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Application of 13C-Labeled Phospholipid Fatty Acid Analysis in Soil Microbial Ecology Research: Postprint

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Date: 2017-11-06T00:00:00+00:00

Abstract

Phospholipid fatty acids (PLFA) are important components of microbial cell membranes. Different microbial communities can synthesize different PLFAs through various biochemical pathways; therefore, certain PLFAs can be selected as biomarkers for changes in microbial community structure. The combination of PLFA with stable isotope 13C labeling (13C-PLFA) technology can not only determine the composition of microbial communities in in situ soil environments, but also enables targeted exploration of microbial communities involved in carbon source metabolic processes in soil ecosystems, providing information on soil microbial interactions within complex communities, and has broad application prospects. Its basic principle is as follows: a substrate enriched with the stable isotope 13C is added to soil, certain microbial communities in the soil utilize the substrate 13C to synthesize PLFAs, PLFAs from soil microorganisms are extracted and purified, their 13C abundance is measured using gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS), and through comparative analysis, direct information linking microbial community composition and function is obtained. Based on introducing the principles of 13C-PLFA, this paper reviews the applications of this technology in rhizosphere microbial utilization of photosynthetically assimilated carbon, priming effects of soil organic matter decomposition, methane oxidation, organic pollutant degradation, and microbial utilization of exogenous simple and complex carbon sources, analyzes the advantages and disadvantages of this technology, and provides an outlook on its future applications.

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Full Text

Application of 13C-Labeled PLFA Analysis in Soil Microbial Ecology Studies

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Abstract

Phospholipid fatty acids (PLFAs) are essential components of microbial cell membranes. Different microbial communities synthesize distinct PLFAs through various biochemical pathways, enabling the use of specific PLFAs as biomarkers for tracking changes in microbial community structure. When combined with stable isotope 13C labeling (13C-PLFA), this technique not only identifies in situ soil microbial community composition but also specifically targets microbial groups involved in carbon metabolism within soil ecosystems, providing valuable insights into microbial interactions within complex communities. The fundamental principle involves adding 13C-enriched substrates to soil, where specific microbial communities assimilate the 13C into their PLFAs during growth and metabolism. After extraction and purification of soil microbial PLFAs, gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) determines their 13C enrichment. Comparative analysis then yields direct information linking microbial community composition to function. This paper introduces the principles of 13C-PLFA and reviews its applications in microbial utilization of photosynthetically assimilated carbon in the rhizosphere, priming effects in soil organic matter decomposition, methane oxidation, organic pollutant degradation, and microbial utilization of both simple and complex exogenous carbon sources. We analyze the advantages and limitations of this technique and discuss its future prospects.

Keywords: Phospholipid fatty acid; Stable isotope labeling technique; Soil microbe; Community structure; Microbial function

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Soil microorganisms constitute a vital component of soil ecosystems, influencing nearly all soil processes either directly or indirectly. Traditional microbial research relied heavily on laboratory cultivation techniques, yet these methods can isolate and identify only 0.1%–1% of the total soil microbial population [1]. Consequently, modern approaches such as phospholipid fatty acid (PLFA) analysis have become widely adopted in soil microbial research. PLFAs are key constituents of living microbial cell membranes, and different microbial taxa produce unique PLFAs through distinct biochemical pathways. Because specific PLFAs consistently occur in particular microbial groups while remaining rare in others, they serve as reliable indicators for characterizing overall microbial com-

munity structure. Moreover, since PLFA synthesis is closely linked to microbial growth, PLFAs can also be used to estimate microbial biomass. Comprehensive reviews have detailed the applications of PLFA analysis in soil microbial research [2]. However, PLFA analysis alone cannot directly link microbial identity to function or provide direct information about microbial interactions and metabolic activities. The integration of PLFA with stable isotope 13C labeling (13C-PLFA) has emerged as a powerful solution to this limitation.

