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# Methylation procedures affect PLFA results more than selected extraction parameters

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ABSTRACT

Microorganisms are key players in organic matter and nutrient cycles of terrestrial ecosystems. The analysis of microbial membrane lipids, phospholipid fatty acids (PLFAs) has strongly improved our understanding of how microbial processes contribute to these cycles. The analysis has proven to yield robust results, but adaptations of analytical parameters to laboratory needs might lead to pitfalls and impede comparability of PLFA results between different studies. Here, we show how a set of four analytical parameters (freeze-drying vs. field moist, amount of sample extracted, age of solvent mixture, and methylation methods) influence the quantitative and qualitative results of PLFA analysis. Freeze-drying vs. field moist samples and the amount of sample extracted had only minor effects on PLFA concentrations and recovery of the microbial community structure. Nevertheless, these parameters are important to consider, especially if treatment effects in an experiment are expected to be low. The use of a four weeks old extraction solution resulted in 12% lower PLFA concentrations as well as significant differences in the relative abundance of functional microbial groups. This suggests that extraction solution should be prepared on the day of extraction or that the different components of the extraction solution should be added sequentially to the sample. Most importantly, the choice of the methylation method led to differences in both, PLFA concentrations (35%) and the relative abundance of functional microbial groups, making comparisons between studies difficult. Our study provides a valuable ranking of parameters that need to be considered during PLFA method implementation in a laboratory and also highlights the fact that comparability of studies using different methylation methods might be limited.

## 1. Introduction

Microorganisms are key players in terrestrial ecosystems, contributing to the biogeochemical cycling of nutrients and often quickly responding to environmental changes because of their fast turnover rates (Fierer 2017; Willers et al. 2015a). A better understanding of the factors shaping microbial communities will improve our ability to predict how microorganisms will respond to upcoming global climate change and how we might be able to manage these organisms to provide desired ecosystem functions like soil health (Willers et al. 2015a). A widely used method that has proven to be useful for the characterization of soil microbial communities is the analysis of phospholipid fatty acids (PLFAs; Apostel et al. 2013; Bird et al. 2011; Fierer et al. 2003; Kallenbach et al. 2016). Keeping methodological limitations in mind, such as the low taxonomic resolution (Frostegård et al. 2011), the analysis of PLFAs remains a valuable tool for the assessment of microbial processes in the environment. Phospholipid fatty acids are rather fast and inexpensive to analyze and yield information on both, the microbial abundance as well as the community composition (Frostegård et al. 2011). A recent study highlights that results from the analysis of PLFAs are largely comparable, but also complementary to 16S rRNA derived results (Orwin et al. 2018). Although the strength of 16S rRNA is related to the high taxonomic resolution, PLFAs were more useful at disentangling differences at broader taxonomic scale and when related to microbial activity (Orwin et al. 2018). Furthermore, PLFAs may be stronger in capturing experimental treatment effects on the abundance and structure of microbial communities compared to PCR-based methods (Ramsey et al. 2006; Willers et al. 2015a, 2015b).

The widespread use of the PLFA analysis brings along many variations of the analytical protocol, as laboratories have different equipment, routines, and research aims (Willers et al. 2015a). The most widely used extraction method for the analysis of PLFAs is based on Bligh and Dyer (1959) with modifications either by White et al. (1979) or Frostegård et al. (1991). After the extraction from soil using a solvent

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mixture, the compounds need to be separated into the operationally-defined fractions of neutral lipids, glycolipids, and phospholipids (Vorbeck and Marinetti 1965). There have been recent suggestions for adaptations of the protocol to improve the separation (Dickson et al. 2009) but complete separation is challenging due to the similar polarity of the different compound classes (Heinzelmann et al. 2014; Warren 2019). If analyzed by capillary gas chromatography, the phospholipid fraction needs to be derivatized before quantification. Derivatization of PLFAs is frequently done by methylation to fatty acid methyl esters (FAMEs) using either acid or base catalysts, whereas other methods like silylation are used less often and might entail problems like low stability of samples (Chowdhury and Dick 2012; Willers et al. 2015a).

Adapting analytical parameters such as sample preparation, amount of sample extracted, solvent mixture or derivatization procedure to the given needs of a laboratory or study is common, but might affect the comparability of results. Previous studies highlighted that the amount of sample extracted plays a minor role for PLFA analysis, whereas drying and storage of samples can cause detectable differences in the PLFA composition (Hamer et al. 2007; Veum et al. 2019) and Wu et al. 2009 observed a decrease of PLFA concentrations (28%) after freeze-drying and storage of 1 year. The ratio of the solvents used for the extraction solution was shown to strongly affect the results of PLFA analysis, with up to 20% less PLFAs extracted depending on the ratio (Bligh and Dyer 1959; Papadopoulou et al. 2011). Finally, it is well known that the methylation method chosen for gas chromatographic analysis of PLFAs can have substantial effects on microbial profiling (Chowdhury and Dick 2012; Willers et al. 2015a). While acid-catalyzed methylation was shown to methylate up to 30% more lipids in general (Chowdhury and Dick 2012; Griffiths et al. 2010), the base-catalyzed methylation was proposed to more specifically methylate diagnostic microorganism-derived PLFAs, only (Chowdhury and Dick 2012).

