UNIVERSITY OF TARTU

FACULTY OF SCIENCE AND TECHNOLOGY

Institute of chemistry

EFFECT OF SOIL SAMPLE PREPARATION ON FATTY ACID BIOMARKER CONTENT

Master's Thesis (30 ECTS)

Applied Measurement Science

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TARTU 2021

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List of Abbreviations

AD	Hot Air Oven
AM	Arbuscular mycorrhizal fungi
ARDRA	Amplified ribosomal deoxyribonucleic acid restriction analysis
Conc.	Concentrations
CLPP	Community-level physiological profiles
DNA	Deoxyribonucleic acid
EL-FAME	Ester-linked fatty acid methyl ester
FAME	Fatty acid Methyl Esters
FID	Flame ionization detector
FL	Freezing and Lyophilization
GC	Gas-chromatography
GC-MS	Gas chromatography Mass spectrometry
HPLC	High-Performance liquid chromatography
M	Molar
IS	Internal Standard
MIDI	Microbial Identification method
Min	Minutes
NLFA	Neutral lipid fatty acid
PCB	Polychlorinated biphenyls
PCR	Polymerase chain reaction
PLFA	Phospholipid fatty acid
RISA	Ribosomal intergenic spacer analysis
RTT	Relative Retention Time

SEs	Steryl esters
T-RFLP	Terminal restriction fragment length polymorphism

1 INTRODUCTION

The most important component of nature is soil, where millions of living organisms remain that can be visible or invisible by our naked eyes. Bacteria and fungi are an essential part of this soil ecosystem for the sustenance of the lifecycle. They regulate the supply of nutrients required by plants, such as carbon, phosphorus, and nitrogen. They convert the organic matter into forms that can be uptake by plants. In addition to that, they improve soil water holding capacity, which benefits plants and improves soil quality. The species of bacteria and fungi found in soil affect conditions and nutrients present in the soil, influencing plant species in soil.

Excessive use of chemical pesticides, insecticides kill targeted microorganisms as well as beneficial bacteria and fungi. Moreover, agricultural practices and human interferences change soil properties, resulting in the rise of unfavorable conditions for the growth of specific plants. This whole ecosystem is interdependent on one another, a slight change in these conditions impacts the whole food-chain system and disturbs the balance of an ecosystem. Therefore, it is crucial to characterize the prevalence of microbial communities in the soil to understand environmental changes.

Methods for characterizing soil microbial communities have been evolving at an increasing pace, from culturing techniques to emphasizing on analysis of lipids (ester-linked fatty acid methyl ester and phospholipid analysis) extracted from living beings or various Deoxyribonucleic acid (DNA) molecular techniques. Most focus has been given to method development, but the conditions crucial for soil preparation after sampling have been neglected. It could be possible that based on pre-treatments of soil, the sample might yield different results. The type of habitat of soil sampling delayed in pre-treatments might influence concentrations of fatty acid biomarkers in soil. Therefore, depending on the focus of a specific study, it is essential to determine the effect that sample pre-treatment and storage can have on the result. Neglecting any influence might lead to false results.

2 LITERATURE OVERVIEW

2.1 Background

Microorganisms present in soil are diverse and essential for the sustainability of plants [1]. They act as modulators for exchanging nutrients from the soil to plants and maintain the life cycle in the ecosystem [2]. They comprise fatty acids used as biomarkers for assessing the microbial communities present in soil [2]. The difference in the prevalence of fatty acids depicts the overall change in the microbial community in soil [3].

Phospholipids and Neutral lipids are essential microbial lipids containing fatty acids [4,5]. Phospholipids are amphiphilic in nature, with two fatty acid chains attached to two hydroxyl groups of glycerol (hydrophobic tail) and one hydroxyl group of glycerol linked to a phosphate group (hydrophilic head), as shown in Figure 1[6].

Neutral lipids such as triacylglycerols (TAGs), steryl esters (SEs), and wax esters (WEs) are stored in the form of energy [7]. They consist of glycerol molecules boned to carbon chains (Figure 2) with no charged group [8].

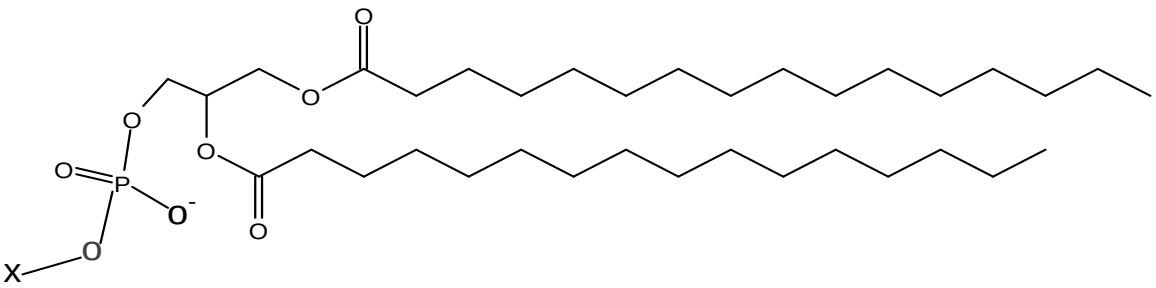


Figure 1. General Structure of phospholipids

X substituents- Hydrogen, ethanolamine, choline, serine, glycerol, phosphatidylglycerol, inositol

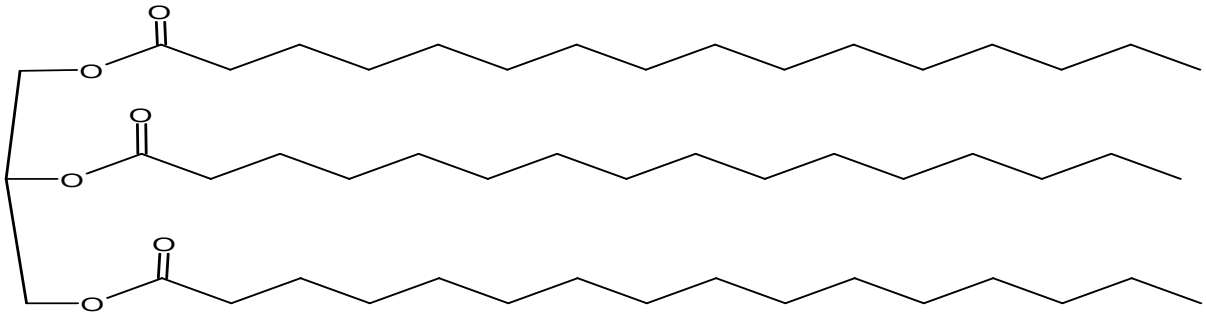


Figure 2. General Structure of neutral lipids (Triacylglycerol).

2.2 Techniques on accessing microbial communities

There are different approaches to accessing soil microbial communities. They are molecular biological, biochemical, and microbial methods.

In the molecular biological method, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) are separated from microbial cells, duplicated, sequenced using polymerase chain reaction (PCR) [9]. PCR produces a large number of targeted microbial DNA copies allowing to analyse sample if present in miniscule amount. Other molecular biological method includes denaturing gradient gel electrophoresis (DGGE), amplified ribosomal DNA restriction analysis (ARDRA), ribosomal intergenic spacer analysis (RISA), terminal restriction fragment length polymorphism (T-RFLP) [9].

Microbiological methods use cell counting, colony counting, cell culture, staining techniques to analyse microorganisms in the soil. These methods include community-level physiological profiles (CLPP) and cell counting techniques [9].

Biochemical methods include techniques such as lipid analyses and DNA composition kinetic and metabolic assays for characterizing microbial communities [9]. Lipid analysis was commonly preferred over other methods before developing molecular methods as they are free from bias arising from culturing techniques [10].

Phospholipid fatty acid analysis (PLFA) is one of the most widely used and popular among these methods. Method of PLFA analysis has been developed based on the procedure developed by Bligh and Dyer in 1959 for analysis of lipid from frozen fish [11]. White further developed this method in 1983 [12]. Many studies have been done to assign PLFA and Neutral Lipid Fatty acid (NLFA) biomarkers to specific microbial classes, but the research relating the influence of pre-treatment on both PLFA and NLFA biomarkers is still insufficient. Therefore, commonly samples are advised to be analysed as soon as possible or immediately lyophilized, followed by freeze storage [12].

The method of PLFA analysis is well-established to characterize the microbial communities in diverse environments, including agricultural soils, grasslands, the aquatic environment, and other systems [13]. Moreover, the results obtained from PLFA analysis are linked to living cells because phospholipids degrade rapidly after cell death [13]. Besides PLFA, Microbial Identification Method (MIDI) and EL-FAME (Ester-linked fatty acid methyl ester) have been devised to make methods simpler and easier [3,14]. However, in these methods, fatty acids are extracted in soil without separating neutral lipids, phospholipids, and glycolipids. Therefore, there are high chances that concentrations of fatty acids obtained from MIDI and EL-FAME are higher than PLFA [13,14].

EL-FAME and PLFA results were comparable in bacteria as a study conducted by Miura *et al*. [15]. However, in contrast to that, the study suggests analysing fungi by the PLFA method as the other methods seem to be affected by conditions such as the biological status of fungi or soil constituents in the EL-FAME method [15].

Similarly, a study by Fernandes *et al*.[3] suggested PLFA over the MIDI method, as in MIDI, extracted fatty acids belong to plants than microorganisms [3]. Therefore, PLFA is still considered the better option to carry out the quantitative estimation of bacterial and fungal biomarkers. Table 1 shows the strengths and weaknesses of different techniques for characterizing microbial communities.

Table 1. Comparison of different techniques for characterizing microbial community [9,10,16–18]

Techniques for characterizing microbial community		
	Strength	**Weakness**
Molecular biological	High specificity. Reliable, reproducible, rapid. Detects structural change in the microbial community. Significant numbers of samples can be analysed at once. Compare differences in the microbial community.	PCR biases. Expensive. Limited to a part of the microbial community. Requires knowledge of target gene sequence beforehand and high skill.
Biochemical	Sensitive detection and accuracy. Quantification of different microbial groups. Inexpensive, reproducible.	Cannot identify individual species. Time-consuming.
Microbiological	Inexpensive. Generates a large amount of data.	Favourable for fast-growing organisms. Time-consuming.

2.3 Bacterial and fungal biomarkers

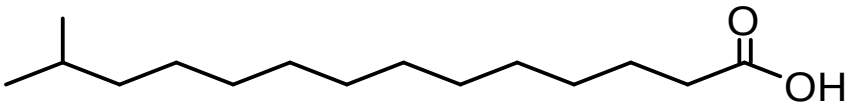
Biomarkers are molecules specific to individual species or microbial communities present in constant quantity related to living organisms [19]. Some of the examples of common fatty acid signatures are presented in Table 2. The chemical structure of fatty acid biomarkers focused on

this study is depicted in Figures 3 and 4. Fatty acids symbol represents a number of carbon atoms, the number of double bonds, the double bond position from the methyl (ω) end of the molecule. The prefixes and suffixes ‘a’ and ‘i’ refer to anteiso and iso branching. Me and OH represent methyl groups and hydroxyl groups, respectively. The symbol cy represents cyclo-propyl saturated fatty acids [20,21]. In this study, bacterial biomass was characterized by eight fatty acid biomarkers (i15:0, a15:0, i16:0, a17:0, 17:0, 10Me17:0, 10Me18:0, cy19:0) [22], total fungal biomass by 18:2ω6,9 [5] and Arbuscular Mycorrhizal Fungi (AM) biomass by group-specific NLFA (16:1ω5) [23].

