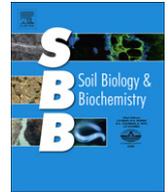




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## Citation Classics

## Use and misuse of PLFA measurements in soils

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## ABSTRACT

The determination of the phospholipid fatty acid (PLFA) pattern of soil organisms has become one of the most commonly used methods to study microbial community structure. Here we recapitulate the background of our work applying the PLFA method to soil in the early 1990s. We also stress that although the PLFA method was, and still is, a rapid and sensitive method to detect changes in the microbial community in soil, as with all popular methods it can be misused. We discuss problems in PLFA interpretation, the extent of turn-over of PLFAs in soil, and the flawed use of diversity indices to evaluate PLFA patterns.

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## 1. Introduction

The 60s and 70s was an era of large scale ecosystem analysis, where among other things the soil system was described in several large programs within the International Biological Program (IPB) or, as a follow-up program in Sweden, the Swedish Coniferous Forest Project (SWECON). One outcome of these projects was a recognition of the short-comings of the traditional plate count technique as a way of reliably describing the community composition of the soil microbes. The above projects were basically regarding the microorganisms as a black-box when attempting to apply mathematical models to the soil system. There was, however, a growing need for methods that could open up the black-box. There was also a hope that such methods would not only provide insights into the composition of soil microbial communities, but also that such knowledge would increase the predictive power of ecosystem models, thereby increasing our ability to detect malfunction of the soil system. This was often phrased that a coupling of function and structure (meaning community composition) was needed. The analysis of phospholipid fatty acids (PLFAs) was seen as one way of getting a handle on the structure of the soil microbial communities.

## 2. Background research and thinking

It is often stated that a well-cited paper is either a review or is describing a new method. Although this SBB Citation Classic originally was neither of these, it has to a large extent been cited as one of the first papers describing the use of the PLFA technique in soil. However, this was not the intention when performing this study.

It all started with some lucky coincidences: having the right people in the right place at the right time. Anders Tunlid had spent a two years post-doctoral period with David White at the Institute for Applied Microbiology, University of Tennessee, USA, before returning to Lund in the end of the 1980s. For a number of years David White had been pioneering the use of molecular markers (including PLFA) for assaying the structure and activity of microbial communities in natural environments, particularly aquatic habitats such as sediments (White, 1983). During the mid-to-late 1980s, the White laboratory was a very active and stimulating environment that attracted a large number of post-docs, students, and collaborating partners, and the laboratory housed the latest and most advanced technology for analyzing biomarkers (GC, LC, MS, MS/MS, FTIR, etc.). David was very keen on applying the lipid methods to soil systems and one of the first studies was published in 1989 (Tunlid et al., 1989). However, the use of lipid markers was met by large degree of scepticism among soil microbial ecologists. For example, we (EB and AT) remember the very critical response of a talk by David White presenting the lipid methods at the 3rd International Symposium on Microbial Ecology in East Lansing in 1983. Many microbial ecologists in the audience, in particular those

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working with soils, raised serious concerns about the methods and some were even provoked by David White's strong assertion that lipid biomarker analyses would provide most of the information needed for characterizing microbial communities in the environment – including biomass, composition, nutritional status and metabolic activity. The idea of determining the biomass of microbial communities by measuring the concentration of specific cellular components was, however, not new among soil microbiologists. For example, ATP had been used as a biochemical marker for microbial biomass since the 1970s. The requirements of a “good” biomass indicator were extensively discussed in the scientific community and were eventually defined by Jenkinson and Ladd (1981) as: (1) the measured component must be present in all parts of the soil biomass in the same (known) concentration at all times; (2) the compound is present only in living organisms and not in dead cells or in the other non-living parts of the soil organic matter; (3) it must be possible to quantitatively extract the compound from soils; and (4) there must be accurate and precise method for determining the compound in soil extracts. It was fiercely debated as to what extent the PLFAs fulfilled these criteria and if the composition of PLFA could provide information into the types and activities of microorganisms present in an environmental sample. Furthermore, the PLFA method was considered as technically very demanding, requiring advanced skills and rather expensive instruments. In addition to all this the generated data was difficult to interpret. With hindsight it is not surprising that even the *potential* of the method for answering significant questions within soil microbiology was questioned.