These studies have contributed to a wealth of knowledge about the sensitivity of the PLFA method to single analytical parameters. Despite this knowledge, we still lack a ranking of how strongly individual parameters may affect the final results. Consequently, the question remains difficult to answer which parameters can lead to the most critical pitfalls and reduce comparability between different studies. To answer these questions, we tested the effects of sample preparation (freeze-drying vs. field-moist), different amounts of extracted soil (3 g, 5 g, 7 g), age of extraction stock solution (4 weeks vs. freshly prepared), and different methylation procedures (base vs. acid-catalyzed) on the quantitative and qualitative results of PLFA analysis. We highlight parameters with major effects on the results compared to those that only have a minor effect.

## 2. Method

### 2.1. Reagents and glassware

In terms of purity, all organic solvents used were Rotisolv®, GC Ultra Grade (Carl Roth GmbH, Germany). All other chemicals were at least per analysis (p.a.) grade. Before use, all glassware was cleaned in a dishwasher and rinsed with deionized water before being dried, followed by heating to 500 °C for 5 h for all non-volumetric glassware. Ultra-purified water (MilliQ Advantage A10, Merck KGaA, Germany) was used for all analytical steps.

### 2.2. Soil

As example for widely distributed soil types, a slightly acidic loam soil was used for all tests performed in this study (soil A; Table 1), except for the comparison to another soil (soil B; Table 1), where we used a soil with neutral pH and clay loam texture. The samples were sieved to 2 mm before conducting subsequent analyses. The soil was kept in a fridge at +4 °C until analysis or freeze-drying with a Christ Alpha 1–4 lyophilisator (Martin Christ Gefriertrocknungsanlagen, GmbH, Germany).

**Table 1**
Properties of the analyzed soils.

| Soil | pH in 0.01 M $CaCl_2$ | Organic carbon [g $kg^{-1}$] | Water content [%] | Sand [%] | Silt [%] | Clay [%] |
|---|---|---|---|---|---|---|
| A | 6.00 | 42.4 ± 1.6 | 21% | 33 | 39 | 28 |
| B | 7.03 | 76.3 ± 4.8 | -n.d. | 33 | 34 | 33 |

n.d. not determined.

### 2.3. PLFA extraction

PLFA analysis was performed following the method by Frostegård et al. (1991), based on protocols by Waldrop and Firestone (2006) and Gunina et al. (2017) with some modifications. We used 4 g of moist sample, except when testing for the parameter 'dry', where we used 4 g of freeze-dried soil and for the test of the parameter 'weight', in which we used 3 g, 5 g, and 7 g of moist sample. We analyzed three replicates of the same sample for each parameter tested, except for the sample weights, where we analyzed only one sample for each weight.

An initial extraction was performed for 2 h in sealed glass centrifuge tubes on a horizontal shaker after adding 4 mL of extraction solution (1:2:0.8 of chloroform ($CHCl_3$): methanol (MeOH): citric acid buffer (pH 4)) per g soil. 50 μg 1,2-dinonadecanoyl-sn-glycero-3-phosphocholine (PC 19:0, Avanti Polar Lipids, USA) was added to the soil/extraction solution mixture as internal standard. The extraction solution was prepared on the day of extraction, except for the test of the parameter 'old solvent' where we used extraction solution older than four weeks. The samples were then centrifuged for 10 min at 800 x *G* and the supernatant pipetted to separation funnels. Three more extractions were conducted on the extraction residues of the first step, each time adding 5 mL of the extraction solution, shaking for 30 min, centrifuging, and transferring the supernatant to the separation funnels. After combination of the extracts of the individual samples, 0.34 times the total volume of extraction solution of both $CHCl_3$ and citric acid buffer were added to the funnels and mixed for 15 min on a horizontal shaker. After phase separation overnight, the lower phases were released. The liquid-liquid extraction was performed three more times, each time adding 10 mL $CHCl_3$, shaking, and phase separation for at least 15 min before releasing the lower phases. After reducing the combined $CHCl_3$ to around 100 μL using a Multivapor (Multivapor™ P-6, Büchi Labortechnik AG, Switzerland), the samples were transferred to a column with activated silica gel (Silica 60, Honeywell Fluka, USA; activated overnight at 110 °C) for separation. The neutral, glyco- and phospholipid fractions were eluted sequentially with 5 mL $CHCl_3$ (sequentially adding five times 1 mL), 20 mL acetone (sequentially adding four times 5 mL), and 20 mL MeOH (sequentially adding four times 5 mL), respectively. After reducing the phospholipid fractions to around 100 μL, remaining water was removed over a column with anhydrous sodium sulfate ($Na_2SO_4$).

The methylation was performed as described in Wiesenberg and Gocke (2017). Briefly, the samples were dissolved in 300 μL dichloromethane ($CH_2Cl_2$), after addition of 5 μg $D_{39}C_{20}$ acid as control standard. After addition of 500 μL boron trifluoride-MeOH solution (10% *v*/v, Sigma-Aldrich, Inc., USA), the vials were placed on a heating block at 60 °C for 15 min. Once the samples cooled down to room temperature, 500 μL ultra-purified water was added. Upon centrifugation, the lower organic phases were pipetted onto anhydrous $Na_2SO_4$ and collected in an autosampler vial after filtration. $CH_2Cl_2$ was added to the derivatization vials several times (5–8 times) again until the organic phases remained colorless.

