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DEVELOPMENT OF A HIGH-THROUGHPUT METHOD FOR SOIL FATTY ACID DERIVATIZATION AND ANALYSIS

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INFORMATION SHEET

DEVELOPMENT OF A HIGH-THROUGHPUT METHOD FOR SOIL FATTY ACID DERIVATIZATION AND ANALYSIS

The Bligh and Dyer method of extraction and separation of phospholipid fatty acids (PLFAs) and neutral lipid fatty acids (NLFAs) has been extensively used in soil microbial community studies. A high throughput method of soil fatty acid analysis was developed which involves the extraction of fatty acids, separation of the fatty acids to PLFAs and NLFAs using solid-phase extraction technique, and subjecting the fatty acids to mild alkaline methanolysis prior to analysis using a GC-FID. Where possible stages were modified to be carried on a 96 well plate multi-tier unit, increasing the sample throughput and decreasing the overall consumption of solvents, cost of analysis, and time. The novel high throughput method showed comparable biomass and community characteristics to that of the standard method and thus, could be proposed as a novel method of fatty acid analysis.

Keywords: PLFA, NLFA, GC-FID, high throughput method, fatty acid analysis, soil

CERCS Code: P300 analytical chemistry

MULLA RASVAHAPETE DERIVATISEERIMISE JA ANALÜÜSI KÕRGE LÄBILASKVUSE MEETODI ARENDUS

Bligh and Dyer meetodit kasutatakse laialdaselt fosfolipiidsete rasvhapete (PLFA) ja neutraalsete lipiidide rasvhapete (NLFAs) ekstraheerimise ja eraldamise jaoks mulla mikroelustiku koosluse uurigutes. Magistritöös töötati välja mulla rasvhapete analüüsiks kõrge läbilaskvuse meetod, mis hõlmab rasvhapete ekstraheerimist, rasvhapete eraldamist PLFA-deks ja NLFA-deks, kasutades tahke faasi ekstraheerimistehnikat. Rasvhapped derivatiseeriti leeliselise metanolüüsiga ja analüüsiti kasutades GC-FID metoodikat. Arendustöös modifitseeriti analüüsi etappe, et viia need läbi 96 kaevuga plaadilsüsteemil, suurendades meetodi läbilaskevõimet ja vähendades lahustite üldist tarbimist, analüüsikulusid ja aega. Uudne kõrge läbilaskvusega meetod näitas standardmeetodiga võrreldavaid biomassi ja koosluste koosseisu hinnanguid ning seetõttu saab seda pakkuda välja uueks rasvhepete analüüsi meetodiks.

Märksõnad: PLFA, NLFA, GC-FID, kõrge läbilaskvusega meetod, rasvhapete analüüs, muld

CERCS kood: P300 analüütiline keemia

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ABBREVIATIONS

Abbreviation	Full form
AM	Arbuscular mycorrhiza
DNA	Deoxyribonucleic acid
EL-FAME	Ester-linked fatty acid methyl ester
F: B	Fungal: Bacterial ratio
FAME	Fatty Acid Methyl Esters
GC	Gas chromatography
GC-FID	Gas chromatography with flame ionization detection
GC-MS	Gas chromatography with mass spectrometry detection
GC-TOF-MS	Gas chromatography with Time of flight mass spectrometry detection
ITS	Internal transcribed spacer region
IUPAC	International Union of Pure and Applied Chemistry
NIST	National Institute of Standards and Technology
NLFA	Neutral Lipid Fatty Acids
NMDS	Non-metric multidimensional scaling
PERMANOVA	Permutational multivariate analysis of variances
PLFA	Phospholipid Fatty Acids
PTFE	Polytetrafluoroethylene
RNA	Ribonucleic acid
SPE	Solid-phase extraction
SSU	Small sub-unit region

1. INTRODUCTION

Phospholipid fatty acids (PLFAs) and neutral lipid fatty acids (NLFAs) have been widely used as microbial biomarkers in soil ecological studies. They can be indicative of main microbial groups and are extensively used in the investigation of soil microbial community structures. The biomarkers provide crucial data for the elucidation of general soil health, using various ecological indices such as fungi: bacteria ratio, and are used in various ordination techniques in distinguishing microbial communities from different soils. This method has been used in studies of both natural and anthropogenic perturbations as well as in the identification of different taxonomic groups in the soil ecosystems.