The 13C-PLFA technique operates through several key steps. First, 13C-enriched substrates are added to soil, where certain microorganisms use them as carbon sources for physiological metabolism and growth, incorporating the 13C into their cellular components, including PLFAs. Second, these 13C-labeled PLFAs are extracted, purified, and analyzed to connect microbial community composition with functional activity. The 13C enrichment of PLFAs is measured using gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS), which separates PLFAs on a capillary column before combustion to CO_2 in a micro-oxidation unit. The resulting CO_2 enters the IRMS for determination of the heavy (13C) to light (12C) isotope ratio (13C/12C). Compared to nucleic acid-based 13C labeling techniques (13C-DNA), GC-C-IRMS can resolve 13C isotopic abundance to one-thousandth, enabling detection of even minute differences in 13C content. This gives 13C-PLFA exceptional sensitivity. Additionally, rapid microbial PLFA synthesis allows sufficient 13C-labeled PLFAs to be obtained within short incubation periods, reducing experimental duration and cost. The technique also involves fewer pretreatment steps, minimizing sample loss during processing. Consequently, 13C-PLFA has been widely applied to identify functional microbial communities in soil and trace carbon fluxes among microbial populations, representing a relatively mature technology. This review elaborates on applications of 13C-PLFA in plant photosynthetic carbon utilization, soil organic matter priming effects, methane oxidation, organic pollutant degradation, and microbial utilization of both simple and complex exogenous carbon sources, while also discussing the technique' s strengths and weaknesses.

1. Microbial Utilization of Photosynthetically Assimilated Carbon in the Rhizosphere

Carbon transport and allocation in the "atmosphere-plant-soil" system represents a critical component of the carbon cycle. Plants release a portion of photosynthetically assimilated carbon into soil as rhizodeposits, which are subsequently transformed by soil microorganisms and either released to the atmosphere as gases or stored as soil organic matter, playing a key role in stabilizing soil carbon pools. Understanding microbial transformation of photosynthetic carbon and identifying the responsible microbial communities are therefore essential for elucidating soil organic carbon cycling. Most studies employ gaseous $13CO_2$ pulse or continuous labeling approaches. Butler et al. [3] first used 13C-PLFA to demonstrate that

labeling duration significantly affected microbial utilization of photosynthetic carbon in ryegrass (*Lolium perenne*) rhizospheres, with Gram-positive bacteria (i15:0 and a15:0) showing reduced activity during the second labeling phase while Gram-negative bacteria (16:1$ 5*c*)*becamemoreactive*, *and*f*ungi*(18 : 2 6, 9*c*)*utilizingphotosynthetic carbonthroughoutbothphases.Environmentalconditionssuchassoilmoisture*[4] *concentration [5] also influence rhizosphere microbial utilization of photosynthetic carbon. Tian et al. [4] found that under continuous flooding, photosynthetic carbon was primarily utilized by Gram-positive bacteria in rice (*Oryza sativa*) rhizospheres, whereas under non-flooded and alternate wetting-drying conditions, Gram-negative bacteria and fungi were the main consumers. In contrast, Wu et al. [6] observed that transgenic rice with inserted cry1Ab and marker genes did not differ from non-transgenic rice in rhizosphere microbial utilization of photosynthetic carbon. Beyond gaseous 13*CO*$_2$ labeling, Kušlienė et al. [7] used Na$*{2}13CO_{3}$* solution to label white clover (*Trifolium repens*) and ryegrass leaves, revealing that different crops host distinct microbial communities that utilize photosynthetic carbon. Multiple studies have detected 13C in microbial communities shortly after labeling, indicating rapid transfer of photosynthetic carbon to soil and immediate microbial assimilation [3–7]. Collectively, these findings demonstrate that plant species, growth stage, and environmental conditions significantly alter the microbial communities utilizing photosynthetic carbon, likely due to changes in rhizodeposit composition [4–5,7].