For the test of the parameter 'base-catalyzed methylation,' the methylation was performed as described by White and Ringelberg (1998). Briefly, the samples were dissolved in 0.5 mL $CHCl_3$ and 0.5 mL MeOH, after addition of 5 μg $D_{39}C_{20}$ acid as control standard. 1 mL methanolic KOH (0.2 M) was added to the samples and incubated at 37 °C for 30 min. After cooling to room temperature, 2 mL hexane

($C_6H_{14}$), 200 µL acetic acid (1 M), and 2 mL ultra-purified water were added. Upon centrifugation, the upper organic phases were transferred to clean vials. Hexane was added two subsequent times for complete sample transfer.

Quantification was performed using a gas chromatograph (GC, Agilent 7890 B, Agilent Technologies, Inc., USA) equipped with a multimode inlet (MMI) and a flame ionization detector. Peak identification was supported using 24 different fatty acid standards (Larodan, Inc., USA; Sigma Aldrich, Inc., USA; Avanti Polar Lipids, Inc., USA) and sample measurements on a GC (Agilent 6890 N, Agilent Technologies, Inc., USA) coupled to a mass spectrometer (Agilent 5973 N, Agilent Technologies, Inc., USA) and comparison to mass spectral libraries Wiley/NIST. Both GCs were equipped with a 50 m × 0.2 mm × 0.32 µm Agilent J&W DB-5MS column (Agilent Technologies, Inc., USA). The GC oven and MMI temperature programs are given in the appendices (Table A.1).

PLFAs can be grouped for a functional differentiation of the microbial community. We used the following grouping according to Willers et al. (2015b): gram positive bacteria (gram$^+$; $iC_{14:0}$, $aC_{14:0}$, $iC_{15:0}$, $aC_{15:0}$, $iC_{16:0}$, $aC_{16:0}$, $aC_{17:0}$), gram negative bacteria (gram$^-$; $C_{16:1\omega5c}$, $C_{16:1\omega7c}$, $C_{16:1\omega9c}$, $C_{18:1\omega5c}$, $C_{18:1\omega11c}$), cyclopropyl bacteria ($cyC_{17:0}$, $cyC_{19:0}$), fungi ($C_{18:2\omega6,9}$), and actinobacteria ($10MeC_{16:0}$, $10MeC_{18:0}$). To calculate the abundance of microorganisms, these diagnostic PLFAs were summed up, additionally including the saturated PLFAs (general bacterial markers, $C_{14:0}$, $C_{15:0}$, $C_{16:0}$, $C_{17:0}$, $C_{18:0}$), which are not diagnostic for a specific functional group of microorganisms, but are generally derived from bacteria and partially from plants. We calculated PLFA concentrations based on the internal standard (PC 19:0). Percent abundance was calculated based on the total area of quantified peaks.

### 2.4. Statistics

All data analysis was conducted using R version 3.6.3 (R Core Team 2020). One-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) test was calculated to compare the influence of four analytical parameters on the PLFA concentrations, as well as the relative abundance of the functional microorganism groups. Principal component analysis was calculated using the built-in R function *prcomp*. The dataset used for PCA consisted of 15 data points with 23 variables, which were centered and scaled.

## 3. Results and discussion

### 3.1. Effect of freeze-drying vs. field moist soil

Even though it is preferable to analyze samples directly after sampling to minimize analytical artifacts, in reality, samples often need to be transported and stored until further analysis (Peterson and Klug 1994). Samples which are used for PLFA analysis are generally stored field-moist in the fridge in the short-term but need to be frozen and preferably freeze-dried for long-term storage. The question arises to which extent moist and freeze-dried samples might differ.

In this study, the PLFA concentration was not different between moist and freeze-dried samples ($p = 0.9$). In moist samples the concentration was 85.2 ± 1.2 µg g$^{-1}$ dwt. Soil and in freeze-dried samples 86.0 ± 7.8 µg g$^{-1}$ dwt. soil (Fig. 1). The standard error was five times higher in the freeze-dried samples compared to the field moist samples, indicating that the sample was less homogenous and might need to be more thoroughly mixed after freeze-drying. The functional microbial groups were not significantly different between the moist and freeze-dried soils (Fig. A.1) and also showed very similar variation. Thus, higher variation in the concentration of freeze-dried samples affected all compounds similarly, not distorting the functional microbial groups.

Multivariate analysis is often used to analyze PLFA datasets, to explore changes in the microbial fingerprint based on all identified PLFAs, not summarized subsets as in the functional microbial groups