The Bligh and Dyer method of extraction and separation of fatty acids has been the gold standard in the fatty acid analysis of soil ecological studies. A plethora of scientific literature in the direct or modified applications of the method proves its versatility and informativity. The standard protocol involves the extraction of soil lipids and the fractionation to their key components depending on their polarity, into PLFAs and NLFAs, using solid-phase extraction. The fatty acids separated are derivatized to their fatty acid methyl esters (FAMEs) to make them suitable for detection and quantification using GC-FID. The method is, however, relatively laborious and requires skilled personnel to perform. The primary drawbacks of the method involve the low sample processing capacity and the use of high volumes of toxic and carcinogenic solvents such as chloroform. High throughput methods have been proposed to address the need of processing a large number of samples, especially in large ecological projects and other applications.

The current research project aimed to establish a high throughput method in the settings of the Plant Ecology Laboratory, following the Bligh and Dyer extractant chemistry of the classical method which is already well established [1], emulating the proposed protocol by Buyer and Sasser [2] as a primary reference. The project aimed to develop the method so that the full potential of the 96 well plate multi-tier system and the GC instrument autosampler functionalities could be utilized to increase the sample throughput. The efficacy of the novel method was tested using various indices extensively used in routine ecological studies.

2. REVIEW OF LITERATURE

2.1. Soil microbial fatty acids and their significance in ecology

2.1.1. Lipids

Lipids are a broad, highly varied, group of biological compounds, present in all living organisms. Lipids play an important role both as an energy source and vital structural components in cell membranes [3]. The two major groups of lipids found in organisms are polar lipids, such as phospholipids, as cell membrane components, and neutral lipids which function as an energy source [4]. In these two types of lipids, fatty acids are found connected to glycerol by ester bonds. The varied enzymatic capabilities in lipid metabolism observed in nature result in great structural diversity and organismal specificity of the fatty acids [5]. Fatty acids derived from the phospholipids are named phospholipid fatty acids (PLFAs) and those derived from neutral lipids are named neutral lipid fatty acids (NLFAs).

2.1.2. Nomenclature of fatty acids

The IUPAC nomenclature provides the possibility to classify fatty acids based on the systematic name, while unsaturated fatty acids are named with the number of carbon atoms from the carboxyl group (Δ end) to the nearest unsaturation [3]. Still, due to the wide variety of possible structures of fatty acids, a compact notation was developed in naming fatty acids. The omega system names fatty acids following the naming pattern A:BωC. Here, A represents the number of carbon atoms in the fatty acid backbone, B represents the number of double bonds, and C is the distance to the nearest unsaturated carbon atom from the aliphatic (ω) end of the fatty acid. A suffix of “c” represents cis form and “t” represents the trans configuration of the double bond. Prefixes “i” represents iso, “a” represents anteiso, and “br” stands for an unknown branching position in the carbon backbone. A “Me” following the carbon atom number with the mid-chain branch point presents a methyl fatty acid. For example, “10Me” denotes a methyl group on the tenth carbon atom from the carbonyl end of the fatty acid. The prefix “cy” represents cyclopropyl fatty acids [6,7].

2.1.3. Fatty acid profiling

The composition of lipids is extensively used to study microbial communities based on their chemical signature [3]. Owing to their abundance and chemical diversity [8] and the huge volumes of research done on elucidating and linking the chemical identity to the microbial groups, fatty acids are extensively used in ecological studies. Some of these chemotypes based on the fatty acid patterns are summarised in Table 1.

