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Review paper

Microbial phospholipid biomarkers and stable isotope methods help reveal soil functions

Andrea Watzinger*

Health and Environment Department, Environmental Resources and Technologies, AIT Austrian Institute of Technology GmbH, Konrad-Lorenz-Strasse 24, 3430 Tulln, Austria

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ABSTRACT

This review targets microbial phospholipid biomarkers, their isotope analysis and their ability to reveal soil functions. The amount and composition of phospholipid fatty acids (PLFAs) measured in environmental samples strongly depend on the methodology. To achieve comparable results the extraction, separation and methylation method must be kept constant. PLFAs patterns are sensitive to microbial community shifts even though the taxonomic resolution of PLFAs is low. The possibility to easily link lipid biomarkers with stable isotope techniques is identified as a major advantage when addressing soil functions. Measurement of PLFA isotopic ratios is sensitive and enables detecting isotopic fractionation. The difference between the carbon isotopic ratio of single PLFAs and their substrate (Δ^{13}C) can vary between −6 and +11‰. This difference derives from the fractionation during biosynthesis and from substrate inhomogeneity. Consequently, natural abundance studies are restricted to quantifying substrate uptake of the total microbial biomass. In contrast, artificial labelling enables quantifying carbon uptake into single PLFAs, but labelling success depends on homogeneous and undisturbed label application. Current developments in microbial ecology (e.g. ^{13}C and ^{15}N proteomics) and isotope techniques (online monitoring of CO_2 isotope ratios) will likely improve soil functional interpretations in the future. ^{13}C PLFA analysis will continue to contribute because it is affordable, sensitive and allows frequent sampling combined with the use of small amounts of ^{13}C label.

1. Introduction

Soil ecologists study the interactions among soil organisms and their abiotic environment. These interactions lead to fluxes of elements and energy in the soil. Many processes resulting from these interactions are well known and play a major role in providing ecosystem services. Many soil processes can easily be monitored by targeting the compounds involved. The dynamic nature and spatial heterogeneity of soil processes are distinct features of soil ecology. Thus, each soil sample yields only a spatial and temporal snapshot of the chemical, physical and biological soil properties. Deducing and quantifying soil processes based on this snapshot remains unreliable. The recommendation is therefore to rely on methods linking fluxes and pools (e.g. Nannipieri et al., 2003). Stable isotope methods have long been used to determine pool size and fluxes. For example, the mean residence time of carbon pools using stable isotope methods can easily be calculated by simply measuring the bulk organic matter in the soil (Balesdent et al., 1987; Balesdent and Wagner, 1988). Some heterogeneity is excluded by using bulk measurements. Nonetheless, a basic understanding of microbial processes and interactions is difficult to achieve using bulk stable isotope techniques. Using stable isotope probing (SIP) of microbial compounds (PLFAs, DNA, RNA, proteins) can achieve the link between soil processes and soil organisms (Boschker and Middelburg, 2002; Evershed et al., 2006; Neufeld et al., 2007; Seifert et al., 2012). Methods which additionally resolve the spatial dimension (e.g. Raman microscopy combined with fluorescence in-situ hybridization (FISH) and multi-isotope imaging mass spectrometry (nanoSIMS)) will further improve our understanding of soil microbial ecology (Neufeld et al., 2007). The failure to link microbial ecology to ecosystem processes is commonly related to the various scales in which these systems operate (Drenovsky et al., 2008). While microbial ecologists operate on a soil aggregate scale (0.0025 m^2), ecosystem functioning is described on a landscape scale (1000 m^2). Connecting these scales is a clear future challenge.

* Tel.: +43 50550 4656.
E-mail address: andrea.watzinger@ait.ac.at.

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For a long time, soil biota was treated like a black box, and processes were monitored mainly by quantifying substrates or products. This led to the development of various standardized methods to describe microbiological activity and to estimate the total microbial biomass; e.g. substrate induced soil respiration, community-level-physiological profile BIOLOG®, and enzyme activity measurements. Early approaches to identify related species relied on culturing them. Importantly, less than 1% of soil microorganisms can be cultured, an unrepresentative subsample, which does not allow conclusions to be drawn about the active soil microbial community. The resistance to culturing also underlined the importance of interactions between soil microorganisms and the specific environment for their well-being and growth (Boon et al., 2014). This calls for studying soil processes within the natural environment, specifically for "meta-", the Greek prefix for "together", studies. The first culture-independent attempts to unravel soil microbial taxonomy focused on determining single metabolites, especially on identifying phospholipid fatty acids and ergosterol to quantify fungi (Grant and West, 1986; Tunlid et al., 1989). Today, the use of metabolites is regarded as one of four "-omic" strategies to study soil microbial communities. Besides applying metabolomics, soil microbial communities are studied using metagenomics targeting the deoxyribonucleic acid (DNA), metatranscriptomics targeting the ribonucleic acid (RNA) and metaproteomics targeting the proteins. Changes in the specificity of the biodiversity from taxonomic towards functional biodiversity and from potential towards actual functions exist when applying strategies from genomic, via transcriptomic and proteomic to metabolomic studies. Each of these methods has been combined with SIP to improve functional interpretations; PLFAs (Boschker et al., 1998), DNA (Radajewski et al., 2000), RNA (Manefield et al., 2002), and proteins (Jehmlich et al., 2008). All of these methods have their strengths and weaknesses, and some reviews have summarized them in the light of linking structure and functions (Torsvik and Øvreås, 2002; Drenovsky et al., 2008; Maron et al., 2011). The following review discusses the (1) the usefulness of phospholipid biomarkers to describe soil microbial communities and (2) the ability to link the microbial community to soil processes by measuring carbon stable isotope ratios in phospholipid fatty acids. It focuses on the methodological aspects of PLFAs and SIP analysis and interpretation, and includes an outlook for further research to improve soil functional interpretation.