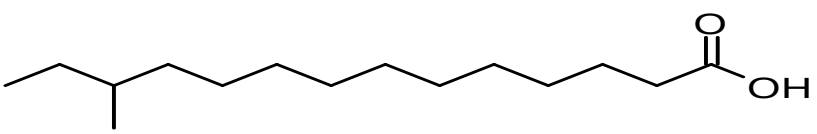
Table 2. Common fatty acid signatures [22,24]

Common bacterial signatures	i17:0, a17:0,17:0, 15:0, i15:0, a15:0, 16:0,
Aerobes	16:1ω7,16:1ω7t, 18:1ω7t
Anaerobes	cy17:0, cy17:0
Sulfate-reducing bacteria	10Me16:0, i17:1ω7
Methane oxidizing bacteria	16:1ω8c,16:1ω8t,16:1ω5c
Cyanobacteria	18:1ω7c
Fungi	18:2ω6,9, 18:3ω3,6,9
Actinobacteria	10Me18:0,10Me17:0
AMF	16:1ω5

i15:0



a15:0



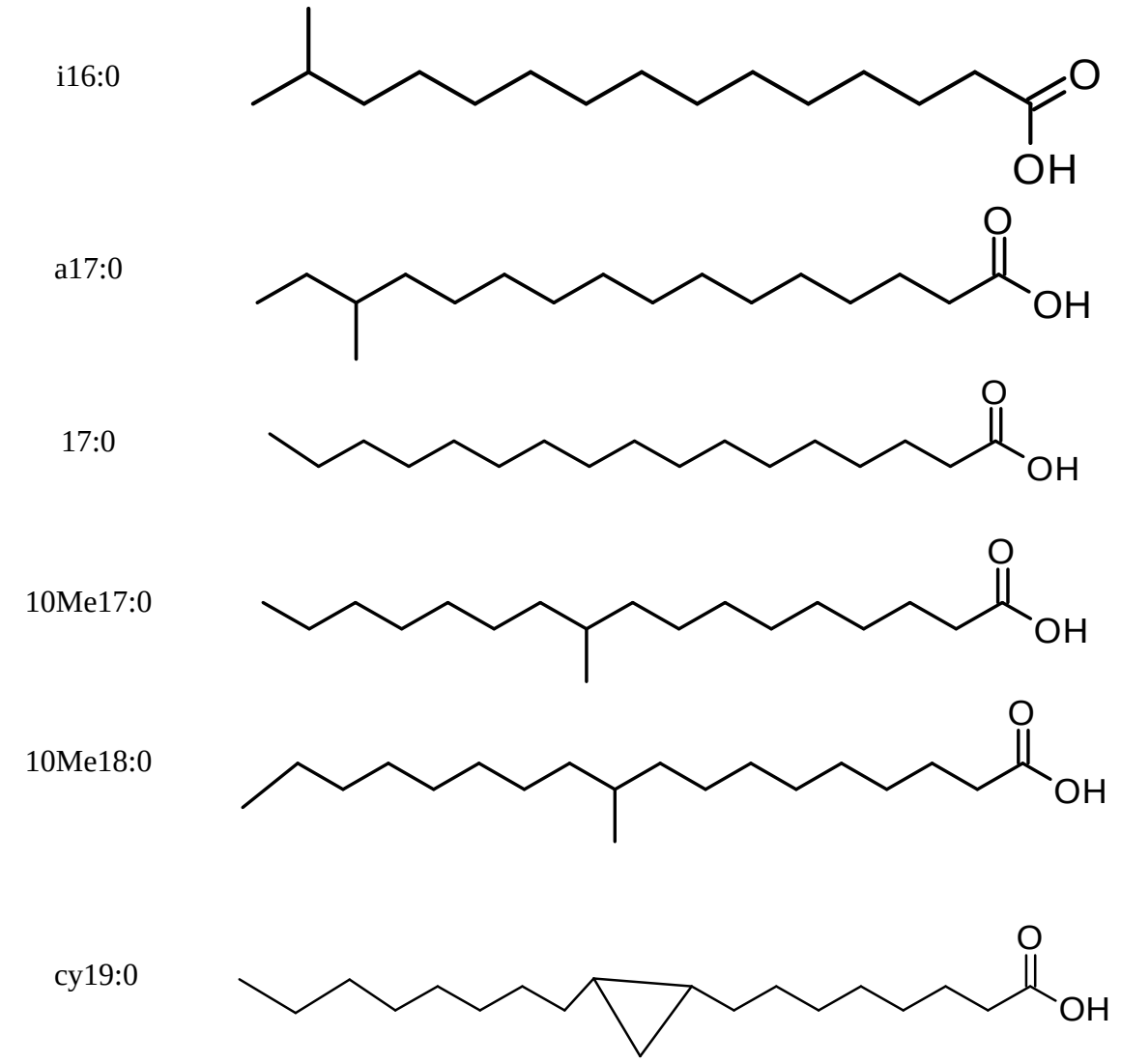


Figure 3. Structures of bacterial biomarker

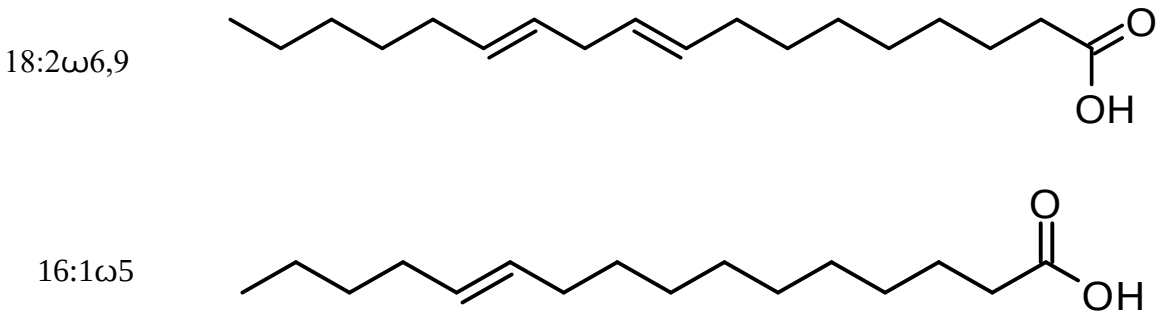


Figure 4. Structures of fungal biomarkers

Microorganisms have distinguishable fatty acids from those of plants and animals in most cases. Some of them are unique to microbial groups, thus selected for identifying microbial communities [25]. In fungi, ergosterol found in the fungal cell membrane is used as a biomarker to determine fungi in the soil. It is the common method for determining fungal biomass, but it

is unable to relate a change in ergosterol content with the shift in environmental conditions [26,27]. Compared to ergosterol measurement, PLFA analysis is beneficial as both bacterial and fungal biomarkers can be covered during analysis and convenient to study simultaneously [24,28]. PLFA 18:2ω6,9,18:1ω9 and 18:3ω3,6,9 are typical fungal biomarkers, PLFA 18:2ω6,9 is more specific to fungi than bacteria [28,29].

2.4 Arbuscular Mycorrhizal Fungi (AM)

AM fungi are a support system of plants under harsh conditions responsible for supplying nutrients required by the host plant and interdepending on one another. In addition, these fungi improve soil quality and texture, making them favourable for the growth of specific plants [30]. Molecular techniques provide good species differentiation, but they are not good at determining the biomass of AM in a soil sample due to PCR biases. Furthermore, the diversity in genes within AM community made it difficult to develop DNA primers that are specific to a group or individual [31,32]. Lipid analysis is commonly used to quantify AM fungal biomass due to analytical difficulties in molecular methods [23].

Signature fatty acid 16:1ω5 is used as a biomarker for the estimation of AM. Though it is possible to use PLFA 16:1ω5 to quantify AM, there are potential levels of PLFA 16:1ω5 that are related to bacteria, so correct biomass estimation for AM fungi is a challenge [23]. Furthermore, the chances of misinterpretation due to interference of bacterial PLFA 16:1ω5 would be increased in soil containing an excessive number of bacteria. Therefore, NLFA 16:1ω5 is a more accurate predictor of AM biomarkers in soil [33].

2.5 Applications of PLFA analysis

The complexities of the ecosystem could be associated with the microbial population in the soil, giving us an understanding of the ecosystem. Furthermore, the characterization of these microbial biotas can act as an indicator of environmental health status. Hence, the application of PLFA analysis is not only limited to the analysis of a microbial community but falls into broad categories as follows.

- Contaminated sites: Soil contaminated by explosives [34], environmental contaminants such as polychlorinated biphenyls (PCB) [35], coal tar [36], olive mill waste [37] can be restored by bioremediation. PLFA method of analysis can be used to identify the conditions of bacterial or fungal species effected by pollutants in soil for bioremediation [38].
- Aquatic environment: The microscopic organisms such as phytoplankton are important for maintaining the food chain cycle in aquatic environments and indicators of the

aquatic ecosystem. PLFA analysis can be used to assess these species important for the maintenance of water bodies [39].

- Waste water Treatment Technology: Waste water treatment plant requires microorganisms to remove organic pollutants in the waste water. The working efficiency of the treatment plant relies on these microorganisms. So, PLFA analysis is required for maintaining waste water treatment plants [40,41].
- Climatic conditions: The status of soil microorganisms can be used to understand the influence of climatic changes such as temperature and humidity on soil microbial communities. PLFA analysis is a useful method to provide the relation between climatic change and microbial shift [42].
- Agricultural practices: Mineral fertilizer and organic compost use in the soil impacts soil fertility and productivity over time. The PLFA method is useful for determining the impact of agricultural activities on the bacterial and fungal patterns in agricultural soil. The findings help recognize the value of organic manure, nitrogen, phosphorus, and potassium fertilizer to improve crop growth and development [43].
- Indicator of environment and soil health: Soil microbial properties are easily affected by disturbances in environmental conditions, so they are used to assess the ecosystem. Microbial patterns can be predicted by PLFA analysis [6].

2.6 Analysis by Gas chromatography Flame-Ionization detector (GC-FID)

Fatty acid profiling is commonly done by GC-FID or Gas chromatography Mass-spectrometry (GC-MS) and is a popular method for lipid analysis [10,44]. For GC analysis, fatty acids in the soil are isolated by a combination of organic solvents followed by centrifugation. First, PLFA and NLFA are separated with solid-phase extraction (SPE) cartridge pre-packed with silica using different eluents. Then, excess methanol and a catalyst are added for transesterification to produce fatty acid methyl esters (FAMEs) [6,10,45]. Finally, the sample is injected into GC, where the sample is carried in a column by carrier gas, usually helium, and separated based on volatility [46]. Detectors such as FID or MS are coupled with GC used for fatty acid analysis. FID is simple, robust, and sensitive to compounds containing carbon and hydrogen bonds. It has a broad linearity range, has a quick response time, and has a detection limit of 10^{-12} g for alkanes [47]. MS, on the other hand, provides structural information and is more specific than FID but still complex in operation than FID. So, GC-FID is still preferred over GC-MS [48]. Besides these analytical methods, High-Performance Liquid chromatography (HPLC) can be considered as an alternative option for GC, but more things are to be considered, such as solvents, mobile, phase, sample preparation.

2.7 Aims of this thesis

Many studies have been carried out comparing different techniques for increasing extraction efficiency, but very little research has been done on soil storage after sampling. It is generally recommended to immediately analyse fresh, field-moist soil or immediate lyophilization with freezer storage which might not be pragmatic under all circumstances. Specifically, freezing and lyophilization, silica gel pre-treatment, and Hot air oven drying are some pre-treatment methods carried out for the storage of soil samples in practice. This study is carried out to:

1. Compare the effects of the pre-treatment method on the fatty acid biomarkers in soil.
2. Compare the effects of type of soil sampling sites on fatty acid biomarkers.
3. Compare the effects of the time factors on fatty acid biomarkers.
4. Compare the effects of time within treatments on fatty acid biomarkers
5. Compare the effects of treatment within the time on fatty acid biomarkers

3 METHODOLOGY

3.1 Experimental Design

A total of 10 L of soil was taken from two sampling sites (field and grassland). For each pre-treatment, 16 sub-samples from each site were divided into four sub-groups based on the pre-treatment time. Thus, there were altogether 96 samples for analysis. In each of the sixteen samples, one of the three pre-treatment methods Freezing and Lyophilization (FL), Silica gel (SG), Hot Air Oven (AD), was chosen to see how they affected the microbial population. Samples were subjected to the selected pre-treatment methods at different time intervals (0, 8, 24, 48). Variations in processing time along with pre-treatment methods were investigated to see what effect they have on fatty acid biomarkers.