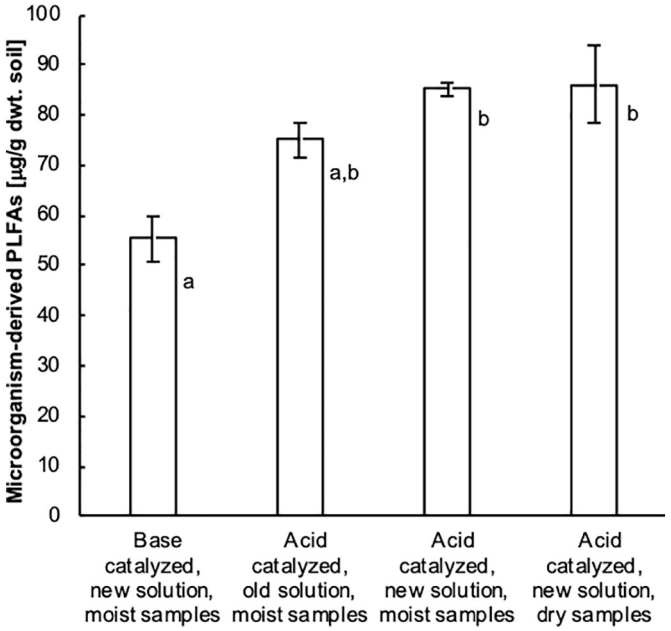


**Fig. 1.** Concentrations of microorganism-derived PLFAs [µg g$^{-1}$ dwt. soil] as affected by four analytical parameters (base catalyzed: base catalyzed methylation, acid catalyzed: acid catalyzed methylation, new solution: extraction solution prepared on the day of extraction, old solution: extraction solution 4 weeks old, moist samples: moist soil, dry samples: freeze-dried soil). Error bars indicate ±SE ($N = 3$) and different letters indicate significant differences between treatments ($p < 0.05$, ANOVA).

(Frostegård et al. 2011). We employed PCA to reduce dimensions of the dataset to those, which explain most variation. Principal component (PC) 1 explained 44.9% and PC2 18.7% of the variation. Both, moist and freeze-dried samples clustered in the same quadrant in Fig. 2a, however they were spatially separated. This highlights that freeze-drying results in a minor difference in the microbial fingerprint, although there was no difference in the concentration (Fig. 1).

Similar to our study, Wu et al. (2009) could discriminate some of their moist and freeze-dried samples after freeze-drying and storage for one year at −70 °C using PCA. Further, they observed a decrease in PLFA concentrations by 28%, which might be related to long-term storage rather than freeze-drying (Wu et al. 2009). We did not observe such an effect in our study, where samples were stored only for 2–4 months before analyses.

To conclude, we showed that the minor impact of drying on the microbial fingerprint observed in the PCA (Fig. 2a) was not reflected in any significant changes in the relative abundance of the functional microbial groups (Fig. A.1). Summarizing single PLFAs within functional groups thus seems to make the results more robust. However, especially when multivariate analysis is used, results can be influenced by freeze-drying. This is important to keep in mind, especially when treatment effects of an experimental setup are expected to be low. Consequently, one should be cautious if PLFA results are compared between sample sets that were dried differently. As long as all samples within a study are treated identically, the error can be expected to have a similar effect on all samples, which ensures their comparability. Thus, freeze-drying of samples provides a good option, if storage over longer periods of time is needed or cold conditions can not be ensured.

### 3.2. Effect of the age of extraction solution

Preparation of larger volumes of solution mixtures as a stock can be beneficial for throughput in a laboratory. If stock solutions are stored, the shelf half-life of these mixtures needs to be known to avoid impacts on the results. This applies to the extraction solution used for PLFA

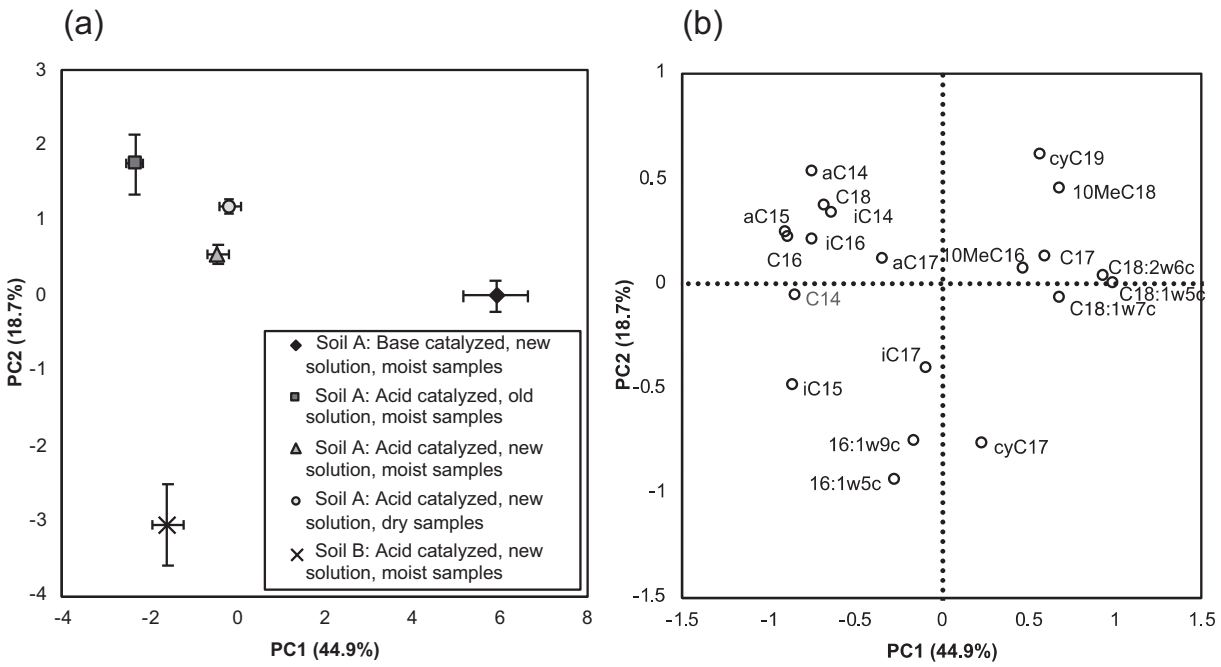


**Fig. 2.** Principal component analysis conducted on the relative abundances of microorganism-derived PLFAs, changing different analytical parameters (base catalyzed: base catalyzed methylation, acid catalyzed: acid catalyzed methylation, new solution: extraction solution prepared on the day of extraction, old solution: extraction solution 4 weeks old, moist samples: moist soil, dry samples: freeze dried soil) and two soil types (Soil A and Soil B). (a) Score plot showing the different parameters and (b) the corresponding loading plot of individual PLFAs. PC1 and PC2 explained 44.9% and 18.7% of the variance, respectively. The scores do not have a unit and error bars indicate ±SE ($N = 3$).