Erlend Bååth had been working with different aspects of soil microbial ecology, including heavy metal effects, and was also into multivariate statistical techniques (for example, describing changes in fungal community composition in polluted soils: Nordgren et al., 1983). We got tipped off that the Swedish Environmental Protection Agency was launching a project, Soil Biological Variables in Environmental Hazard Assessment (MATS), which in 1993 was followed by the research program Integrated Soil Analysis (ISA). We thought that this would be the perfect time to test the potentials of the PLFA technique in soil by comparing its sensitivity to detect toxicity effects caused by heavy metals with other common endpoints such as respiration and biomass. We submitted a grant application in which in addition to using the PLFA to detect heavy metal effects, we proposed evaluating the PLFA data using multivariate statistical techniques – which hitherto hadn't been commonly applied in soil microbiology. Although we didn't obtain funding within the MATS project, we were awarded another grant that covered the purchase of a gas chromatograph and the salary of a PhD student. Åsa Frostegård, who just had completed her master thesis, was then hired as a PhD student on the project. Thus, the setting for the work and the researchers involved were in place.

The GC took some time to arrive, and during this time ÅF worked on using the phosphate group in phospholipids as a biomass indicator: a method that had earlier been described for sediments (Findlay et al., 1989). Although the P-lipid method worked reasonably well (Frostegård et al., 1991), it was too complicated and lacking in precision compared to other biomass measurements that were commonly used at that time including Fumigation–Extraction, ATP content and Substrate-Induced-Respiration. Incidentally the P-lipid method has recently been used as a simple way of determining incorporation of P into microbial biomass using  $^{33}\text{P}-\text{PO}_4^{3-}$  (Rousk et al., 2007).

When the GC arrived we decided to focus on the PLFA method as originally planned. After setting up the equipment, some initial tests and the determination of the most common PLFAs in soil using GC–MS, we began to perform a large laboratory experiment using five different metals at several concentrations and with two

different soils, taking samples regularly over a long time (18 months). This work resulted in two other well-cited papers (Frostegård et al., 1993b, 1996). However, during the initial period of the experiment, samples were only collected and frozen with a view to future processing. We then heard about two forest areas, where the soil pH had been altered by liming (a ‘hot’ subject in Sweden at that time due to possible ongoing acidification due to atmospheric pollution causing acid rain), and took the opportunity to analyze these soils. Since all the tedious testing and determination of the different PLFAs were already done, the work was actually very rapid. The results (analyzed using Principal Component Analyses, PCA, and Partial Least Squares, PLS) clearly differentiated between soils with different pH, demonstrating the potential of the PLFA method to detect environmental effects. We wrote it up as one of the first examples of using the PLFA technique together with multivariate statistics to explore environmental effects on the soil microbiota (Frostegård et al., 1993a).

Although, in retrospect, our application of the PLFA method to soil was successful in that we could detect heavy metal and pH effects, it is interesting that we were not entirely confident that it actually would work! Within the original grant application to the Swedish Environmental Protection Agency an alternative approach to detect heavy metal effects was mentioned. This was the use of the PICT (Pollution Induced Community Tolerance) method, successfully applied later as a complement to the PLFA method to detect heavy metal effects and resulting in several well-cited papers (Díaz-Raviña et al., 1994; Díaz-Raviña and Bååth, 1996). PICT in combination with the PLFA technique was described when the MATS and ISA projects were summarized in a special issue of *Ambio* in 1998 (Bååth et al., 1998).

### 3. Use and misuse of the PLFA method

The publication of our paper in 1993 stimulated many scientists to use the PLFA method for analyzing the biomass and structure of microbial communities in soils. Indeed, these studies have shown that PLFA-based methods have several advantages, being rapid and inexpensive, and usually being both sensitive and reproducible. The number of papers using PLFA, thereby increasing our understanding of the soil ecosystem, is too numerous to review here. We only refer to the early and most recent reviews on this subject (Olsson, 1999; Zelles, 1999; Boschker and Middelburg, 2002; Ruesch and Chamberlain, 2010).

There are also limitations to the PLFA method and here we present three examples of its misuse or misinterpretation. Notably, several of the issues covered in these examples, such as the specificity of signature PLFAs and to what extent such lipids are found in living microorganisms, were already debated during the late 1980s.

#### 3.1. Indicator PLFAs

The interpretation of PLFAs as indicative of different groups of organisms or indicating physiological state of the microorganisms is not straightforward. There are two approaches to analyzing PLFA data. One relies on using the whole PLFA pattern, filtered through a multivariate statistical technique: although this can be followed by comparing individual PLFAs, this is often not the main purpose. With this way of analyzing the data the only question that is answered is: have there been changes in the community due to a specific treatment?