3.2 Collection of soil samples

Soil samples were collected from Suurmetsa, Põlva County, Estonia. Two sites were selected for the sampling of soil in this area; one is an arable field (58°07'08.5"N 27°16'05.5"E) and the other an organically managed grassland (58°07'07.2"N 27°16'02.6"E).

Soil samples were collected with a 2 cm diameter soil drill up to a depth of 20 cm. Samples were collected from an approximately 30 x 50 meters area and then pooled to form a composite sample of 10 L in volume.

After soil samples were collected, they were sieved and homogenised in a rotary mixer for an hour. The composite soil sample was then sub-sampled according to the method of pre-treatment into separately labelled plastic bags: -

i) Freezing and lyophilization (FL) ii) Silica-gel (SG)iii) Hot air oven (AD) at 50°C.

A detailed description of pre-treatment methods is given in Table 3. A time factor was added for each treatment, indicating the delay in sample pre-treatment after collection, as shown in Figure 5. Four-time intervals were used: processing immediately after homogenisation in the rotary mixer; processing after 8 hours; processing after 24 hours; processing after 48 hours. When a time factor was introduced, samples were kept in closed plastic bags at room temperature prior to processing.

Table 3. Description of sample processing

Freezing and Lyophilization	Homogenized samples for immediate processing were placed in the portable freezer maintaining the temperature at -17° C. The rest of the samples were placed in a freezer (-17° C) in the laboratory after 8 hours, 24 hours, and 48 hours. Then, after 48 hours, all the samples were subjected to lyophilization for 24 hours.
Silica gel	Homogenized samples were separately collected in the thin paper bag[1] and placed in the zip-lock bag containing silica gel for immediate processing. The rest of the samples received silica gel after 8 hours, 24 hours, and 48 hours. Silica gel was changed as soon as the color of the silica gel changed.
Hot air oven	Homogenized samples for immediate processing were placed in the hot air oven at 50°C in the laboratory on immediate arrival (1 hour). The rest of the samples were placed in the hot air oven at 8 hours, 24 hours, and 48 hours after collection. Samples were dried for 24 hours in the oven.

[1] Thin paper bags are used as tea-bags

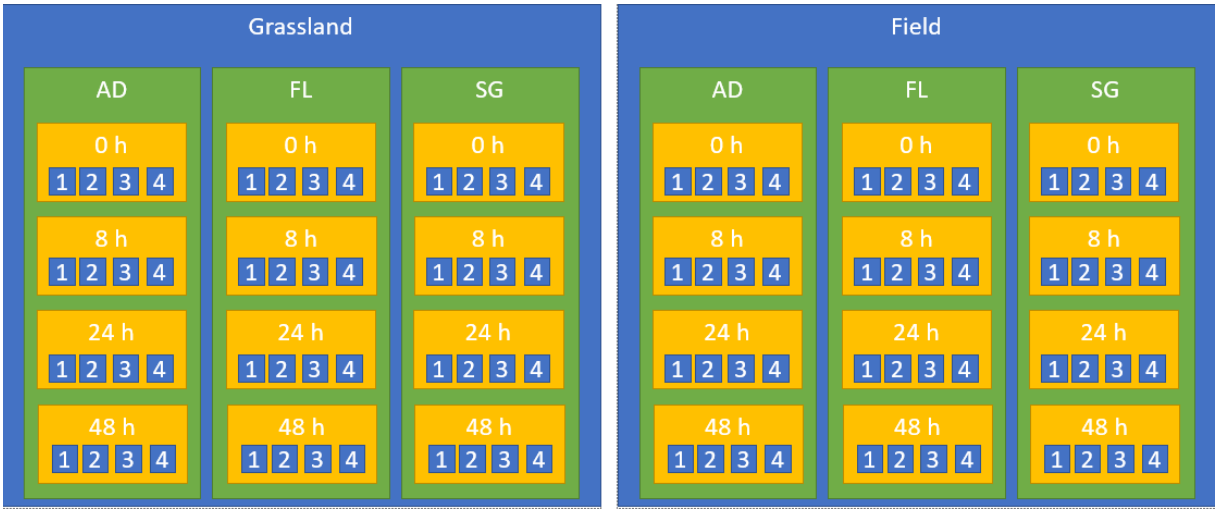


Figure 5. Schematic representation of sample processing

3.3 Chemicals and Solvents

Chemicals and solvents were of analytical grade. Methyl nonadecanoate (Sigma Aldrich) was used as an internal standard (IS). Chloroform and Methanol were produced by Fisher Scientific (UK). Toluene, Acetone, Hexane, and Acetic acid were produced by Merck (Germany). Citric acid anhydrous, Potassium Hydroxide, and Sodium Hydroxide were produced by Merck (Germany). Distilled water is used for preparing aqueous solutions. All the solvents and chemicals and their physical properties are presented in Annex 1.

3.4 Preparation of Solutions

Citrate buffer was prepared by dissolving 31.25 g citric acid in 1 litre distilled water, and pH was adjusted to 4 using sodium hydroxide pellets (0.15 M). Bligh and Dyer [20] solution was prepared by mixing chloroform, methanol, citrate buffer in the proportions 1:2:0.8 (by volume). Toulene and methanol mixture was prepared in proportions 1:1(by volume). Potassium hydroxide was prepared by dissolving 0.89 g potassium hydroxide in 80 ml methanol (0.2 M). Hexane and chloroform mixture was prepared by mixing in proportions 4:1(by volume). Acetic acid was prepared by diluting 6 ml acetic with distilled water in 100 ml volumetric flask. 0.023 mg/ml concentration of methyl nonadecanoate (IS) was prepared in chloroform.

3.5 Sample Preparation

The procedure given below was followed for the extraction of fatty acids [20].

3.5.1 Extraction

a) Approximately 2 g of soil was taken in the Teflon test tube with a screw cap. The weight was noted for the calculation of results afterward. Next, 10 ml of Bligh and Dyer solution was added, and the mixture was vortexed for 15 seconds. The sample was then incubated for 2 hours at room temperature for lipid extraction.

b) The test tube was centrifuged at 3000 rpm for 10 minutes to separate the solid and liquid phases. Then, the supernatant containing extracted lipids was transferred to a glass test tube (30 ml size).
c) 5 ml Bligh and Dyer was again added to the Teflon test tube, and step (b) was repeated to increase the yield of extraction further.
d) 4 ml of chloroform and 4 ml of citrate buffer were added to the supernatant and capped. It was left overnight at room temperature for phase separation.
e) 3 ml of chloroform phase (lower) was transferred to the small glass test tube (8 ml size) with a glass pasteur pipette.
f) Under the stream of nitrogen, the solvent was evaporated on a heating block at 40°C.
g) In the refrigerator, at 4°C, the evaporated samples were stored until ready to proceed for the next step.

3.5.2 Lipid Fractionation

a) SPE column (silica, 500 mg, 6 ml) was placed into spigots on the vacuum manifold.
b) The samples stored in a glass test tube were re-dissolved in 100 µl of chloroform, vortexed for 15 seconds, and transferred to an SPE column. This step was repeated twice (total 200 µl of chloroform), allowing maximum fatty acid residue to transfer into the SPE column.
c) The neutral lipids, glycolipids, and phospholipids were eluted from the SPE column with 1.5 ml chloroform, 6 ml acetone, 1.5 ml methanol, respectively, and collected in the small glass test tube using vacuum and running cartridge dry for few seconds.
d) The collected samples were evaporated to dryness under nitrogen at 40° C.
e) The dried residues were stored in the refrigerator at 4°C until ready to proceed for the next step.

3.5.3 Mild Alkaline Alkanolysis

a) 100 µl of IS (19:0) solution was added to dried residue in the small test tube and evaporated under nitrogen with no heating.
b) The samples were re-dissolved in 1 ml toluene:methanol mixture (1:1) then vortexed for 5 seconds.
c) 1 ml of freshly made KOH (0.2 M) was added, and samples were incubated at 37° C for 15 minutes and cooled for 20 minutes at room temperature. Here, a mild alkaline trans-methylation process occurred to produce fatty acid methyl esters (FAMEs) [49].

d) 2 ml hexane: chloroform (4:1), 0.3 ml 6% acetic acid, and 2 ml water were added and vortexed for 1 min.
e) Two phases were separated by centrifuging the sample at 3000 rpm for 5 minutes.
f) 2 ml of top phase containing FAMEs was transferred into a test tube and dried under a stream of nitrogen without heating.
g) The residue was dissolved in 100 µl hexane and vortexed for few seconds.
h) Small GC vials were filled with 200 µl hexane. Then, the glass insert was placed, and 100 µl of the sample was added to the insert.

3.6 GC-FID analysis

Samples were analysed using Gas chromatography with a flame ionization detector (Agilent 7890A). The operating parameters of GC-FID are presented in Tables 4-6.

Table 4. Inlet Parameters

Parameters	**Setpoint**
Heater	300° C
Pressure	3.5381 psi
Total Flow	47 ml/min
Septum Purge Flow	3 ml/min

Table 5. Parameters of oven temperature

Rate (°C/ min)	**Temp (°C)**	**Hold Time(min)**	**Run Time(min)**
	80	1	1
20	160	0	5
5	270	10	37

Column : DB-5ms, 30m x 530 µm x 1.5 µm

Column Flow rate: 4 ml/min

Carrier gas: Helium

Injection volume: 1 µl

Table 6. Parameters of FID

Parameters	**Setpoint**
Heater	300℃
Air Flow	400 ml/min
Hydrogen (H_2) Fuel Flow	30 ml/min
Nitrogen (N_2) Flow	25 ml/min

Quantitative analysis of the fatty acid was performed based on GC-FID response relative to that of internal standard (19:0). Blanks were included in each analysis. The concentration of fatty acid was calculated using the following equation

$$\text{Concentration(nM/g)} = \frac{A_S}{A_I} \times \frac{IS\ added \times V(7.95\ ml)}{Vs(3\ ml) \times s_\omega \times 312}$$

Where,

A_S = peak area of specific fatty acid,

A_I= peak area of internal standard,

S_w = weight (g) of the sample,

IS = internal standard added (ng),

Vs= volume taken for analysis,

V=volume of chloroform taken in Bligh and Dyer extraction

3.7 Statistical Analysis

R software for windows 4.0.3 was used for statistical analysis. Effects of soil sample preparation on fatty acid biomarkers were tested using one-way Analysis of variance (ANOVA), followed by Tukey's post hoc test. In all cases, differences of $p < 0.05$ were regarded as statistically significant.

4 RESULTS

4.1 Peak identification

Peaks of the fatty acid biomarkers were identified relative to the position of internal standard in both PLFA (Figure 6) and NLFA (Figure 7) fractions. In the NLFA fraction, the peak of AM biomarker (16:1ω5) was analysed.

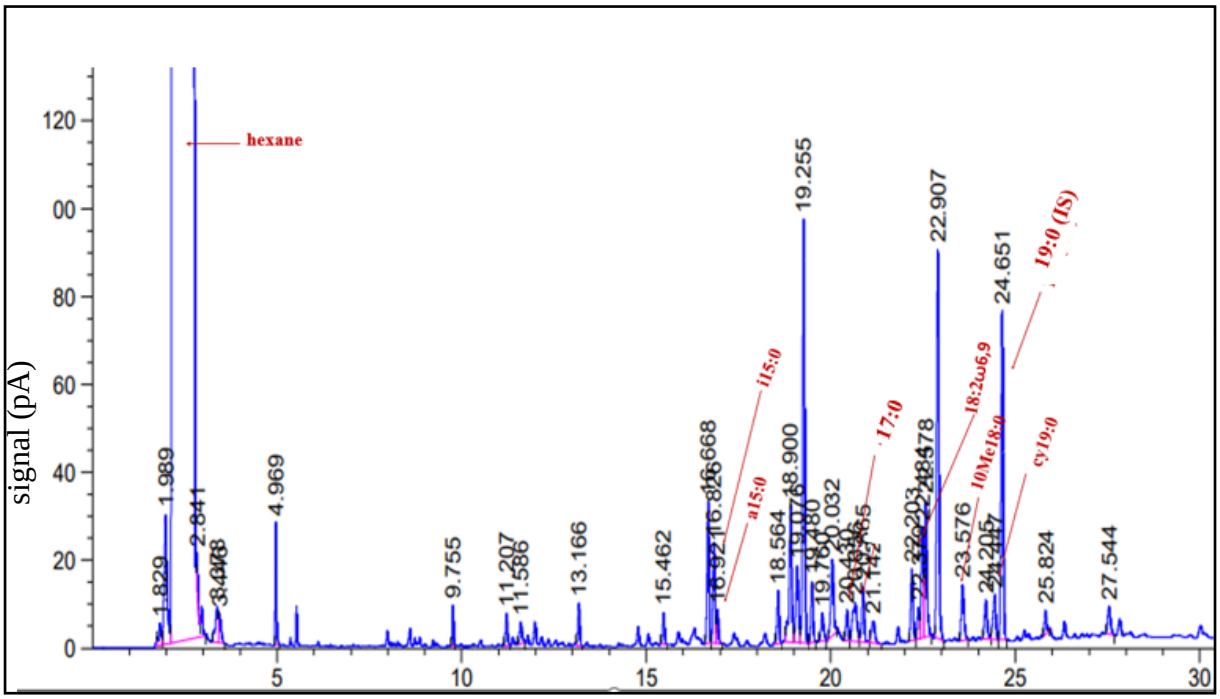


Figure 6. Chromatogram of PLFA fraction

The hexane peak has a retention time of around 2.1 min, the peak of the internal standard elutes from the column around 24.6 min.