analysis, where some laboratories prefer to prepare solution stocks, while others prepare fresh extraction solution for every batch of samples or add different components of the extraction solution sequentially to the sample. We traced the effect of readily prepared extraction solution for PLFA analysis vs. a mixed solution of an age of 4 weeks.

The PLFA concentration extracted with a new solution was 85.2 ± 1.2 μg $g^{-1}$ dwt. soil and 75.2 ± 3.5 μg $g^{-1}$ dwt. soil with an old solution (Fig. 1). This trend to lower PLFA concentrations with the old extraction solution as compared to the new solution was not significant ($p = 0.2$). On the other hand, the relative abundance of several groups of diagnostic PLFAs was significantly affected, where gram$^-$ bacteria accounted for 33.1 ± 1.0% relative abundance with the new solution and 26.5 ± 1.3% with the old solution, actinobacteria 7.8 ± 0.4% and 4.1 ± 0.2%, and the general bacterial markers 22.3 ± 1.4% and 33.4 ± 2.7%, respectively ($p < 0.05$ for all; Fig. A.1). Since the PCA is based on the relative abundance of all PLFAs, samples extracted with the new solution were clearly separated from those samples extracted with the old solution (Fig. 2a). Even if there was no significant effect on the total amount of PLFAs extracted, the extraction efficiency was different for individual compounds, which affected the relative abundance. The lower abundance of gram$^-$ bacteria indicates that less monounsaturated PLFAs were extracted with the old solution, whereas more straight-chain saturated PLFAs were extracted with the old solution, which we grouped as general bacterial markers (Table A.2). This is also reflected in the loading plot of the PCA (Fig. 2b), where the samples extracted with the old solution plot more to the left, which positively correlates with most straight-chain PLFAs, but negatively with the monounsaturated $C_{18}$ PLFAs.

We have two hypotheses, why we observed these differences. First, the composition of the extraction solution might have changed during storage due to the evaporation of volatile constituents (e.g., $CHCl_3$), shifting the extraction solution mixture to a less ideal ratio. In the initial characterization of the method Bligh and Dyer (1959) demonstrated that the mixture of the different solvents changed the extraction efficiency for total lipids. For example a shift from the extraction solution ratio 1:2:0.8 ($CHCl_3$:MeOH:$H_2O$) to a ratio of 0.5:2:0.8 ($CHCl_3$:MeOH:$H_2O$) yielded 20% less total lipids (Bligh and Dyer 1959). We hypothesize that already smaller shifts in the solution ratio can have an impact on the extraction efficiency and might discriminate between PLFA compounds. Second, decreasing stability of the solvents in the mixture, especially $CHCl_3$, could cause a lower extraction efficiency. For example, the use of 2-Methyl-2-butene instead of ethanol as stabilizer of $CHCl_3$ reduced the extraction efficiency of PLFAs by up to 65% and especially monounsaturated fatty acids were less efficiently extracted (Fuhrmann et al. 2009), which is in line with our observations. Thus, mixing $CHCl_3$ with the other solvents and storing the mixture as a stock solution might inhibit proper stabilization of e.g. $CHCl_3$, causing degradation to phosgene, chlorine, and hydrogen chloride (Maudens et al. 2007). Inhibition of stabilization can cause unpreceded reactions between the solvent constituents, leading to lower extraction yields or discrimination between different organic substances.

We highlight the importance of preparing fresh extraction solution or even sequentially adding all solvent constituents directly to the sample. If a batch of samples is extracted with the solution being prepared for another batch a few weeks ago, results might be biased by a changing composition of extracted PLFAs.

### 3.3. *Effect of sample amount*

Depending on the experimental design, the amount of sample material available for PLFA analyses can be limited. Down-sizing samples might result in high variability of results as a consequence of sample heterogeneity. We investigated the reproducibility of the analysis in the typical range of available sample material for samples obtained from, e.g. soil coring or soil pit sampling.

The amount of PLFAs extracted from 3 g, 5 g, or 7 g of soil increased proportionally from 250.9 μg, to 437.7 μg and 590.3 μg, respectively. The average concentration was 85.2 ± 1.2 μg $g^{-1}$ dwt. soil (Fig. A.2). Similarly, the relative abundance of functional microbial groups was not affected by changing soil weights and averaged at 21.4 ± 0.6% for

gram$^+$ bacteria, 33.1 ± 1.0% for gram$^-$ bacteria, 11.6 ± 0.4% for fungi, 7.8 ± 0.4% for actinobacteria, 3.7 ± 0.1% for cyclopropyl bacteria, and 22.3 ± 1.4% general bacterial markers (Fig. A.3).