The second approach involves trying to elucidate the effects on specific groups of microorganisms, assuming that certain PLFAs are markers for a particular group or at least indicative of changes in that group. The presence of indicator PLFAs unique to certain taxa is inferred from pure culture studies. Many papers list the connection

between different PLFAs and different groups of organisms, but these reports do not always coincide. There are several examples where the same PLFAs are stated to indicate very different groups. For instance, the PLFAs cy17:0 and cy19:0, usually considered to be indicators of Gram-negative bacteria are also found in large amounts in some Gram-positive bacteria (Schoug et al., 2008). The PLFA 16:1 $\omega$ 5, common in arbuscular mycorrhiza (Olsson et al., 1995), and sometimes used as a marker of that group, is also found in bacteria (Nichols et al., 1986). We believe that these discrepancies indicate that caution must be taken in interpreting PLFAs, and that good marker PLFAs only exist in very specific cases (see Ruess and Chamberlain, 2010 for a recent discussion on this). Examples of such cases from our own studies are in heated peat, with alicyclic PLFAs being indicative of thermophilic *Alicyclobacillus* (Ranneklev and Bååth, 2003) or in soil for the PLFAs 16:1 $\omega$ 8 and 18:1 $\omega$ 8, indicative of methanotrophic bacteria (Sundh et al., 2000).

The PLFAs 18:2 $\omega$ 6,9 and 18:1 $\omega$ 9, both common in fungi and described as marker PLFAs for that group, are other examples of problems in associating individual PLFAs to specific microbial groups. First, these two PLFAs are not exclusive to the fungi, but common in many eukaryotic organisms, including plants. However, fungal biomass in soil is higher than root biomass, and the often high correlation of 18:2 $\omega$ 6,9 to the fungal marker ergosterol indicates that this PLFA is actually a good fungal indicator, as reported recently by Kaiser et al. (2010). The concentration of 18:1 $\omega$ 9 is usually well correlated to 18:2 $\omega$ 6,9 (Fig. 1) and, if fungal biomass increases in soil, both PLFAs should increase in parallel. However, as also seen in Fig. 1, when there are no fungi (zero-levels of 18:2 $\omega$ 6,9), there is still around 4–5% of the total PLFAs that is 18:1 $\omega$ 9, probably due to its presence in bacteria (e.g. Schoug et al., 2008). Thus, in some soils 18:1 $\omega$ 9 is a relatively good indicator of fungi (e.g. forest soils in Fig. 1) while in other soils (agricultural soils) it is a poor indicator. In the same way PLFA 16:1 $\omega$ 5 can be a good indicator of arbuscular mycorrhizal within roots, when bacterial biomass is low, but a poor one in soil with high bacterial biomass. In the latter case one has to rely on the neutral lipid fatty acid (NLFA) 16:1 $\omega$ 5 to indicate arbuscular mycorrhiza (Olsson, 1999).

Ratios of specific PLFAs have also been used to indicate stress or starvation, like trans/cis (e.g. 16:1 $\omega$ 7t/16:1 $\omega$ 7c) or cyclo/mono-unsaturated precursor (cy17:0/16:1 $\omega$ 7c and cy19:0/18:1 $\omega$ 7c). In this case it is presumed that the bacteria alter the composition of

their cell membrane in response to the environmental conditions. Although that may be correct, the change may equally be due to a shift in the species composition of the microbial community. At present we have no way of differentiating between an altered membrane composition and altered species composition as the explanation for a change in PLFA pattern. Recent work also indicates that the trans/cis ratio is not a good indicator of long-term environmental stress (Fischer et al., 2010). Another example of potential misinterpretation of PLFA ratios indicating stress is during re-growth of bacteria after a heating event which releases ample, additional supplies of carbon and nutrients. The microorganisms are, therefore, not expected to be experiencing either starvation or stress. Still, this situation is often found to be characterized by an increase in the PLFA cy17:0 (Bárceñas-Moreno and Bååth, 2009), i.e. an increased ratio cy17:0/16:1 $\omega$ 7c, which may erroneously be interpreted as stress conditions.