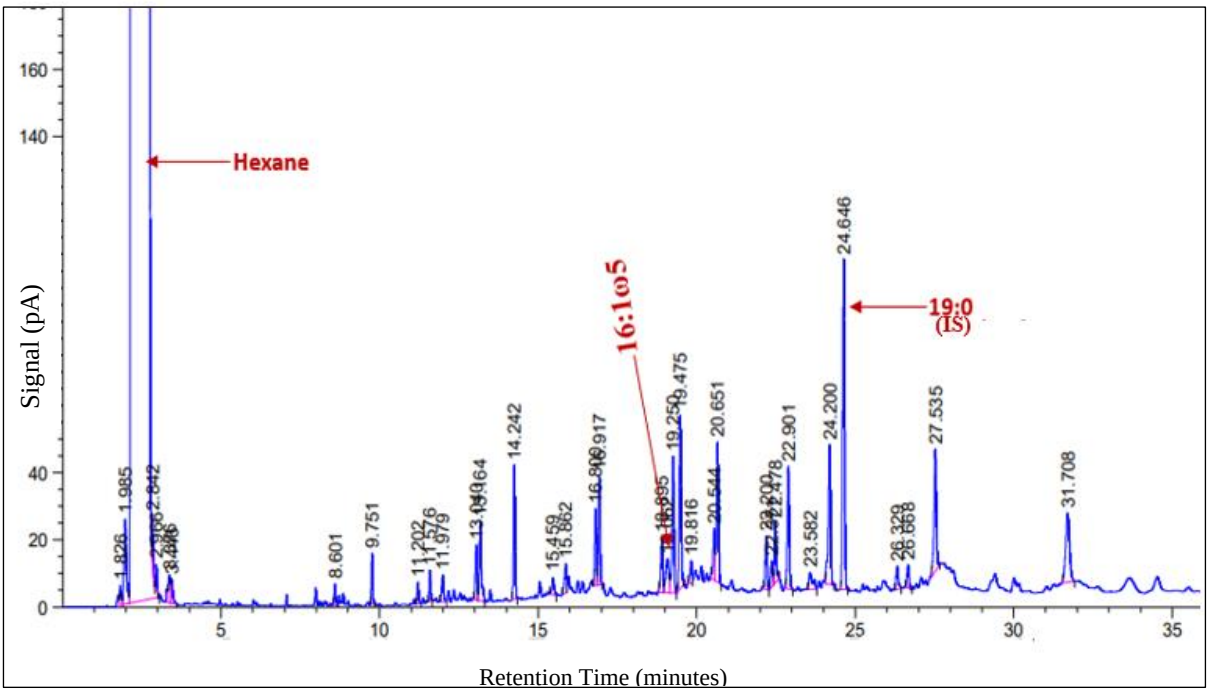


Figure 7. Chromatogram of NLFA fraction

4.2 Fatty acid biomarkers concentrations in soil

The concentrations of fatty acid obtained in grassland samples subjected to pre-treatment FL in PLFA fraction are presented in Table 7. The rest of the results are presented in Annex 3-7. The results of fatty acid concentrations show variation. In the grassland sample (Table 7), though there is inconsistency in results, fatty acid concentrations do not change significantly with delay in pre-treatments. In the case of bacterial biomarkers 10Me18:0, the concentrations range from 2.19 nM/g to 2.26 nM/g for samples immediately, which are freeze and lyophilized and those delayed after 48 hours have concentrations ranging from 2.13 nM/g to 2.57 nM/g.

Table 7. Concentrations of fatty acid biomarkers in grassland sample (FL)

Pre-treatment time	Concentrations (nM/g)								
	i15:0	**a15:0**	**i16:0**	**a17:0**	**17:0**	**10Me17:0**	**18:2ω6,9**	**10Me18:0**	**cy19:0**
0 hour									
Sample 1	2.18	0.00	1.42	1.03	2.34	2.12	1.12	2.26	2.67
Sample 2	1.78	0.00	1.60	1.19	1.84	1.10	1.48	2.58	3.57
Sample 3	2.72	2.70	1.78	1.18	2.10	1.92	1.07	2.84	2.95
Sample 4	2.50	0.00	1.56	1.07	2.21	1.31	0.96	2.19	2.99
8 hours									
Sample 1	3.17	3.06	1.93	1.20	2.06	1.76	1.06	2.70	2.82
Sample 2	1.00	1.36	0.93	0.77	1.15	2.36	0.71	2.36	2.21
Sample 3	1.75	0.00	1.50	1.09	1.47	0.00	1.43	2.15	2.75
Sample 4	3.54	0.89	2.00	1.23	2.39	1.27	0.85	2.15	2.98
24 hours									
Sample 1	2.73	2.79	1.92	1.24	1.84	1.61	0.00	2.55	2.66
Sample 2	1.81	2.14	1.44	1.08	1.66	2.55	0.93	2.50	2.33
Sample 3	2.66	0.00	1.91	1.31	2.32	1.38	0.82	2.28	3.37
Sample 4	2.34	0.00	2.03	1.47	2.78	1.08	1.11	2.60	4.03
48 hours									
Sample 1	3.77	0.79	2.36	1.52	2.84	1.28	1.08	2.57	3.45
Sample 2	2.88	2.74	2.05	1.40	2.26	1.67	0.83	2.91	3.19
Sample 3	1.46	0.00	1.46	1.12	1.40	0.85	0.85	2.19	2.96
Sample 4	2.20	0.00	1.60	1.19	4.20	0.00	1.07	2.13	2.39

The concentrations of AM fungi biomarkers decreased as the delay in pre-treatment time increased in most cases (Table 8). Fatty acids are sensitive to stress factors such as temperature and humidity, leading to degradation or change of fatty acid composition in AM fungi, resulting in the decline of fatty acid concentrations. The concentrations of fatty acids for AM fungi are higher than in the field might be due to these stress factors. In the case of grassland samples, immediately freeze-dried AM concentrations (16:1ω5) range from 6.36 nM/g to 8.41 nM/g, while field samples treated similarly range from 0.00 nM/g to 1.42 nM/g. This shows the effect of agricultural practice on fungal habitats. In this study, only 50℃ was used to dry in the oven, so the concentrations did not drastically change. In the study done by Veum *et al.* [12], the soil sampled from the Golden Prairie Natural area in Missouri was analysed for fatty acid profiles under various storage conditions. Among these samples, the immediately lyophilized showed that the mean concentration of AM was 10.75 nM/g. While samples that were air-dried at 105℃ showed a remarkable decline in fatty acid concentrations [12].

Table 8. Fatty acid concentrations of AM fungi biomarkers in grassland and field

Sites	Grassland			Field		
	FL	**SG**	**AD**	**FL**	**SG**	**AD**
0 hour						
Sample 1	7.82	7.39	6.85	1.42	0.00	1.49
Sample 2	6.36	5.63	6.48	0.00	0.00	0.00
Sample 3	7.23	7.00	5.13	0.87	0.00	0.78
Sample 4	8.41	7.38	9.52	0.00	0.00	0.00
8 hours						
Sample 1	5.40	5.79	6.64	1.25	1.47	0.00
Sample 2	8.36	5.61	5.43	0.00	1.19	1.48
Sample 3	1.91	9.97	4.34	0.97	3.10	0.77
Sample 4	7.31	8.41	7.79	0.00	0.63	0.00
24 hours						
Sample 1	4.60	4.17	5.85	1.25	1.14	0.00
Sample 2	6.44	6.52	4.39	0.00	0.00	0.00
Sample 3	6.28	4.49	5.96	0.00	0.00	0.00
Sample 4	5.49	2.84	4.20	9.56	0.65	0.00
48 hours						
Sample 1	4.19	8.63	7.12	3.80	1.30	0.00
Sample 2	7.02	6.29	3.07	11.68	0.00	0.94
Sample 3	3.55	4.96	2.79	0.00	0.00	0.79
Sample 4	2.32	2.22	2.19	0.00	0.00	1.80

4.3 Effects of sites, pre-treatments, and time

Tables 9-13 show the results of ANOVA. The bold asterisk (*) sign represents statistically significant differences ($p < 0.05$), the df column represents the degree of freedom, the F column represents test statistic from F- test, and the *p* column represents the p-value of F-statistic. In most bacterial and fungal biomarkers, $p < 0.001$ indicates that the location and soil sample type significantly effect these biomarkers (Table 9). Two bacterial biomarkers (i15:0, a17:0) have $p < 0.05$, and total fungal biomarkers (18:2ω6,9) $p < 0.001$ indicating significant effect by the pre-treatment methods (Table 10), while AM biomarkers did not seem to be significantly influenced by pre-treatment methods. Only one bacterial biomarker (10Me17:0) has $p < 0.01$ indicating the effect of delay in pre-treatments (Table 11).

Table 9. ANOVA-test results for the overall influence on fatty acid biomarkers

	Site (Type)			
	Biomarkers	**df**	**F**	***p***
Bacteria	i15:0	1	40.54	**< 0.001***
Bacteria	a15:0	1	0.001	0.971
Bacteria	i16:0	1	96.33	**<0.001***
Bacteria	a17:0	1	74.36	**<0.001***
Bacteria	17:0	1	32.46	**< 0.001***
Bacteria	10Me17:0	1	0.31	0.576
Fungi	18:2ω6,9	1	14.30	**<0.001***
Bacteria	10Me18:0	1	108.70	**< 0.001***
Bacteria	cy19:0	1	54.47	**< 0.001***
AM fungi	16:1ω5	1	125.00	**<0.001***
Fungal:Bacterial Ratio (F:B)		1	78.02	**< 0.001***

Table 10. ANOVA-test results for the overall influence of pre-treatments on fatty acid biomarkers

		Treatments		
		df	**F**	***p***
Bacteria	i15:0	2	3.57	**0.032***
Bacteria	a15:0	2	0.22	0.081
Bacteria	i16:0	2	3.05	0.052
Bacteria	a17:0	2	3.19	**0.046***
Bacteria	17:0	2	2.75	0.069
Bacteria	10Me17:0	2	0.38	0.069
Fungi	18:2ω6,9	2	8.96	**< 0.001***
Bacteria	10Me18:0	2	4.99	**0.009 ***
Bacteria	cy19:0	2	0.12	0.885
AM fungi	16:1ω5	2	0.63	0.533
	F:B Ratio	2	0.34	0.711

Table 11. ANOVA-test results for the overall influence of time on fatty acid biomarkers

			Time	
		df	**F**	***p***
Bacteria	i15:0	3	0.55	0.648
Bacteria	a15:0	3	0.73	0.538
Bacteria	i16:0	3	0.09	0.964
Bacteria	a17:0	3	1.15	0.335
Bacteria	17:0	3	0.80	0.496
Bacteria	10Me17:0	3	4.07	**0.009***
Fungi	18:2ω6,9	3	0.78	0.508
Bacteria	10Me18:0	3	0.18	0.912
Bacteria	cy19:0	3	0.01	0.999
AM fungi	16:1ω5	3	0.28	0.839
	F:B Ratio	3	0.60	0.617

In the grassland samples, any pre-treatment methods, if applied immediately, do not influence concentrations of fatty acids in most cases. (Table 12), but in field, bacterial biomarker (i15:0) showed $p < 0.001$.