Table 1. PLFA and NLFA biomarkers used in microbial taxonomy. The prefixes i, a, and cy stands for iso, anteiso, and cyclopropyl ring respectively. A Me proceeded by a number represents a methyl group at a specific branch point.

Taxonomic group	PLFA or NLFA biomarker group	Specific examples	Reference
All organisms	Saturated straight-chain PLFAs	12:0, 13:0, 14:0, 15:0, 16:0, 17:0, 18:0	[6]
Bacteria	Multiple groups of PLFAs	i15:0, a15:0, i16:0, i17:0, a17:0, cy17:0, cy19:0, 16:1ω7, 17:1ω9, 18:1ω7	[7]
Gram-positive bacteria	Branched PLFAs	i14:0, i15:0, a15:0, i16:0, i17:0, a17:0	[6,7]
Gram-negative bacteria	Cyclopropyl and mono PLFAs	cy17:0, 16:1ω7, 18:1ω7, 17:1ω9	[7]
Actinomycetes	10Me-PLFAs	10Me16:0, 10Me17:0, 10Me18:0	[6,7]
Fungi	Polyunsaturated PLFAs	18:2ω6,9	[7]
Arbuscular mycorrhizal (AM) fungi	NLFA	16:1ω5	[4]

2.1.4. Applications of fatty acid profiling

Soil bacterial community structure is commonly measured using PLFA and NLFA profiling [9]. The method has been applied in a range of studies from agricultural and farming research [10–12],

ecological studies [13–15], archeology [16], bioremediation studies [17], and other research activities [18]. The studies also vary in the soil sampling sites that span natural habitats such as forests [15], mangroves [19], and floodplains [20] to anthropogenic sources such as compost [21] and genetically modified corn fields [22].

2.1.5. Ecological indices

The difficulty of measuring soil microbial activity in the natural environment has been hampering ecological studies and remained a black box even after the advent of methods to quantify soil microbial biomass [23]. Biological indicators and indices are commonly used to assess the interactions of soil microbial communities and their abiotic environment [24]. These interactions are well documented and have been observed to have a pivotal role in ecosystem functions [24]. These functionalities can be monitored by the analysis of various chemicals directly involved in these processes and by analysis of key microbial players in the soil community [24]. Changes in soil properties such as pH, depth of the soil, moisture, and temperature can greatly impact the soil microbial community composition and biomass [25]. The fungal: bacterial (F: B) ratio has been proposed as a method to evaluate the response of the soil microbiota to effects such as pH, soil carbon and nitrogen dynamics, and other environmental changes [25]. These indices measured via changes in soil microbial populations provide an early indication of ongoing changes in the soil which cannot be easily obtained by other means. The total PLFA amount in soil has a direct strong correlation with the soil microbial biomass [26] and has been used in various studies [20,27]. The association of fungi in soils with low decomposition rates and low nutrient availability and the converse association of bacteria to soils with high decomposition rates and nutrient availability means that an increase in the F: B ratio should reflect a slow growth rate and a reduction in nutrient availability [9]. It can be indicative of soil pH changes as fungi are noted to be more acid-tolerant than bacteria which will be reflected in the dominance of fungi in acidic soil [25].

Other applications of PLFAs in the calculation of ecological indices involve Gram-positive: Gram-negative ratio [28] and microbial physiological or nutritional stress indicators such as saturated: monosaturated PLFAs ratio and the sum of cyclopropyl PLFAs to the sum of their monoenoic precursors [7]. The PLFA and NLFA analysis can provide insight into the prevailing bacterial community structure as fatty acids are quickly degraded in nature and the analysis would predominantly measure the fatty acids derived from living organisms [29]. The application of these

chemotypes in profiling various microbial groups has been proposed by several authors (refer the Table 1). But the use of fatty acids as species proxies has been cautioned against in their retrospective article on the use and misuse of this analysis by Frostegård *et al* [30].