### 3.2. Rate of turn-over of PLFAs

The idea that PLFA patterns always change rapidly in response to altered environmental conditions should be viewed with caution. There are few reports actually measuring the turn-over of PLFAs in soil. It is, of course, very difficult to measure the turn-over of PLFA of the indigenous microorganisms in soil. Adding pure PLFA or pure cultures of organisms, or labelling soil microorganisms through addition of labelled substrate, has a limited value in indicating the real turn-over of indigenous microorganisms. PLFA quantification is probably suitable for detection of increases in biomasses after for example, the addition of substrates, and appears to be at least as sensitive as most other biomass related methods. Detection and interpretation of decreases in PLFA abundance are much more difficult since this assumes the death of the microorganisms and the subsequent degradation of their PLFAs. However, these two processes can be independent of each other. For example, a toxic substance, that kills the microorganisms, may also inhibit enzymes degrading the PLFA, resulting in a delayed decrease in PLFAs. In a study of the fate of specific PLFAs produced by thermophilic bacteria in heated peat (Ranneklev and Bååth, 2003), decreasing temperature below the minimum temperature for growth of these bacteria led to a decrease in their specific PLFAs. The decrease was, however, slower at 5 °C than at 25 °C. The reason for this is probably not that the thermophiles survived better at the lower temperature, but instead that the degradation of the dead bacteria was slower at low temperatures. Thus, environmental conditions are crucial for the turn-over rate of PLFAs, and the PLFA method will not, under all conditions, be a method indicating rapid changes.

### 3.3. Diversity indices and PLFA

Last but not least, the use of PLFA data to calculate diversity indices and then trying to interpret these are flawed approaches and should not be used. The most common way of doing these calculations is by treating each PLFA as a “species” and the size of the chromatogram peak as equivalent to the frequency of that species. One then calculates Shannon diversity (H), species richness (S) or Evenness (E) or any other diversity related index. Here we will resist inserting numerous references to those who have taken this erroneous approach. Suffice to say that there are several reasons why diversity calculations should not be based on PLFA data. First, how do we actually interpret “diversity of PLFAs”? Fungi have very few different PLFAs in their membranes and, even if there were thousands of different fungal species in a soil sample, there would be less than ten major types of fungal PLFAs. The same PLFAs could, theoretically, derive from a sample with only ten or less fungal species. For bacteria there is more variation, but some have few

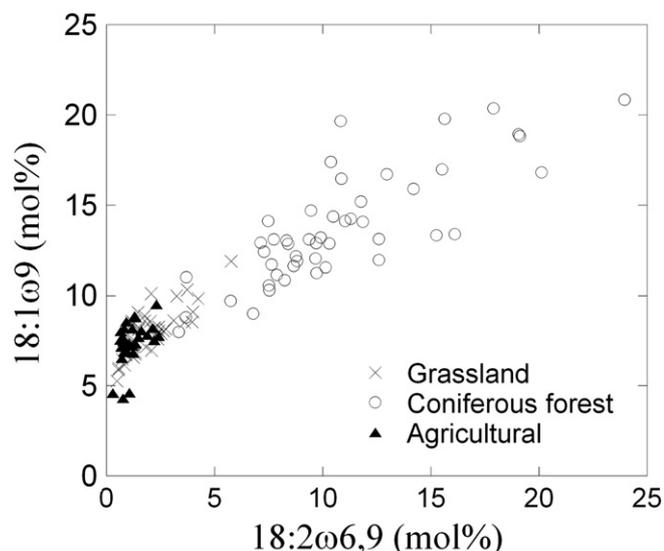


Fig. 1. The relative concentration (mol%) of the PLFAs 18:2 $\omega$ 6,9 and 18:1 $\omega$ 9 in different soils. Both PLFAs are supposed to indicate fungal biomass in soil.

## Published Items in Each Year

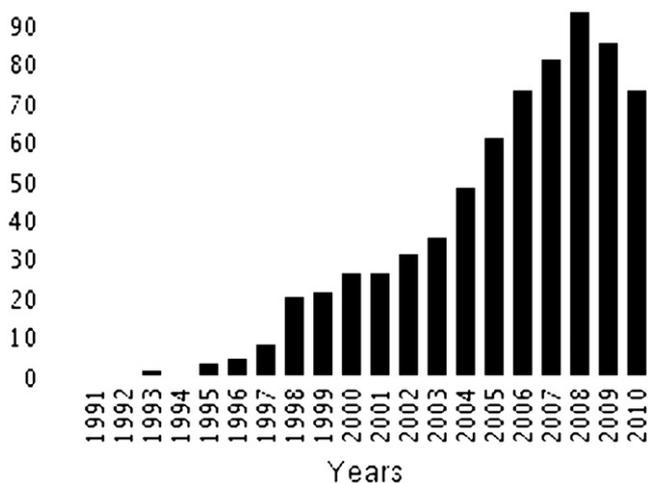


Fig. 2. Number of publications found when searching ISI Web-of-Science for "PLFA AND Soil" 2010-11-16. There was one in 1991 and three in 1993. Totally there were 708 hits.