Table 12. ANOVA- test results for comparing among pre-treatments considering the time factor

Grassland samples (Treatment within time)												
Time	**0 hour**			**8 hours**			**24 hours**			**48 hours**		
	df	**F**	***p***	**df**	**F**	***p***	**df**	**F**	***p***	**df**	**F**	***p***
i15:0	2	0.25	0.781	2	0.35	0.622	2	0.97	0.415	2	1.82	0.216
a15:0	2	0.02	0.981	2	0.06	0.944	2	0.19	0.832	2	0.01	0.989
i16:0	2	0.58	0.580	2	0.33	0.725	2	0.86	0.455	2	2.32	0.154
a17:0	2	0.61	0.565	2	0.32	0.733	2	2.78	0.115	2	4.63	**0.041***
17:0	2	0.64	0.552	2	0.13	0.877	2	2.12	0.176	2	0.36	0.705
10Me17:0	2	0.70	0.52	2	0.18	0.840	2	1.16	0.355	2	0.23	0.803
18:2ω6,9	2	1.73	0.231	2	10.27	**0.004***	2	3.59	0.072	2	1.25	0.332
10Me18:0	2	1.37	0.302	2	2.21	0.166	2	2.85	0.110	2	2.80	0.114
cy19:0	2	0.15	0.865	2	0.31	0.739	2	0.74	0.503	2	0.53	0.604
16:1ω5	2	0.42	0.669	2	0.67	0.669	2	1.11	0.371	2	0.59	0.573
F:B	2	0.38	0.693	2	0.55	0.546	2	0.13	0.883	2	0.10	0.902

Field samples (Treatment within time)												
Time	**0 hour**			**8 hours**			**24 hours**			**48 hours**		
	df	**F**	***p***	**df**	**F**	***p***	**df**	**F**	***p***	**df**	**F**	***p***
i15:0	2	44.59	**< 0.001***	2	0.49	0.629	2	1.57	0.260	2	1.02	0.398
a15:0	2	0.10	0.908	2	0.24	0.791	2	0.03	0.972	2	0.48	0.633
i16:0	2	8.72	**0.008***	2	3.33	0.083	2	1.28	0.324	2	1.96	0.197
a17:0	2	2.60	0.129	2	0.56	0.589	2	0.32	0.733	2	3.34	0.083
17:0	2	5.20	**0.003***	2	1.80	0.220	2	1.79	0.221	2	0.80	0.477
10Me17:0	2	0.02	0.985	2	0.02	0.984	2	0.01	0.991	2	0.53	0.606
18:2ω6,9	2	1.13	0.366	2	1.39	0.299	2	1.58	0.258	2	3.35	0.081
10Me18:0	2	3.72	0.066	2	0.95	0.424	2	1.87	0.210	2	3.01	0.091
cy19:0	2	2.02	0.188	2	0.42	0.669	2	0.69	0.528	2	1.17	0.353
16:1ω5	2	1.30	0.320	2	2.12	0.177	2	1.17	0.354	2	1.05	0.281
F:B	2	0.16	0.861	2	0.95	0.437	2	1.37	0.362	2	0.289	0.763

Time delay in pre-treatment did not show significant differences in concentrations of bacterial and fungal biomarkers in FL samples in the case of grassland and field samples. In the case of SG, $p < 0.05$ in AM biomarker, the same was obtained in bacterial biomarker a17:0 in AD sample (Table 13).

Table 13. ANOVA-test results for the influence of delay in treatments

Grassland samples (Time within treatment)									
	FL			**SG**			**AD**		
	df	**F**	***p***	**df**	**F**	***p***	**df**	**F**	***p***
i15:0	3	0.08	0.968	3	0.57	0.644	3	0.33	0.804
a15:0	3	0.20	0.891	3	0.29	0.829	3	0.06	0.981
i16:0	3	0.70	0.573	3	0.29	0.834	3	0.03	0.994
a17:0	3	1.90	0.183	3	0.44	0.728	3	0.04	0.989
17:0	3	1.12	0.378	3	1.33	0.309	3	1.07	0.397
10Me17:0	3	0.77	0.531	3	0.94	0.454	3	2.23	0.137
18:2ω6,9	3	1.33	0.310	3	1.62	0.236	3	0.57	0.645
10Me18:0	3	0.22	0.879	3	1.17	0.360	3	0.93	0.456
cy19:0	3	0.51	0.680	3	0.17	0.915	3	0.27	0.846
16:1ω5	3	2.02	0.165	3	1.78	0.204	3	2.59	0.101
F:B Ratio	3	2.70	0.093	3	1.18	0.359	3	1.78	0.204
Field samples (Time within treatment)									
	FL			**SG**			**AD**		
	df	**F**	***p***	**df**	**F**	***p***	**df**	**F**	***p***
i15:0	3	2.23	0.137	3	0.39	0.760	3	1.56	0.251
a15:0	3	0.25	0.859	3	0.55	0.656	3	0.10	0.957
i16:0	3	0.39	0.765	3	0.71	0.562	3	0.25	0.862
a17:0	3	0.57	0.648	3	0.15	0.931	3	4.29	**0.02***
17:0	3	0.40	0.758	3	0.39	0.764	3	1.26	0.331
10Me17:0	3	0.05	0.987	3	0.33	0.808	3	1.05	0.406
18:2ω6,9	3	0.01	0.998	3	0.14	0.935	3	-	-
10Me18:0	3	0.56	0.655	3	0.37	0.776	3	0.89	0.474
cy19:0	3	0.60	0.627	3	0.26	0.853	3	0.38	0.771
16:1ω5	3	0.82	0.506	3	4.20	**0.030***	3	1.25	0.336
F:B_Ratio	3	0.88	0.498	3	0.45	0.729	3	0.03	0.968

4.4 Comparison Results

The results with $p < 0.05$ after one-way ANOVA were statistically analysed using Tukey's post hoc test to confirm significant differences in fatty acid biomarkers concentration. Sign* signifies a significant effect in the boxplots, and symbol 'ns' signifies non-significant. The lower line in the boxplot represents Q1 (25th Percentile), the line inside the box represents the median, and the upper line of the boxplot represents Q3 (75th Percentile). The distance between the lower and the upper line is the Interquartile range (IQR). The lower whisker represents the minimum (Q1- 1.5*IQR), and the upper whisker represents (Q1+ 1.5*IQR). The points outside the lower and upper whiskers area are outliers.

4.4.1 Comparison of results among sites, pre-treatments, and time

The results from grassland samples were compared with the results of field samples. Grassland samples showed higher fatty acid biomarker concentrations than the field; the same case was obtained with fungal: bacterial (F:B), except for bacterial biomarkers (a15:0 and Me17:0) (Figure 8).

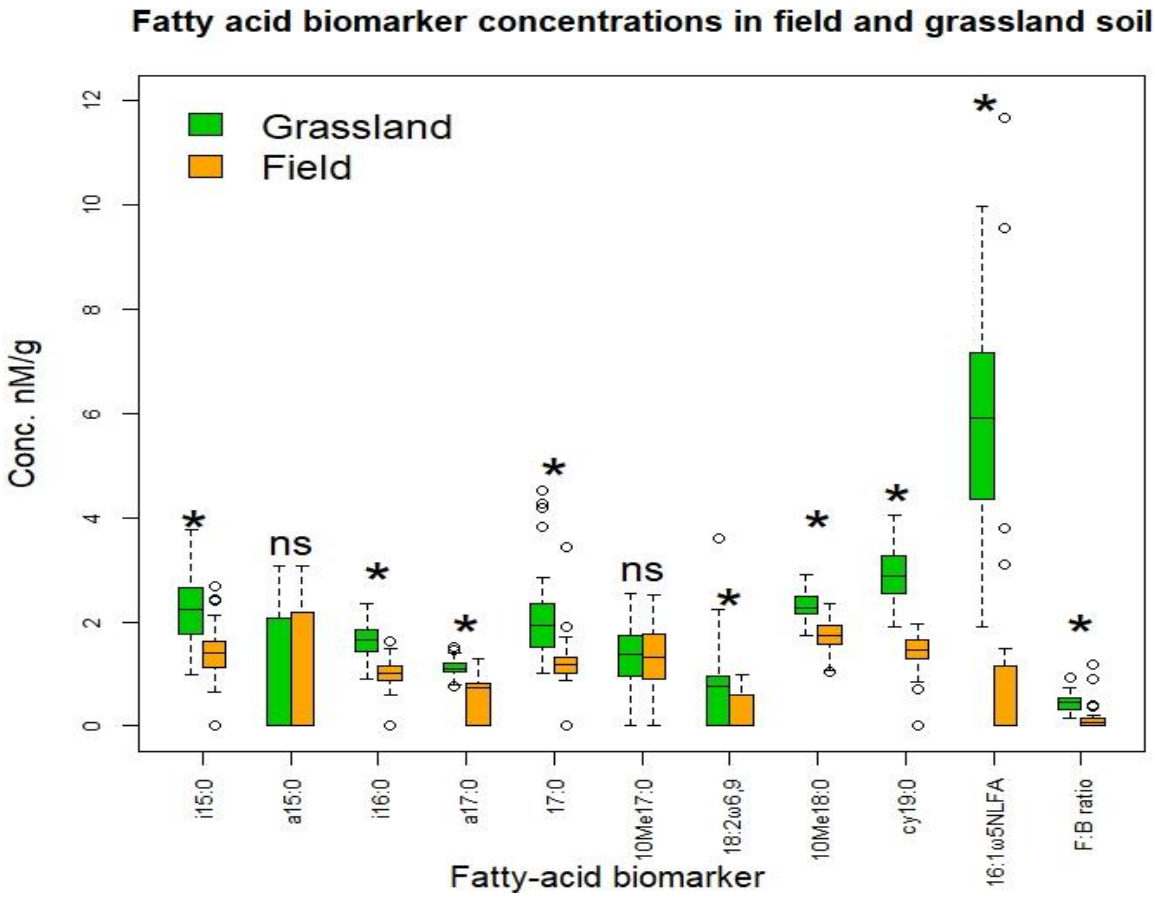


Figure 8. Comparison of the effect of sites on fatty acid biomarker content (* significant effect, ns- non-significant effects)

According to the method of pre-treatments, samples from FL, SG, and AD were compared separately without considering soil types. The concentrations of biomarkers in FL and SG pre-treatment samples did not vary significantly when pre-treatments were compared.

However, when FL and AD were compared, the concentrations of bacterial biomarkers (i15:0 and a17:0) and total fungal biomarkers (18:2ω6,9) were significantly different. Total fungal biomarker(18:2ω6,9) showed a significant difference between concentrations when the influence of two pre-treatment methods (AD and SG) are compared. The F:B ratio did not indicate a noticeable difference (Figure 9).