2. Microbial Communities Involved in Priming Effects on Soil Organic Matter Decomposition

Soil organic matter (SOM) represents the largest carbon pool and profoundly influences global carbon cycling. Numerous studies have shown that adding fresh organic materials accelerates SOM decomposition, a phenomenon known as the priming effect [8]. Researchers have extensively investigated the microbial communities responsible for priming effects under various environmental conditions using different 13C-labeled organic substrates. Garcia-Pausas and Paterson [9] added 13C-labeled glucose to grassland soils and found that Gram-negative bacteria (cy17:0 and cy19:0) were the primary glucose utilizers, while fungi and actinomycetes directly induced positive priming effects. However, Bastida et al. [10] observed that Gram-negative bacteria controlled priming effects initially after glucose addition, with fungi and actinomycetes becoming dominant later. Nottingham et al. [11] used 13C-natural abundance sucrose and corn straw to identify specific Gram-negative bacteria (16:1$ 5*c*and16 : 1 $7c) as the main SOM carbon utilizers directly linked to priming effects. Blagodatskaya et al. [12] found that priming induced by 13C-labeled cellulose was driven primarily by fungi and Gram-negative bacteria within short periods (<14 days), but by fungi and Gram-positive bacteria during longer incubations (14–60 days). Rhizodeposit inputs also trigger priming effects, with Gram-positive bacteria being the main controlling group [13]. Additionally, environmental conditions [14], soil depth [15], and nutrient additions [16] affect both the magnitude of

priming effects and the underlying mechanisms that alter participating microbial communities. Synthesizing these studies reveals that organic substrate type, incubation duration, and environmental conditions significantly modify the microbial communities involved in SOM priming. The underlying mechanisms include: (1) altered microbial community structure [7–9] or enhanced microbial activity through energy provision [11,13] under certain conditions; and (2) increased production of decomposition enzymes without changes in community structure or activity under other conditions [12].

3. Methane-Oxidizing Microorganisms

Methane (CH_4) is a potent greenhouse gas with 23 times the global warming potential of CO_2 on a 100-year timescale. Atmospheric CH_4 concentrations have risen continuously over the past two centuries, and soil methanotrophic bacteria represent the sole biological sink, accounting for approximately 10% of the atmospheric methane sink. Consequently, researchers have intensively studied the microbial communities involved in methane oxidation. Boschker et al. [17] first applied 13C-PLFA to report microbial CH_4 oxidation in freshwater sediment, identifying characteristic PLFAs of Type I methanotrophs (16:1$ 9c, 16 : 1 7c, and 16 : 1 5c) enriched in 13C, conclusively linking Type I rather than Type II methanotrophs to environmental CH{4}$ oxidation. Subsequently, Nold et al. [18] confirmed these results in lake sediments and found that increased ammonium concentrations significantly reduced microbial utilization of 13CH_4. In contrast, Maxfield et al. [19] observed that CH_4-13C primarily enriched the Type II methanotroph characteristic PLFA 18:1$ 7c in grasslands soils. Mohanty et al.[20] found both Type I (16 : 1 8c and 16 : 1 5t) and Type II (18 : 1 8c) methanotrophs participated in CH{4}$ oxidation in German beech (*Fagus sylvatica*)-oak (*Quercus robur*) forest soils, with nitrogen fertilizer addition (NH_4Cl and KNO_3) stimulating Type I while inhibiting Type II methanotroph activity. Maxfield et al. [21] studied CH_4 oxidation in non-flooded arable soils at Broadbalk, revealing Type II methanotrophs dominated in fertilized plots, whereas Type I methanotrophs prevailed in manure-amended soils. These studies collectively demonstrate that both Type I and Type II methanotrophs can oxidize CH_4, with nitrogen form influencing which type dominates. However, most research has focused on high-affinity methanotrophs under conventional CH_4 concentrations. Chowdhury and Dick [22] proposed that Type II methanotrophs function under high CH_4 concentrations, while Type I methanotrophs may be more important under low CH_4 conditions.