Wu et al. (2009) also report that the amount of sample extracted does not strongly affect the microbial community composition and concentration in the soils they analyzed. Nevertheless, they observed that the concentration of PLFAs was markedly lower in one of their soil types when they only extracted 1 g and that with decreasing amount of sample extracted, fewer PLFAs could be identified (Wu et al. 2009).

In conclusion our results show that if the ratio between soil and extraction solution is kept constant, the amount of PLFAs extracted per g soil is not affected by a change in the amount of sample extracted in the range of soil weights tested here. Nonetheless, we recommend to test this parameter, especially if one wants to work with very low sample weights or if PLFA concentrations are low as indicated by Wu et al. (2009). Otherwise, there might be the risk that certain biomarkers can end up being below the detection limit.

### 3.4. Effect of the methylation method

Methylation of PLFAs to FAMEs can be conducted using base or acid catalysts (Chowdhury and Dick 2012). Both methods have advantages and disadvantages in terms of availability, speed, working temperature, or disposal (Willers et al. 2015a). But more importantly, the choice of method can have strong impacts on concentrations and composition of FAMEs (Willers et al. 2015a). We investigated whether the choice of base vs. acid methylation method might impede comparability of results of the PLFA analysis.

The concentration of PLFAs was reproducible within each methylation method but was 35% lower with the base-catalyzed (55.3 ± 4.5 μg g$^{-1}$ dwt. soil) compared to the acid-catalyzed method (85.2 ± 1.2 μg g$^{-1}$ dwt. soil; $p < 0.05$; Fig. 1). Furthermore, the relative abundance of all microbial functional groups was significantly different between the two methylation methods ($p < 0.05$, Fig. A.1), except for the cyclopropyl bacteria. While the general bacterial markers and gram$^+$ bacteria were relatively more abundant with the acid catalyzed method, actinobacteria, fungi and gram$^-$ bacteria were more abundant with the base catalyzed method. Differences in the functional microbial groups are reflected in the score plot of the PCA where the results of the two methods did not plot in the same quadrant (Fig. 2a). They were separated along PC1, and the distance between the methylation methods was similar to the distance to a different soil (Soil B, Fig. 2a). This distance indicates that the difference of the microbial fingerprint of the same sample caused by the choice of the methylation method was of a similar magnitude as when extracting a different soil sample. However, the distance has to be interpreted with care, as the variation explained by the two axes is different, as well as the scale. The loading plot of the PCA indicates that mainly the monounsaturated $C_{18}$, the mid-chain-branched, as well as straight-chain saturated and some terminally branched PLFAs separated samples methylated with base- or acid-catalysts along PC1 (Fig. 2b). Total concentrations indicated that all compounds except cyclopropyl saturated PLFAs were more efficiently extracted with the acid-catalyzed method, with the largest differences in straight-chain saturated and terminally branched PLFAs (Table A.2).

Chowdhury and Dick (2012) also found higher total yields using an acid-catalyzed compared to a base-catalyzed method, despite using a variation of the acid-catalyzed method employed in this study. They suggest two reasons for the differences. The first reason is the hydrolysis of PLFAs to free fatty acids instead of FAMEs in the base-catalyzed methylation, thus resulting in a loss of these compounds (Chowdhury and Dick 2012). The second reason Chowdhury and Dick (2012) mention is that the polar lipid fraction might be contaminated by free fatty acids due to incomplete separation on the silica gel column. Since free fatty acids are not methylated efficiently with the base-catalyzed methylation (Griffiths et al. 2010) they would remain unnoticed with this method but would contribute to the FAMEs with the acid-catalyzed method which fully methylates free fatty acids. Incomplete separation due to very similar polarity of glycol- and phospholipids has already been reported in the early studies on PLFA analysis (Vorbeck and Marinetti 1965) and has been repeatedly confirmed more recently (Dickson et al. 2009; Heinzelmann et al. 2014; Warren, 2019). Thus, we would like to call to mind that the fractions are merely operationally defined and that both, acid- and base-catalyzed methods likely contain additional compounds than only phospholipids. Chowdhury and Dick (2012) further recommended the use of base-catalyzed methods, as they could not find mid-chain-branched PLFAs using their acid-catalyzed method. This was clearly not the case with the acid-catalyzed method in our study, where we recovered even slightly higher concentrations of mid-chain-branched PLFAs with the acid- as compared to the base-catalyzed method (Table A.2).

To summarize, we showed that results obtained with different methylation methods are not entirely comparable. With the above-mentioned knowledge gaps, it is premature to select a preferred methylation. Furthermore, we might need to rethink whether the methanolic fraction should really be called phospholipids as mentioned by Warren (2019).

### 3.5. Ranking the tested extraction parameters and conclusion

As we discussed in this study, the effect of single extraction parameters on the results of PLFA analysis can vary strongly. Therefore, ranking the parameters according to their effect on the composition and concentration of PLFAs will be even more helpful to users of the method, which we try for the first time. However, one has to be aware that not all parameters could be tested and that varying other parameters might have even stronger effects than the ones described here.

According to both, the PCA (Fig. 2a) and the overall concentrations of PLFAs (Fig. 1), the choice of methylation method was clearly the most important factor tested in this study. With the acid-catalyzed method, the concentration of PLFAs was 35% higher and the microbial fingerprints of replicates of the same sample, but analyzed after base- vs. acid-catalyzed methylation could be as different as different soil samples (Fig. 2a). This aligns well with previous findings, where acid catalyzed methylation was reported to be more efficient by up to 30% (Griffiths et al. 2010) and the composition of PLFAs was strongly altered (Chowdhury and Dick 2012).