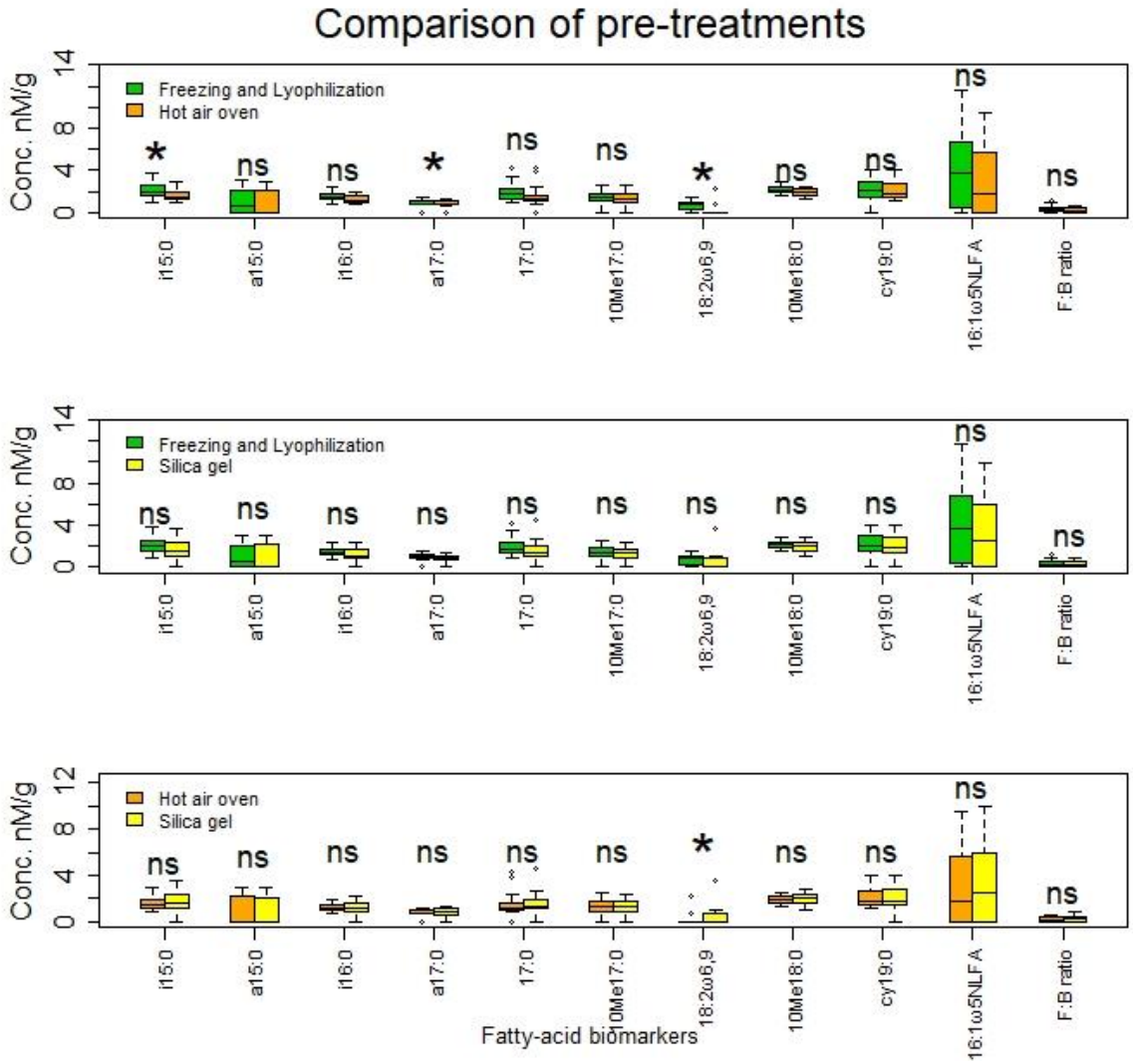


Figure 9. Comparisons of effects on pre-treatment on fatty acid biomarkers (*significant effects, ns- non-significant effects)

The comparison among samples was carried in which pre-treatments were delayed by the same time. Regardless of the choice of pre-treatment, whether the time is delayed or not did not seem to influence most bacterial and fungal biomarkers, except for the 10Me17:0 fatty acid biomarker (Figure 10).

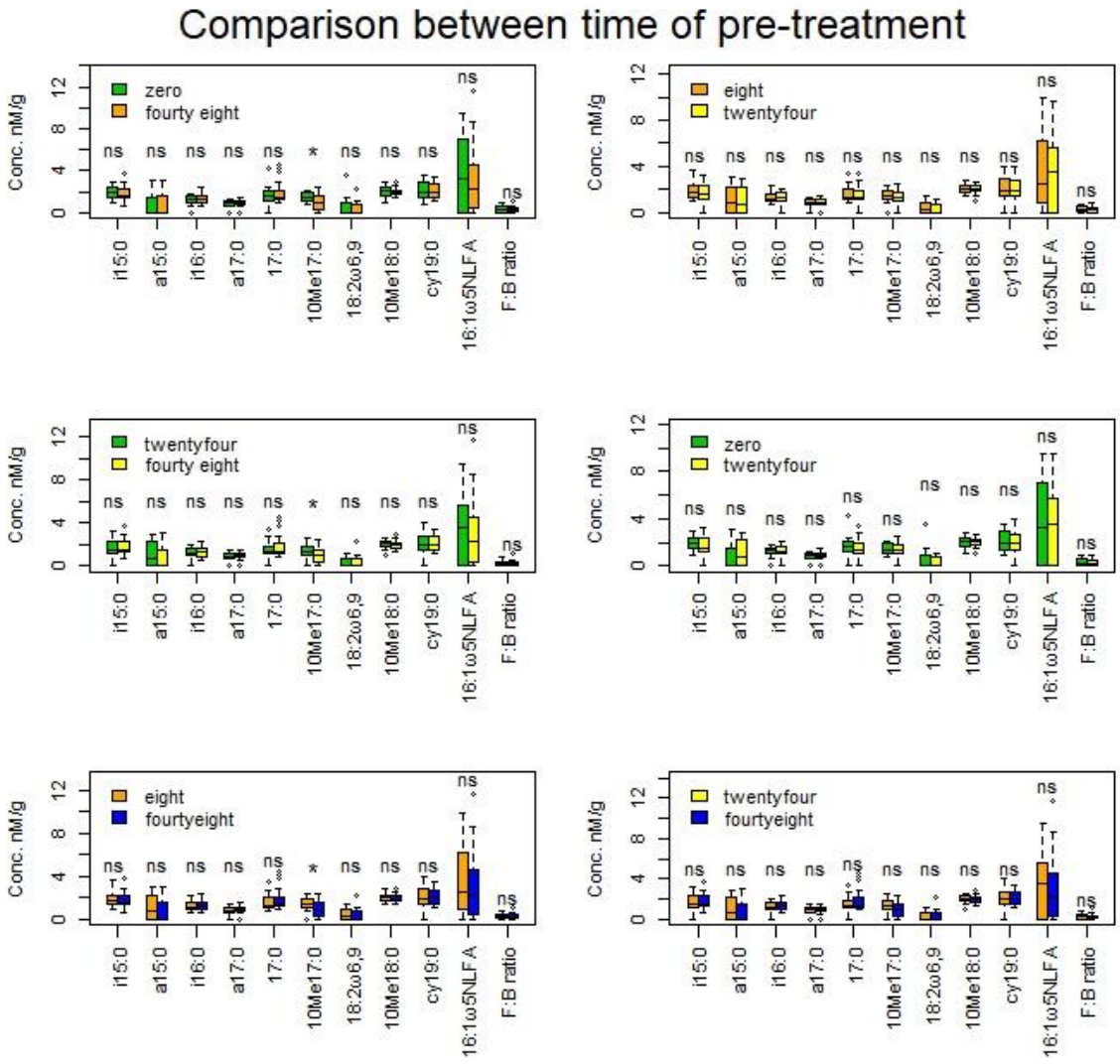


Figure 10. Comparison of the effect of delay in the processing of sample on fatty acid biomarkers (*significant effects, ns- non-significant effects)

4.4.2 Comparisons of treatment within the time

Samples from the field and grassland were subjected to three pre-treatment methods. In each pre-treatment method, samples were grouped according to delay in pre-treatment methods. Then, the results from the same delayed time among pre-treatment methods were compared. The fatty acid concentrations compared with pre-treatment delayed by eight, twenty-four, and fourty-eight hours did not show any significant differences in the case of the field sample. However, the fatty acid concentrations in immediately pre-treated samples showed a significant difference in some cases of bacterial biomarkers (i15:0 and i17:0). When FL and AD were compared, the same was found when comparing FL and SG (Figure 11).

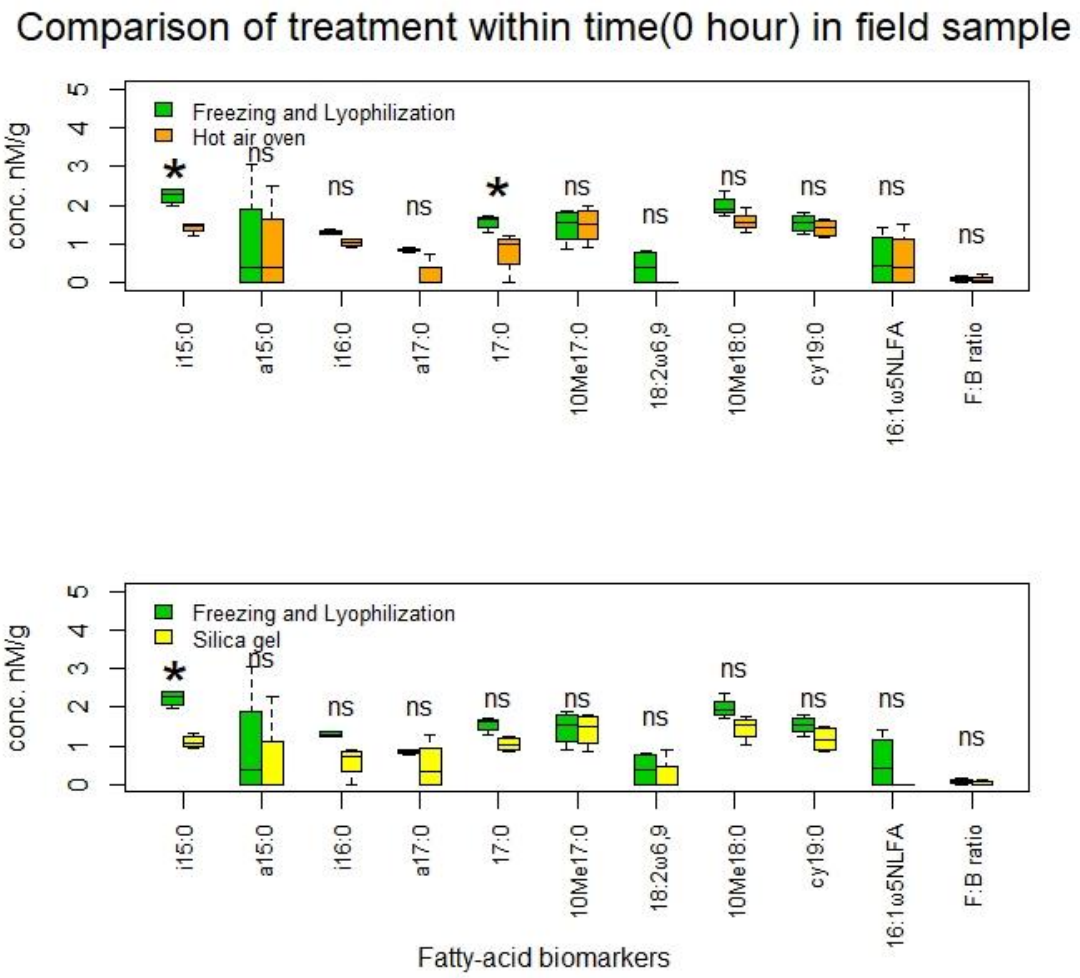


Figure 11. Influence of immediate processing of sample on fatty acid biomarkers (*significant effects, ns- non-significant effects)

In grassland, the fungal biomarker(18:2ω6,9) showed a substantial effect of pre-treatments FL and AD carried out at 8 hours, and the same finding was observed between pre-treatments AD and SG (Figure 12). The choice between pre-treatments FL and AD impacted the bacterial biomarker (a17:0) after 48 hours in the grassland sample (Figure 13).