4. Microbial Degradation of Organic Pollutants

Soil serves as a final sink for various environmental organic pollutants, making soil contamination a critical global environmental issue. Natural soil microbial populations degrade organic pollutants, representing a fundamental elimination pathway. This approach offers cost-effectiveness, complete mineralization to

CO_2 and water without secondary pollution, and high treatment efficiency, making it the most promising remediation method. Hanson et al. [23] first used 13C-PLFA to study microbial utilization of 13C-labeled toluene, finding that after 119 hours of incubation, approximately 27% of total PLFAs were labeled with toluene-derived 13C, demonstrating the technique' s applicability in complex soil environments. Mauclaire et al. [24] then employed 13C-PLFA to investigate toluene metabolism by *Pseudomonas* sp. and the bacterivorous flagellate *Vahlkampfia* sp., highlighting the technique' s unique advantage in tracing toluene-carbon through microbial food chains. Tillmann et al. [25] used 13C-labeled 2,2 -dichlorobiphenyl to study polychlorinated biphenyl (PCB) degradation, observing 13C enrichment in characteristic fatty acids of *Burkholderia* (16:0, 17:0cyclow7c, 18:1$ 9c, 19 : 0cyclo 8c)butnotinthemostabundant * Methylobacterium * fattyacid(18 : 1 7c), identifying * Burkholderia * astheprimaryaerobicPCBdegraderinthetestedsoil.Mellendorfetal.[26]foundthatPLFAsi14 : 0, 15 : 0, 18 : 0, 18 : 1 5c, andfungalPLFA18 : 2 $6,9c incorporated phenanthrene-13C, with rapeseed oil addition enhancing this enrichment and accelerating phenanthrene degradation. Researchers have also investigated microbial degradation of pentachlorophenol [27], clomazone (a herbicide) [28], and polycyclic aromatic hydrocarbons (PAHs) [29]. Notably, while microorganisms can degrade organic pollutants, these compounds are also toxic to microbes, significantly affecting microbial community activity and structure and inhibiting phospholipid biosynthesis, which complicates result interpretation [30].

5. Microbial Utilization of Exogenous Simple Carbon Sources

Glucose is a simple organic carbon source that can be directly utilized by most anaerobic and aerobic microorganisms, making it a standard substrate for studying microbial carbon utilization. Ziegler et al. [31] found that Gram-positive bacteria were the primary glucose-13C utilizers during early incubation, while actinomycetes (10me18:0) played important roles in glucose carbon recycling after glucose depletion, emphasizing that sampling time critically influences interpretation of glucose utilization. Dungait et al. [32] similarly observed that sampling timing affected which microbial groups utilized glucose-13C, with Gram-positive bacteria dominating glucose utilization throughout incubation (approximately 30%) while fungal utilization remained low (<1%). They also noted that increasing glucose concentration enhanced 13C utilization by cropland soil microbial communities, whereas glucose concentration had minimal effects on 13C utilization by Gram-positive bacteria and actinomycetes in grassland soils. Zhang et al. [33] reported that glucose-13C initially appeared primarily in bacterial PLFAs, but fungal and actinomycete utilization increased over time, with soils receiving organic fertilizer showing significantly higher 13C-PLFA contents than those receiving chemical fertilizer or no amendment. Microbial glucose utilization is also influenced by soil type [34], while grassland plant community composition does not affect microbial glucose utilization [35].