The second strongest effect on the results was caused by the age of the extraction solution. After storage of a stock solution for only four weeks, the microbial fingerprint of samples extracted with this solution were significantly different compared to samples extracted with fresh solution (Fig. A.1). We hypothesize this might be related to changes in the solution ratio or stabilization. These parameters have been shown to strongly affect results of PLFA analysis, for example decreasing yields by up to 65% when choosing the wrong stabilizer (Fuhrmann et al. 2009). The simple solution to avoid a storage effect is the preparation of fresh solutions and not relying on stock solutions that were stored for several weeks.

A lower effect as compared to the methylation procedure and the storage issue is the sample preparation and here especially the freeze-drying. As it is sometimes unavoidable to store and transport samples without any reliable cooling options, it seems comforting that freeze-drying did not significantly affect the concentration, nor the composition of the functional microbial groups. Nevertheless, the PCA did reveal that fresh samples were distinct from freeze-dried samples. As it is common to always treat samples identically within an individual study, the comparability of results within one study should entail always the same systematical error related to drying, which does not hamper the comparability of results. However, long-term storage might cause significant loss of PLFA recovery by up to 28%, as observed by Wu et al. (2009).

Finally, sample weights in the tested range (3–7 g) did not affect the amount, nor the composition of the functional microbial groups.

However, Wu et al. (2009) did observe that certain compounds were below the detection limit at very low sample amounts. It is important to assess the ideal sample amount for individual studies.

We are aware that other parameters, such as sample storage or separation procedure are missing in this ranking and should also be carefully evaluated. Phospholipid fatty acids are often used in experiments to discriminate between treatment effects. As the replication was good when extraction parameters were kept constant, the analysis of PLFAs remains a valuable tool to assess the microbial response to various treatments. However, it is crucial to keep analytical parameters constant to capture subtle experimental treatment effects, and comparability between studies is limited, especially if different methylation methods are used. With the presented ranking of the impact of different extraction parameters on PLFA results, future PLFA users can better estimate the impact of single extraction parameters on the analytical results and also better judge PLFA results observed in different studies.

## Author contributions

Cyrill U. Zosso: Investigation; Formal analysis; Validation; Visualization; Writing - original draft. Guido L.B. Wiesenberg: Conceptualization; Investigation; Methodology; Project administration; Resources; Writing - review & editing.

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## Declaration of Competing Interest

The authors have no conflict of interest to declare.

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## Appendix A. Appendices

**Table A.1**
GC oven and multimode inlet temperature programs.

| GC oven temperature program | | | Multimode inlet temperature program | | |
|---|---|---|---|---|---|
| Rate [°C min$^{-1}$] | Temperature [°C] | Hold time [min] | Rate [°C min$^{-1}$] | Temperature [°C] | Hold time [min] |
| – | 50 | 4 | – | 60 | 0.5 |
| 10 | 150 | 0 | 850 | 400 | 5 |
| 2 | 160 | 0 | 50 | 250 | – |
| 0.5 | 170 | 10 | | | |
| 0.2 | 175 | 10 | | | |
| 0.2 | 180 | 10 | | | |
| 0.2 | 185 | 5 | | | |
| 0.2 | 190 | 5 | | | |
| 2 | 210 | 0 | | | |
| 5 | 320 | 15 | | | |

**Table A.2**
Concentrations of specific groups of microorganism-derived PLFAs [μg g$^{-1}$ dwt. soil] as affected by four analytical parameters (base catalyzed: base catalyzed methylation, acid catalyzed: acid catalyzed methylation, new solution: extraction solution prepared on the day of extraction, old solution: extraction solution 4 weeks old, moist samples: moist soil, dry samples: freeze-dried soil; mean ± SE, $n = 3$).

| PLFA biomarker | Base catalyzed, new solution, moist samples | Acid catalyzed, old solution, moist samples | Acid catalyzed, new solution, moist samples | Acid catalyzed, new solution, dry samples |
|---|---|---|---|---|
| Terminally branched (iso) | 2.5 ± 0.4 | 7.4 ± 0.5 | 7.8 ± 0.4 | 7.3 ± 0.6 |
| Terminally branched (anteiso) | 2.6 ± 0.7 | 7.8 ± 0.4 | 8.5 ± 0.3 | 8.2 ± 0.7 |
| Mid-chain-branched | 4.3 ± 0.4 | 2.7 ± 0.2 | 6.0 ± 0.3 | 6.6 ± 0.9 |
| Cyclopropyl | 2.2 ± 0.2 | 2.6 ± 0.1 | 2.8 ± 0.1 | 2.4 ± 0.2 |
| Unsaturated | 36.6 ± 2.0 | 32.2 ± 1.9 | 43.2 ± 0.5 | 41.8 ± 3.3 |
| Straight-chain saturated | 7.1 ± 0.9 | 22.4 ± 2.1 | 17.0 ± 1.2 | 19.7 ± 2.9 |