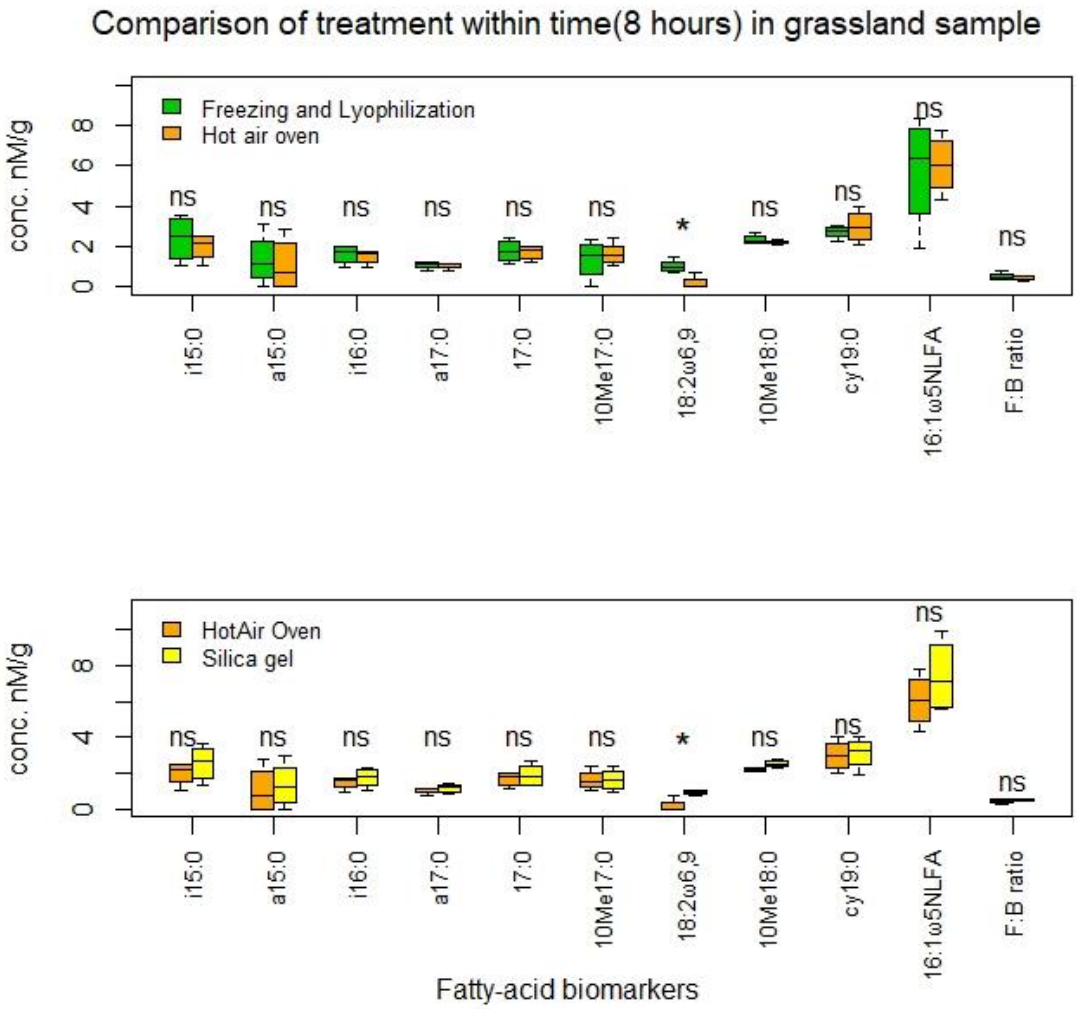


Figure 12. Influence in fatty acid biomarkers for sampled processed after 8 hours (*significant effects, ns- non-significant effects)

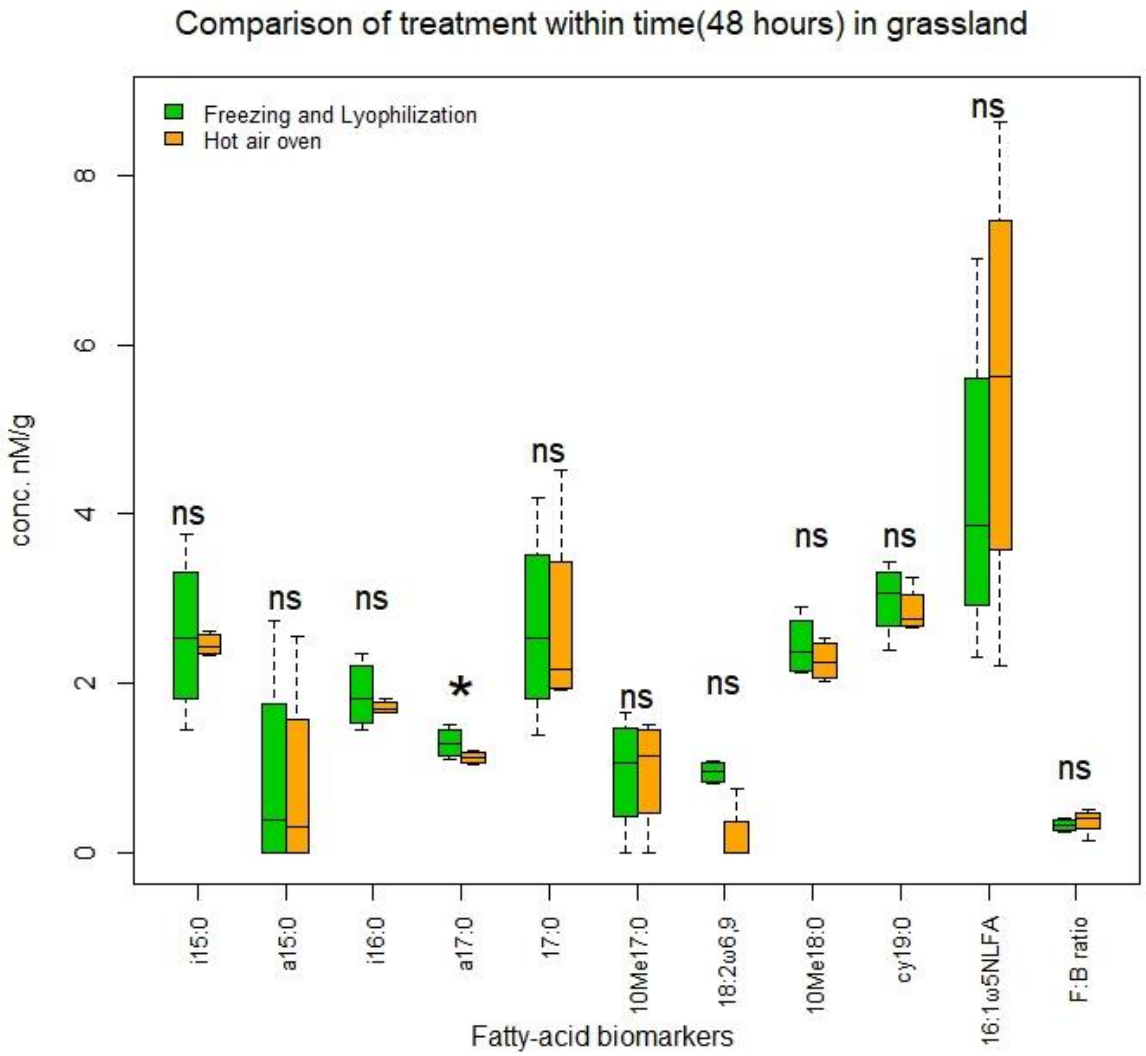


Figure 13. Influence of fatty acid biomarker for sample processed at 48 hours (*significant effects, ns-non-significant effects)

The effect of time within individual treatments were compared, but there was no substantial impact in the concentrations of bacterial biomarkers and total fungal in both field except for AM fungi.

5 CONCLUSIONS

Traits of soil mainly influence the concentrations of fatty acid biomarkers. The microbial communities required favourable conditions for survival. The use of fertilizers and farming might negatively affect some microorganisms due to changes in nutrient content, soil moisture, and soil texture. This is also presented by the study results as biomarkers concentrations of some of the bacterial biomarkers, especially in AM, are relatively higher in grassland than in the arable field. Therefore, it is worth thinking about where the soil is being collected, targeted biomarkers, and the influence in the delay of pre-treatment time before carrying out analysis.

Soil is immediately freeze-dried after sampling, and this is the standard procedure followed in most cases. However, this is not practical in all cases where sampling locations are far. Comparisons of the various pre-treatment methods indicated that both the FL and SG methods are adequate enough to identify and quantify fatty acid biomarkers. In addition, the results obtained from both pre-treatments methods are similar. So, SG can be chosen as the alternative to FL for the farthest sampling locations and convenience.

When the soil pre-treatment was delayed, a comparison between pre-treatments FL and SG showed that concentrations of biomarkers did not have significant differences. However, total fungal biomarkers were influenced when AD samples are compared with FL and SG samples. Surprisingly, the fatty acid concentrations in samples that had undergone immediate pre-treatment were effected. In contrast, the longer delay did not show varying results among pre-treatment methods. This indicates the chance of degradation in a longer time frame. Therefore, if it is FL or SG, prompt pre-treatment could be the only way to achieve the best results.

If pre-treatments were delayed by eight hours, total fungal biomarkers (18:2ω6,9) were influenced by this delay in the grassland sample. Therefore, though it might not require immediate pre-treatment, it is best to carry out immediate pre-treatments. Hot air oven might not be the best choice for evaluating total fungal biomarkers, showing inconsistent results with both SG and FL. AM biomarkers, on the other hand, were not effected by pre-treatment methods or pre-treatment time, but still, there is a decline in concentrations of fatty acids though not significantly. So, it is best to carry out pre-treatment as soon as possible.

The fungal and bacterial biomarkers ratio did not seem to be influenced by treatments or time, giving us the flexibility to use whatever approach or treatments we choose if research on the general ecological pattern is required. In conclusion, SG could be the safest alternative to FL-pre-treatment in terms of simplicity and effectiveness, but still, it is safe to carry out pre-treatment methods as immediately as possible.

6 ACKNOWLEDGEMENT

I would like to express my appreciation and warm thanks to everyone who has assisted and supported me during my study.

First and foremost, I want to express my gratitude to Tanel Vahter and Professor Koit Herodes, my superiors, for their unwavering encouragement and patience. Thank you very much to both of my supervisors for finding time despite the busy schedules to help me out anytime I needed it and keeping me focused during this period.

Professor Ivo Leito, my program coordinator, for his tremendous encouragement and patience during my academic years at this university. I would like to express my gratitude to all my AMS (Applied Measurement Science) and EACH (Excellence in Analytical Chemistry) colleagues.

I would like to express my gratitude to my parents (Mahendra and Saraswati Kasaju) and sister (Samjhana Kasaju) for believing in me and being pillars of my growth. Finally, without my husband (Binay Bikram Thapa), whose words and relentless encouragement have given me strength during my academic studies, my path would have been incomplete.