Beyond glucose, researchers have examined microbial utilization of other simple carbon sources under various conditions. Arao [36] found that acetate-13C primarily incorporated into PLFAs 18:2$ 6, 9c, 16 : 0, and18 : 1 9c, withmicrobialacetateutilizationincreasingwithsoilpH.Glycine — 13Cwasmainlyutilizedby Gram−positivebacteria(i16 : 0)inundisturbedheathsoils, andelevatedCO_{2}$ concentration reduced glycine utilization by Gram-positive bacteria (i17:0) while increasing utilization by non-specific PLFAs 16:0 and 18:0 [37]. Wang et al. [38] demonstrated that soil type significantly affected microbial utilization of urea-13C, with 13C primarily incorporating into PLFAs 16:0, 16:1$ 5c, andcy19 : 0inredsoils; 16 : 0, 16 : 1 5c, and16 : 1 7cinfluvo − aquicsoils; and16 : 0, 18 : 1 $7c, and i15:0 in paddy soils, with total PLFA-derived 13C from urea increasing with soil pH. Several studies have compared microbial utilization of different simple carbon sources simultaneously. Brant et al. [39] compared forest soil microbial utilization of glucose, glutamic acid, oxalic acid, and phenol under different management practices. Dungait et al. [40] examined microbial utilization of glycine, amino acid mixtures, and root extracts. Rinnan et al. [41] compared microbial assimilation of 13C-labeled glucose, acetate, glycine, starch, and vanillin. Gunina et al. [42] simultaneously assessed microbial community utilization of amino acids (alanine and glutamate), monosaccharides (glucose and ribose), and carboxylic acids (acetate and palmitic acid). Collectively, these studies reveal that microbial utilization of simple carbon sources is significantly affected by sampling time. Furthermore, microbial utilization of exogenous simple carbon sources depends both on the inherent soil microbial community structure, where dominant groups are primary utilizers [32–33], and on the carbon source type, as the same community may utilize different substrates with varying efficiency [39–42].

6. Microbial Utilization of Exogenous Complex Carbon Sources

Plant residues are the most common complex organic carbon sources, and their decomposition plays a major role in maintaining soil nutrient cycling, preserving SOM content, and providing carbon and energy sources for soil biota. Williams et al. [43] first applied 13C-PLFA to study microbial decomposition of 13C-labeled crimson clover (*Trifolium incarnatum*) and ryegrass (*Lolium multiflorum*) roots and shoots, finding that some PLFAs (16:1$ 5cand10me17 : 0)barelyutilizedaddedrootandshootcarbon, whileothers(16 : 0, 18 : 1 9c, and18 : 2 6, 9c)activelyincorporatedcarbonfrombothresiduesthroughoutincubation, concludingthatbothplantresidue positiveandGram−negativebacteriawerethe primaryutilizersof13Cfromhairyvetch(∗Viciadasycarpa∗)roots 13CenrichedinGram − positivebacteria(i15 : 0anda15 : 0)andGram − negativebacteria(16 : 1 7cand16 : 1 5c), withheavymetalmixtureadditionpromotingthisenrichment[45].Elfst 0, Gram−negativebacteria(16 : 1 7cand18 : 1 7c), and fungi(18 : 1 9c)werethemainconsumersofcrimsonclove KuceraandDick[47]foundthatbacteriadominatedearly−stage(< 1month)decompositionofDouglasfir(∗Pseu 5months).Patersonetal.[48]furtherdemonstratedthatwater−solublecomponentsofryegrassresidueswerepri negativebacteria, whereasfungimainlydecomposedinsolublecomponents.However, McMahonetal.[49]foundt

2 $6,9c) could utilize water-soluble, non-water-soluble, and whole ryegrass residue components, while PLFAs i15:0, 10me16:0, and cy19:0 poorly utilized any component, with fungal utilization increasing over incubation time.

Biochar, a solid product of low-temperature pyrolysis of biomass under anoxic or micro-oxic conditions, has high organic carbon content, porosity, and alkalinity, and can significantly increase soil carbon storage when applied to soil. Understanding microbial biochar utilization is therefore crucial for long-term soil carbon stabilization. Steinbeiss et al. [50] first studied microbial utilization of biochar produced from glucose and yeast via hydrothermal carbonization, finding that fungi were the primary utilizers of yeast-derived biochar, while Gram-negative bacteria dominated glucose-derived biochar utilization, attributing this difference to distinct surface properties and chemical functional groups. Watzinger et al. [51] observed that only actinomycetes (10me18:0) utilized wheat husk and willow (*Salix*) biochar in incubation studies, while Gomez et al. [52] found that soil type significantly influenced microbial biochar utilization from oak (*Quercus*), with Gram-positive bacteria, Gram-negative bacteria, and fungi being the main utilizers in haplic gleysol and gleyic phaeozem, Gram-positive bacteria, Gram-negative bacteria, and actinomycetes in haplic luvisol, and no significant utilizers in luvic phaeozem. Farrell et al. [53] reported that Gram-positive bacteria dominated wheat (*Triticum aestivum*) and eucalyptus (*Eucalyptus robusta*) biochar utilization during early decomposition, with fungal utilization increasing over time. These findings indicate that microbial utilization of complex carbon sources exhibits temporal dynamics, likely related to different microbial communities specializing in specific carbon components, with some communities not participating in decomposition of particular complex carbon sources or their components under specific conditions.