PLFAs, or few dominant PLFAs, while other bacteria have many different PLFAs in their membrane. Are the former species less diverse than the latter ones? Of course not! Second, although diversity indices, like Shannon, are less influenced by the sample size than are estimates of species richness, they are still affected. This can easily be shown by taking a PLFA sample and injecting different amounts into the GC, where all diversity indices will be correlated to the amount injected. Thus, in many cases apparent changes in diversity reported are actually only a consequence of different amounts of PLFA in different samples. Third, one part of Shannon's index is evenness, which has high values for communities with equal amounts of each species. Thus, one would have the highest diversity when all PLFAs are found in equal amounts, which would only be the case when a standard mixture is used. Of course, the fact that a totally unnatural sample will show the highest diversity makes any such interpretation flawed. Lastly, any calculation of diversity indices uses only a small part of all the information found in the PLFA pattern and, as such, is a misuse of the large amount of information gathered with the PLFA method.

#### 4. Concluding remarks

A search on Web-of-Science for "Soil and PLFA" (708 hits) clearly shows that the PLFA method rapidly became, and still is, a very popular method when assessing soil microbial community composition (Fig. 2). In 2007–2009 more than 80 papers per year fell into this category, indicating that it is still considered a useful method. Only four hits were found for 1993 (the year of the citation classic) and earlier. It is thus clear that this SBB Citation Classic, together with other papers on the use of the PLFA technique in soil by Frostegård et al. (1991, 1993a, 1993b), has mainly been cited as a methodological paper, being one of the first to show the potential of the PLFA technique for answering important questions in soil microbiology. The paper was also cited for the actual method used to extract and analyze PLFA in soil. Although our method was based primarily on the Bligh & Dyer extraction technique (Bligh and Dyer, 1959), with the modifications used in David White's laboratory, we also introduced some of our own modifications, e.g. the use of citrate buffer instead of phosphate buffer. A key feature of our method was that it was rapid, allowing for more samples to be processed in a week than, for example, the more elaborate method

introduced by Zelles et al. (1992) at the same time although, to be fair, their method detects more PLFAs. Last, we believe that our paper exemplified the powerful use of multivariate statistical techniques in exploring and presenting the data.

In addition to the methodology, the result, that is the effect of pH on soil microorganisms, was also of value. The canonical importance of pH in determining microbial community composition in soil has recently been re-emphasized using molecular techniques (Lauber et al., 2009; Rousk et al., 2010a). Our work was actually one of the first to show the importance of pH, although we were reluctant to stress this at the time since liming of the soil that we studied was made several years before sampling and it is known that this increases the availability of soil organic matter – and thus microbial activity. This introduces a possible confounding factor that is difficult to separate from the direct pH effects. However, the main changes in PLFA pattern in response to pH reported in our 1993 paper (e.g. an increase in the abundance of 16:1 $\omega$ 5 and some other mono-unsaturated PLFAs, and a decrease in cy19:0, with increasing pH), have been found in many other soils and were recently suggested to be general indicators of direct pH effects (Rousk et al., 2010b).

Nowadays, the PLFA method for assaying the composition of microbial communities has to a large extent been replaced by techniques based on nucleic acid extraction and analysis, particularly genes coding for ribosomal RNA (rRNA). However, the PLFA- and the rRNA-based methods have different strengths and weaknesses and thus complement one another. The PLFA method is a rapid and inexpensive way of assaying the biomass and composition of microbial communities in soils, and may even be more sensitive in detecting shifts in microbial community composition when compared to nucleic acid based methods (Ramsey et al., 2006). The use of PLFA analysis is an efficient way to rapidly screen whether the fungal or bacterial part of the soil community has been affected by a treatment. The relative abundance of fungi and bacteria, often referred as the fungal/bacterial ratio, can be calculated from the amounts of PLFAs specific to these respective groups (Frostegård and Bååth, 1996) and has been used widely for comparing soils and treatments. The conversion factors established to estimate fungal and bacterial biomass-C from amounts of PLFA show good correlation with other ways of determining the biomass of these groups (Klamer and Bååth, 2004; Frostegård and Bååth, 1996) and give reliable determinations of the amounts of different carbon substrates incorporated into soil bacterial biomass (Jia et al., 2006). Although being informative in many investigations, the PLFA method cannot compete with the rRNA methods in the phylogenetic resolution by which a given community can be characterized. However, rRNA analyses provide little information on the phenotype and the activity of the microorganisms in the environment. On the other hand, the lipid biomarker methods may provide such information by analyzing membrane (PLFA) and storage (NLFA) lipids of microorganisms (Bååth, 2003). Combined with the use of isotope ( $^{13}\text{C}$  or  $^{14}\text{C}$ ) labelled substrates, the lipid methods can also be used to identify the metabolically active part of the microbial community (Boschker and Middelburg, 2002; Ruess and Chamberlain, 2010).

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