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Annex 1

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Annex 2

Reagent	**Producer**	**Purity, %**	**Molecular weight [g/mol]**	**Density [g/cm^3]**	**Boiling point [°C]**	**Melting point [°C]**	**CA**
Acetone	Merck	≥ 99.8	58.08	0.8	56	-95	67-64-1
Acetic acid	Merck	≥ 99.8	60.05	1.05	118	16.7	64-19-7
Chloroform	Fisher Scientific	99.8	119.38	1.7	62	-64	67-66-3
Hexane	Merck	≥ 97	86.18	0.7	69	-95	110-54-3
Methanol	Fisher Scientific	32.04	32.042	0.79	65	-98	67-56-1
Toulene	Merck	≥ 99.9	92.14	0.87	111	-95	108-88-3
Citric acid anhydrous	Merck	≥ 99	192.12	1.66	310	153	77-92-9
Potassium Hydroxide	Merck	≥ 85.0	56.11	2.04	1324	380	1310-58-3
Sodium Hydroxide	Merck	≥ 99.0	40.00	2.1	1388	318	1310-73-2

Annex 3

Fatty acid concentrations in grassland soil (SG)

Pre-treatment time	Concentrations (nM/g)								
	i15:0	**a15:0**	**i16:0**	**a17:0**	**17:0**	**10Me17:0**	**18:2ω6,9**	**10Me18:0**	**cy19:0**
0 hour									
Sample 1	2.26	0.00	1.48	0.97	1.97	0.72	3.62	2.16	2.16
Sample 2	2.46	2.62	1.77	1.09	1.87	1.88	0.00	2.71	2.89
Sample 3	1.82	0.00	1.50	1.06	1.46	1.05	0.95	2.35	3.26
Sample 4	2.75	0.00	1.75	1.17	2.41	1.35	0.86	2.35	3.57
8 hours									
Sample 1	2.13	0.00	1.64	1.14	1.28	0.96	0.91	2.45	4.06
Sample 2	1.35	1.65	1.05	0.82	1.39	2.37	0.78	2.26	1.91
Sample 3	3.17	2.96	2.03	1.26	2.15	1.87	1.08	2.79	2.99
Sample 4	3.63	0.79	2.28	1.38	2.69	1.32	0.93	2.52	3.44
24 hours									
Sample 1	1.61	2.01	1.38	0.99	1.61	1.69	0.00	2.48	2.53
Sample 2	3.19	0.78	2.06	1.24	2.41	1.39	0.82	2.29	3.39
Sample 3	1.10	0.00	1.10	0.79	1.24	0.90	0.66	1.85	2.60
Sample 4	1.92	0.00	1.65	1.11	1.48	0.00	0.00	2.26	2.79
48 hours									
Sample 1	2.36	2.55	1.73	1.10	1.92	1.52	0.00	2.55	2.69
Sample 2	2.62	0.00	1.82	1.22	2.36	1.37	0.00	2.39	3.25
Sample 3	2.53	0.61	1.67	1.05	1.96	0.94	0.76	2.02	2.67
Sample 4	2.33	0.00	1.66	1.16	4.53	0.00	0.00	2.12	2.85

ANNEX 4

Fatty acid concentrations in grassland sample (AD)

Pre-treatment time	Concentrations (nM/g)								
	i15:0	**a15:0**	**i16:0**	**a17:0**	**17:0**	**10Me17:0**	**18:2ω6,9**	**10Me18:0**	**cy19:0**
0 hour									
Sample 1	1.84	0.00	1.23	0.87	1.69	1.97	0.00	1.75	2.15
Sample 2	2.06	2.50	1.54	1.05	1.73	1.91	0.00	2.49	2.55
Sample 3	1.48	0.00	1.30	1.03	4.28	0.97	0.00	2.14	3.11
Sample 4	2.99	0.77	1.85	1.20	2.36	1.48	0.80	2.23	3.55
8 hours									
Sample 1	1.93	0.00	1.56	1.15	1.99	1.06	0.72	2.22	4.00
Sample 2	1.06	1.45	0.91	0.75	1.18	2.38	0.00	2.17	2.03
Sample 3	2.53	2.82	1.69	1.05	1.55	1.64	0.00	2.33	2.56
Sample 4	2.43	0.00	1.74	1.15	1.99	1.38	0.00	2.08	3.32
24 hours									
Sample 1	0.97	1.41	1.02	0.81	1.15	2.36	0.00	2.22	1.94
Sample 2	2.32	2.75	1.78	1.07	1.57	1.60	0.00	2.34	2.41
Sample 3	2.31	0.00	1.90	1.21	2.05	1.33	0.00	2.21	3.15
Sample 4	1.21	0.00	1.38	0.96	1.01	0.95	0.00	2.03	2.90
48 hours									
Sample 1	2.24	2.98	1.72	1.14	1.65	1.72	0.00	1.91	3.25
Sample 2	1.36	0.00	1.28	0.96	1.54	0.88	0.00	1.91	2.65
Sample 3	2.24	0.00	1.55	1.06	1.22	0.00	0.00	1.97	2.72
Sample 4	1.19	0.00	1.28	1.03	3.82	0.00	0.00	2.01	2.71

Annex 5

Fatty acid biomarkers concentrations in field (FL)

Pre-treatment time	Concentrations (nM/g)								
	i15:0	**a15:0**	**i16:0**	**a17:0**	**17:0**	**10Me17:0**	**18:2ω6,9**	**10Me18:0**	**cy19:0**
0 hours									
Sample 1	2.42	0.75	1.24	0.78	1.57	1.87	0.74	1.74	1.45
Sample 2	2.14	3.06	1.34	0.88	1.29	1.74	0.00	2.36	1.26
Sample 3	1.99	0.00	1.23	0.86	1.72	1.33	0.00	1.89	1.81
Sample 4	2.42	0.00	1.37	0.89	1.67	0.88	0.81	1.95	1.66
8 hours									
Sample 1	1.83	0.51	1.13	0.75	1.39	1.73	0.64	1.60	0.00
Sample 2	1.24	1.97	1.26	0	1.06	1.81	0.00	2.05	1.01
Sample 3	1.81	0.00	1.28	0.87	1.41	1.38	0.00	1.81	1.79
Sample 4	1.37	0.00	1.14	0.86	3.43	0.90	0.98	1.92	1.81
24 hours									
Sample 1	2.67	0.69	1.62	1.13	1.91	1.20	0.85	2.09	1.96
Sample 2	1.04	1.92	1.27	0	0.92	2.27	0.00	1.89	0.96
Sample 3	1.85	2.89	1.27	0.91	1.15	1.88	0.00	2.10	1.42
Sample 4	1.43	0.00	1.15	0.95	3.43	0.83	0.87	1.99	1.94
48 hours									
Sample 1	1.14	1.77	0.89	0.78	1.11	2.12	0.59	2.08	1.51
Sample 2	1.81	0.00	1.23	0.98	1.65	1.22	0.66	1.80	1.75
Sample 3	1.94	3.02	1.49	1.06	1.28	1.63	0.00	2.15	1.38
Sample 4	0.88	0.00	0.87	0.81	1.22	0.66	0.54	1.73	1.48

Annex 6

Fatty acid concentrations in field sample (SG)

Pre-treatment time	Concentrations (nM/g)								
	i15:0	**a15:0**	**i16:0**	**a17:0**	**17:0**	**10Me17:0**	**18:2ω6,9**	**10Me18:0**	**cy19:0**
0 hours									
Sample 1	0.94	0.00	0.68	1.29	0.94	1.75	0.00	1.03	0.95
Sample 2	1.12	2.26	0.80	0.00	0.86	1.80	0.00	1.74	0.85
Sample 3	1.33	0.00	0.92	0.00	1.13	1.28	0.00	1.49	1.37
Sample 4	1.03	0.00	0.88	0.63	1.24	0.84	0.90	1.63	1.51
8 hours									
Sample 1	1.43	2.25	1.05	0.63	1.00	1.74	0.00	1.99	1.37
Sample 2	1.04	2.40	0.69	0.70	0.87	2.31	0.00	1.72	0.72
Sample 3	1.37	0.00	0.98	0.00	1.18	1.22	0.00	1.50	1.65
Sample 4	1.60	0.00	1.11	0.00	1.30	0.82	0.69	1.59	1.53
24 hours									
Sample 1	1.57	2.39	1.20	0.73	1.09	1.55	0.63	2.15	1.44
Sample 2	0.00	2.38	0.00	0.78	0.00	2.34	0.00	1.06	0.00
Sample 3	1.51	0.00	1.02	0.00	1.20	1.21	0.00	1.52	1.43
Sample 4	1.09	0.00	0.90	0.72	1.29	0.86	0.59	1.70	1.73
48 hours									
Sample 1	0.64	1.32	0.59	0.57	0.93	2.41	0.61	1.91	1.33
Sample 2	1.38	0.00	0.91	0.00	1.21	1.16	0.00	1.48	1.46
Sample 3	0.74	0.00	0.72	0.65	1.09	0.75	0.00	1.46	1.35
Sample 4	1.61	0.00	1.07	0.82	1.35	0.00	0.00	1.60	1.45

Annex 7

Fatty acid concentrations in field sample (AD)

Pre-treatment time	Concentrations (nM/g)								
	i15:0	**a15:0**	**i16:0**	**a17:0**	**17:0**	**10Me17:0**	**18:2ω6,9**	**10Me18:0**	**cy19:0**
0 hours									
Sample 1	1.53	0.76	0.95	0.00	0.00	1.99	0.00	1.30	1.14
Sample 2	1.48	2.50	1.07	0.00	0.91	1.71	0.00	1.92	1.31
Sample 3	0.00	0.00	1.14	0.73	1.21	1.29	0.00	1.55	1.65
Sample 4	1.23	0.00	0.91	0.00	1.03	0.91	0.00	1.54	1.53
8 hours									
Sample 1	1.37	2.19	0.99	0.00	0.87	1.53	0.00	1.82	1.30
Sample 2	1.03	2.11	0.78	0.67	0.86	2.26	0.00	1.74	1.39
Sample 3	1.64	0.00	1.13	0.00	1.21	1.19	0.00	1.61	1.66
Sample 4	1.77	0.00	1.06	0.73	1.27	0.92	0.00	1.61	1.72
24 hours									
Sample 1	1.39	0.00	1.11	0.79	1.33	1.14	0.00	1.67	1.61
Sample 2	1.19	2.51	0.97	0.74	1.07	2.53	0.00	2.09	1.38
Sample 3	1.26	2.15	1.03	0.70	0.94	1.56	0.00	1.89	1.27
Sample 4	0.92	0.00	0.83	0.68	1.15	0.78	0.00	1.59	1.72
48 hours									
Sample 1	1.48	2.90	0.96	0.73	1.01	1.52	0.00	1.91	1.30
Sample 2	1.49	0.00	0.99	0.77	1.31	1.04	0.00	1.52	1.52
Sample 3	1.44	0.00	0.93	0.82	1.38	0.84	0.00	1.70	1.56
Sample 4	1.40	0.00	0.93	0.73	1.07	0.00	0.00	1.37	1.17

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INFORMATION SHEET

Effect of soil sample preparation on fatty acid biomarker content

Different techniques for the pre-treatments were applied at various time frames to evaluate their impact on fatty acid biomarkers content for the soil sampled from Suurmetsa, Põlva County, Estonia, one from the arable field the other from grassland. The fatty acids were extracted by the PLFA method and analysed by using GC-FID. The study included eight bacterial biomarkers (i15:0, a15:0, i16:0, a17:0, 17:0, 10Me17:0 ,10Me18:0, cy19:0),total fungal biomass 18:2ω6,9 and AM fungal biomass by group specific NLFA (16:1ω5). Three pre-treatment methods (Freezing and Lyophilization, Silica gel, and Hot Air Oven) on four different time frame (0, 8, 24, 48) was carried out. Comparing different pre-treatment methods showed both freezing and lyophilization, and the silica gel method is adequate to identify fatty acid biomarkers content. Still, soil pre-treatment should be done as early as possible to get better results. However, for studying general ecological patterns, whichever approaches, or treatment methods can be chosen.

Keywords: Phospholipid fatty acids, Neutral lipid fatty acids, Fatty acid biomarkers, Pre-treatment methods

CERCS: P300 analytical chemistry

INFOLEHT

Pinnaseproovi ettevalmistamise mõju rasvhapete biomarkeri sisaldusele

Mullaproovi ettevalmistamise metoodika mõju rasvhapete biomarkerite sisaldusele.

Töös viidi läbi katsed hindamaks mullaproovi ettevalmistamise metoodika mõju rasvhapete biomarkerite sisaldusele. Kuna mulla rasvhapete biomarkeri sisaldused võivad ka pärast proovi kogumist organismide elutegevuse tõttu muutuda, on proovi ettevalmistuse metoodika usaldusväärsete tulemuste jaoks oluline faktor. Katsetati kolme eeltöötluse meetodit (külmutamine ja lüofiliseerimine, kuivatamine ränigeeli abil, kuivatamine pöördõhuga ahjus) ja nelja erinevat ajaviibe intervalli (kohene töötlemine, töötlemine 8h, 24h ja 48h pärast proovi kogumist). Mullaproovid koguti kõrvuti asetsevalt põllu- ja rohumaalt. Meetodite võrdlemine näitas, et kõik katsetatud ettevalmistuse meetodid on rasvhapete analüüsiks sobivad, kuid mulla eeltöötlus tuleks läbi viia võimalikult kiiresti pärast proovi kogumist. Ajavahemikul proovi kogumisest kuni eeltöötlemiseni jätkuvad muutused mõnede rasvhapete biomarkerite sisaldustes.

Märksõnad: fosfolipiidsed rasvhapped, rasvhapete biomarkerid, eeltöötlusmeetodid

CERCS: P300 analüütiline keemiaCERCS: P300 analüütiline keemia