2.2. Methods of fatty acid analysis

2.2.1. Method evolution and current status.

Early quantification of microbiota involved staining and microscopical analysis which resulted in the observation of both living and dead organisms [4]. The study of microbial communities by culture methods has the drawback of poor or varied growth rates between organisms and the non-representativeness of the prevailing community [24,29]. Various biomolecular analyses were later introduced to study the microbial community of soil such as the use of chitin and ergosterol to measure the biomass of ectomycorrhiza and arbuscular mycorrhiza [4], 16S rRNA gene metabarcoding [9], and analysis of many other microbial cell constituents. With the discovery of the possibility to use fatty acids as biomarkers, novel methods were proposed for microbial analysis. Several methods of fatty acid methyl ester (FAME) profiling were introduced such as the analysis via phospholipid fatty acid (PLFA), ester-linked fatty acid methyl ester (EL-FAME) [31,32], and other techniques [8]. The extraction methods have seen further developments [33] along with the improvements in the techniques for detection [34,35] but the use of Gas chromatography with flame ionization detection (GC-FID) dominates the research studies. This is owing to GC-FID's simplicity in operation and maintenance, its greater linear range, and the sensitivity, reliability, and reproducibility in comparison with other analytical instruments [6]. It is relatively a cheaper investment for most ecology laboratories compared to other proposed analytical instruments such GC-TOF-MS [36].

The sample extraction method, initially performed for fatty acid extraction from fish tissues [37], eponymously called Bligh and Dyer method, gained traction with its application proposed for the analysis of soil microbiota by Frostegård *et al* [38,39]. Here they made changes such as the use of citrate buffer and further modifications were made by multiple other researchers, such as the use of solid-phase extraction (SPE) and use of methanolic KOH [40], making the Bligh and Dyer method the gold standard of fatty acid profiling. The method itself is advantageous by being relatively rapid compared to the original method by Bligh and Dyer [37] and typically also being sensitive and reproducible [30] compared to other methods.

2.2.2. High throughput method.

Although the Bligh and Dyer method have its pros, making it a mainstay in soil ecological studies, it is still underpinned by the use of carcinogenic, toxic, and environmentally unfriendly solvents such as chloroform and methanol. This led to various studies being carried out to find eco-friendly "greener" substitutes for these solvents [41]. Yet another developmental direction adopted by other researchers was towards miniaturizing the process by the use of 96 well plate systems [2,31]. This approach has the added benefit of increasing the sample throughput making it suitable for the application in large-scale studies [2] at the same time reducing the solvent usage per sample, the overall costs, and the time consumed. A key point of improvement that could be made on the protocol proposed by Buyer and Sasser [2] is the continued use of the 96 well plate system throughout, including the GC analysis phase, compared to the use of classical GC vials, as proposed by Buyer and Sasser. This would tap the true potential of the 96 well plate system, further cutting down on the costs and time consumed.

3. RESEARCH AIMS

The primary goal of the current study was to establish a high throughput method for soil fatty acid analysis adhering to the Bligh and Dyer extraction chemistry and using the protocol already well established in the Plant Ecology Laboratory [1], as closely as possible. The method was developed using the protocol developed by Buyer and Sasser [2] as a primary reference. The method implemented was evaluated for its performance efficacy by comparing with parallelly run classical method of lipid analysis and by evaluating the microbial biomass and microbial community.

The sub-objectives of the study encompass the selection of a suitable solvent to be used during the instrumental analysis stage, due to the inferior practical applicability of the classical solvent, n-hexane, in the high throughput method. Another objective was to upgrade the current laboratory and instrumental settings to efficiently accommodate the implementation of a high throughput method.