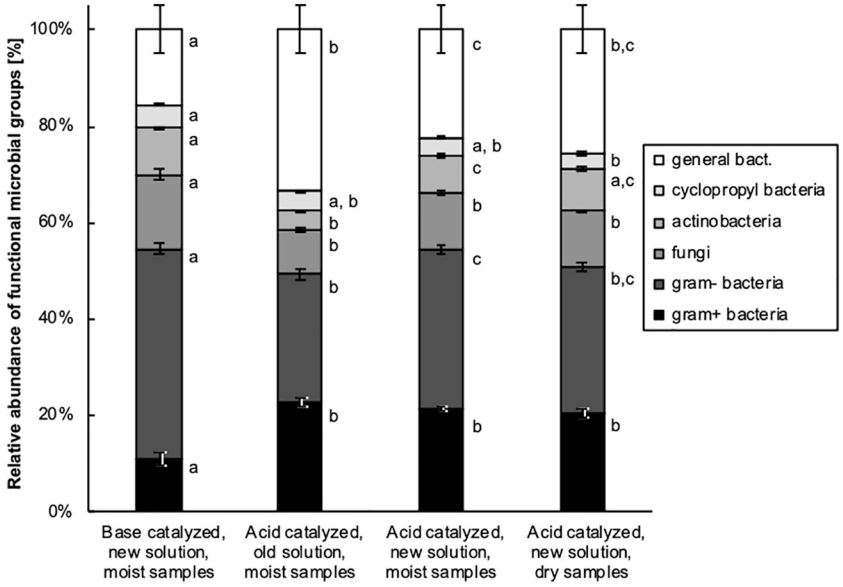


**Fig. A.1.** The relative abundance of functional microbial groups based on PLFA analysis as affected by different analytical parameters (base catalyzed: base catalyzed methylation, acid catalyzed: acid catalyzed methylation, new solution: extraction solution prepared on the day of extraction, old solution: extraction solution 4 weeks old, moist samples: moist soil, dry samples: freeze dried soil). Error bars indicate ±SE ($N = 3$) and different letters indicate significant differences between treatments ($p < 0.05$, ANOVA).

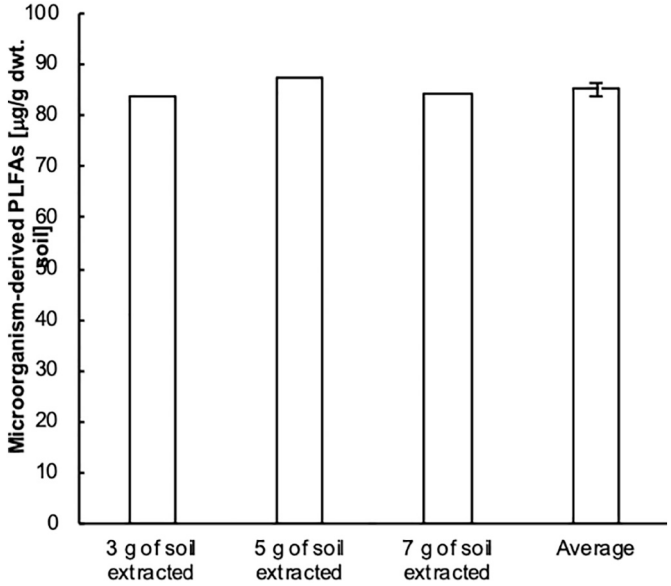


**Fig. A.2.** Concentrations of microorganism-derived PLFAs [µg $g^{-1}$ dwt. soil] as affected by different amounts of extracted soil (3 g, 5 g and 7 g; $N = 1$). The last column represents the average of all different treatments with the error bar indicating ±SE ($N = 3$).

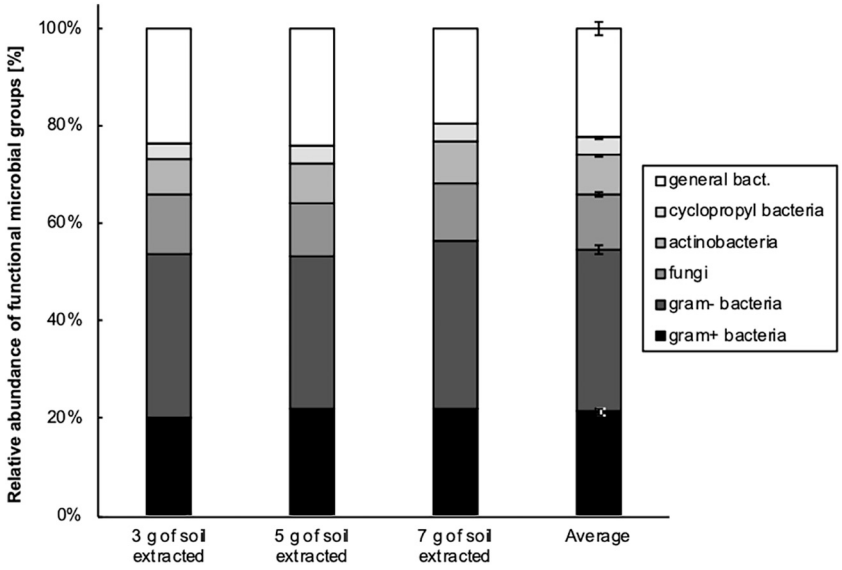


**Fig. A.3.** The relative abundance of functional microbial groups based on PLFA analysis as affected by different amounts of extracted soil (3 g, 5 g, 7 g). The last column represents the average of all different treatments with the error bar indicating ±SE ($N = 3$).

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