2. Microbial phospholipid biomarkers in soils

2.1. Characteristics and nomenclature

The use of microbial lipids as biomarkers reflects the distinct and characteristic lipid composition of microbial families (Koga and Morii, 2005; Christie, 2011). Additionally, many microbial fatty acids (FA) are different from those of plant and animal tissues, and some are even specific for single microbial groups (Zelles, 1999). Consequently, lipids have been used for taxonomic evaluation of microorganisms. In bacteria, FAs are mainly bound to larger molecules such as phospholipids (Zelles, 1999). An ester phospholipid is characterized by a hydrophobic tail consisting of two fatty acids (FAs) with various length, saturation, ring structures and substituted groups. These two FAs are linked via an ester bound to a glycerol and hydrophilic head. Even though methods are available to determine intact phospholipids from environmental samples (Rütters et al., 2002; Sturt et al., 2004), many researchers focused on extracting and determining the FAs from the phospholipids, i.e. phospholipid fatty acids (PLFAs), and measurement by gas chromatograph (GC) (Frostegård et al., 2011).

FAs are designated by their total number of carbon atoms, the number of double bonds, their position and geometry, and side chains. According to the IUPAC nomenclature (IUPAC-IUB Commission, 1968) unsaturated FAs are counted from the carboxylic group (Δ end) to the nearest carbon bond. Nonetheless, many researchers count the carbon atoms from the terminal methyl group (ω end) as proposed by Frostegård et al. (1993). This is biologically more meaningful because the position of the double bond dictates the biosynthesis route (Ruess and Chamberlain, 2010).

2.2. Simple PLFA extraction – critical steps and common problems

The early method of lipid extraction using an organic solvent water mixture was developed by Bligh and Dyer (1959). White et al. (1979) modified the method and used it to identify the microbial community in ecological samples. This method was then further developed and optimized for applications in soil science (Frostegård et al., 1991, 1993; Zelles, 1999). Work to increase the sample throughput by omitting the lipid separation step and examining the whole cell fatty acid pattern was published but has mostly failed to convince the scientific community (Zelles, 1999; Fernandes et al., 2013). Whole cell fatty acid detection methods do not fulfil the need to exclusively detect the living microbial soil organisms and include the storage lipids of eukaryotes, shifting the FA pattern. Extended PLFA methods developed by Zelles et al. (1992) detected more PLFAs because non-ester linked fatty acids are included and pre-separation before GC/MS measurements increased resolution. The protocol, however, was apparently too laborious to be adopted for routine microbial lipid analysis. Consequently, the most frequently cited methods still rely on the extraction developed by White et al. (1979) and Frostegård et al. (1993) (Frostegård et al., 2011). The following paragraphs summarize the critical steps and common problems associated with the PLFA extraction method based on the paper of Frostegård et al. (1993).

The abundance and spatial inhomogeneity of microorganisms in the soil determine the sample amount. In natural soils, 1–3 g of soil is usually sufficient to obtain a representative microbial PLFA fingerprint or reliable PLFA content (Frostegård et al., 1991; Drenovsky et al., 2004). Sample pre-treatment (e.g. sieving) and storage/transport conditions (cooling, freezing, freeze drying, …) will influence the PFLAs analysis (Frostegård et al., 1991; Petersen and Klug, 1994; Wu et al., 2009). Some environmental samples such as compost, landfill leachate and waste material might require a larger sample size and special sample preparation such as freeze drying and hand sorting before PLFA analysis (Watzinger et al., 2008; Mellendorf et al., 2010).

PLFAs are extracted from the soil using a chloroform:methanol:buffer solution. This first step was subject to some variation in the literature. Phosphate, citrate and acetate buffer solutions, to achieve better solution of PLFAs in the organic phase, were tested (White et al., 1979; Frostegård et al., 1991; Nielsen and Petersen, 2000). Dichloromethane was used instead of chloroform (Nielsen and Petersen, 2000). Soil weight to extractant ratio and extraction time, cycle and conditions (e.g. shaking) were altered by various authors (Frostegård et al., 1991; Wu et al., 2009; Papadopoulou et al., 2011). It became apparent that changes in the extraction procedure will change the amount and pattern of extractable lipids (including PLFAs).

As already noted, PLFAs are only one fraction of extracted lipids. Separation of the different lipid fractions is frequently achieved by separation with silica solid phase extraction (SPE) columns. This step is vital. The dehydration, the conditioning of the SPE column, as well as the amount and solvents used to add, wash and elute the PLFA fraction were relevant (Frostegård et al., 1991; Fuhrmann et al.,