatty acid content (Salvioli et al. 2010), as can the particular type of chloroform used to elute NLFA (Drijber and Jeske 2019). Shifts in AM fungal community composition may also influence measurements because different taxa can vary considerably in their fatty acid concentration (Graham et al. 1995) and some AM fungal genera (e.g., *Gigaspora*, *Paraglomus*, and *Ambiospora*) may produce little or no 16:1ω5. These genera may instead produce more 20:1ω9 (Graham et al. 1995; Bentivenga and Morton 1996), which has prompted some researchers to include this fatty acid in order not to underestimate AM fungal biomass (Vořišková et al. 2017). Combining community characterization with fatty acid analyses may be a good idea wherever possible to estimate the relative abundance of AM fungal families.

Still another approach would be to identify AM fungi-specific compounds with a greater complexity than fatty acids, such as phosphatidylcholines (PC) with acyl combinations in specific positions, such as 20:5–20:5-PC, 20:5–20:4-PC, or 20:5–20:3-PC (Wewer et al. 2014). Although greater specificity towards the target organisms could be achieved due to their unlikely presence in plants and bacteria, concerns remain about variation in quantity of such compounds per unit biomass among the different AM fungal species/families. Furthermore, much more sophisticated and expensive biochemical analyses would be required to identify and quantify such compounds in biological samples as compared to fatty acid methyl ester analyses, limiting the throughput and cost-effectiveness of such an approach.

Conclusions

To achieve greater consistency and the ability to compare results across studies, it is imperative that soil ecologists discuss pros and cons of various methods. Here we outline what we know about 16:1ω5 PLFA and NLFA and under which conditions each of them can be used to assess AM fungal biomass. Both PLFA and NLFA 16:1ω5 are reliable biomarkers for AM fungal biomass in roots. However, NLFA 16:1ω5 is preferred in soils as it is more specific and more responsive than PLFA 16:1ω5, especially if non-mycorrhizal controls are difficult or impossible to include in studies. More information about congruence between fatty acid analyses and other methods used to estimate viable AM fungal biomass, the persistence of lipids and fatty acids in soil, and the degree of specificity would further improve applicability and interpretation of these methods.

Supplementary information The online version contains supplementary material available at https://doi.org/10.1007/s00374-022-01670-9.

Declarations

Conflict of interest The authors declare that no competing interests.

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