7. Other Applications

Beyond these applications, 13C-PLFA has been used in other contexts. Apostel et al. [54] employed position-specific 13C labeling of alanine and glutamate to study microbial metabolism of specific carbon atoms, revealing that carboxyl carbons were rapidly lost through oxidation while reduced carbons (C3-5) were preferentially incorporated into PLFAs. Alanine C2 carbon primarily entered Gram-negative bacterial membranes, whereas glutamate C2 carbon was rapidly lost from PLFAs, suggesting glutamate C2 incorporation into PLFAs occurs via the glyoxylate cycle or conversion to aspartate before entering the tricarboxylic acid cycle. Kramer and Gleixner [55] studied microbial decomposition of organic carbon after vegetation change from C3 to C4 plants, finding that Gram-positive bacteria primarily utilized C3-derived carbon while Gram-negative bacteria preferentially used C4-derived carbon. Streit et al. [56] investigated warming effects (+4°C) on alpine soil microbial decomposition, observing that warming decreased fungal (18:2$ $6,9c) utilization of newly formed soil organic carbon while increasing their use of older carbon.

8. Problems and Prospects

The 13C-PLFA technique has been primarily applied to carbon cycling studies in plant-soil systems, including investigations with $13CO_2$ or $13CH_4$ gases and other 13C-labeled organic substrates. Researchers have made significant advances, such as demonstrating rapid utilization of plant-fixed carbon by rhizosphere microbial communities [3–7], identifying Type I methanotrophs as the dominant CH_4 oxidizers in freshwater sediments [17–18], and showing that dominant microbial communities are the primary utilizers of simple carbon sources [32–33]. Despite providing valuable insights into biogeochemical processes, the technique has inherent limitations. First, PLFAs are imperfect biomarkers, as characteristic fatty acids have not been established for all soil organisms, and specific PLFA-microbe relationships remain uncertain. For example, PLFA 16:1$ 5*ciscommonlyusedasanarbuscularmycorrhizalfungalbiomarkerbutalsooccursinGram−negativebacteria.Second, cross−feedingpresentsacommonchallenge.Duringlong−termincubations, somemicroorganismscanutilizeothermicrobialbiomassormetabolitesassubstrates*[57], *whil*
respired by other microbes. Some studies have attempted to mitigate this by adding $12CO_2$, but ideally, sampling should occur soon after labeling to avoid cross-feeding. However, this creates practical difficulties: sampling too early may result in incomplete 13C labeling, complicating accurate measurement, while secondary heterotrophic consumption may outpace primary autotrophic assimilation. Therefore, analyzing soil samples at multiple time points is recommended to compare primary assimilation and secondary consumption rates, enabling assessment of cross-feeding risks.

Because PLFAs disappear rapidly after cell death, 13C-PLFA can identify active microbial biomass involved in substrate metabolism. Compared to DNA/RNA-based techniques, PLFA more sensitively reflects changes in soil microbial community structure. However, 13C-PLFA alone cannot provide detailed information on taxonomic composition or phylogenetic relationships. Therefore, simultaneous application of 13C-PLFA and 13C-DNA/RNA techniques offers a promising approach for comprehensively understanding soil microbial functions in ecosystem processes.

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