4. EXPERIMENTAL

4.1. Chemicals

All solvents and chemicals used were of analytical grade or above. Solvents used in the primary research work were chloroform (Fischer Scientific UK), methanol (Fischer Scientific UK), acetone (Merck KGaA, Honeywell, Germany), toluene (Merck KGaA), and n-hexane (Honeywell, Germany). Chemicals used were citric acid (Merck KGaA), acetic acid (Merck KGaA), sodium hydroxide (Merck KGaA), and potassium hydroxide (Merck KGaA). Additional solvents used in the preliminary evaporation experiment were 2,2,4-trimethylpentane (Lachner, Slovakia Republic), toluene (Sigma-Aldrich, USA), octane (PEAHIM, Russia), and dimethyl sulfoxide (Sigma chemical company, USA). Further information regarding the solvents and chemicals used is presented in Annex 1. A detailed description of reagents and solvent mixtures used in this research is provided in Annex 2.

4.2. Instruments

An Agilent (Agilent Technologies, Inc. USA) 7890A Gas Chromatography System equipped with an Agilent GC Sampler 80 autosampler was used. Vortexing was done using Vortex-Genie 2 (Scientific Industries, USA) vortex machines, all centrifugations were performed using an Eppendorf 5804 R Centrifuge (Eppendorf, Germany), a Reacti-Therm III Heating Module (Thermo Scientific, USA) was used to dry under nitrogen, and an ultrasonic cleaner (Telsonic Ultrasonics Inc., USA) was used for sonication processes and as well as a hot water bath. All pH adjustments were performed using a Hanna HI 98191 (Hanna Instruments, UK) pH meter. A 96 well plate nitrogen evaporator, built in-house (see Annex 4), was used for the drying stages of the 96 well multi-tier unit. Pipetting was performed using 50 – 200 µL, 200 – 1000 µL automatic pipettes (Gilson, Inc. USA), 30 – 300 µL multichannel pipette (Biohit, Finland), 100 -1200 µL multichannel pipette (Finnpipette electronic pipette, Thermo Fischer Scientific, USA) and 1 – 5 mL automatic pipette (Thermo Fischer Scientific, USA). Distilled water was prepared using a Stillo distiller (Mocom, Italy).

4.3. Materials

Solid-phase extractions were performed in Hypersep Silica (500 mg) syringe barrel columns (Thermo Scientific, USA) in the classical method and Discovery DSC SI SPE (50 mg) 96 plates

(Supelco Inc., USA) in the high throughput method. Sample extractions were initially done in 50 mL polytetrafluoroethylene (PTFE) tubes with screw caps. Subsequent stages were performed in 16 mm x 150 mm (approximately 35 mL) and 13 mm x 100 mm (approximately 8 mL) glass tubes with screw caps and PTFE liners. Gas chromatography vials, glass inserts (250 µL) with polymer feet, and screw caps with PTFE septa (Agilent, USA) were used for the sample analysis using the classical method. The 96 well multi-tier unit (MTP System ABS Plate) with prescored PTFE/Silicone mats were used along with 9 mm x 30 mm (1.0 mL) and 9 mm x 44 mm (1.5 mL) glass conical vials (Qmx Laboratories, UK). All glassware was washed in running tap water, then in distilled water before heating in an oven at 400˚C for 4 hours to incinerate all organic matter. A PTFE multichannel reservoir/trough, manufactured in-house, was used for all multichannel pipetting purposes (see Annex 4).

4.4. Method outline

The method thus far implemented in the Plant Ecology Laboratory for the analysis of soil fatty acids will be referred to as the classical method [1] in the following text. The method established in the current research work, referring to the work done by Buyer and Sasser [2], with modifications, will be referred to as the high throughput method.

Sampling needs to be done under suitable conditions before analysis, adhering to appropriate sampling protocols. In the current research work, the samples used in the analysis were procured from a batch of samples already appropriated for the DNA analysis studies (EIC Environmental program project SLTOM19092 [42]), and thus, the sampling stage for this project was not performed. The samples collected were dried and stored in permeable paper bags placed inside zip lock bags containing dry silica gel. Out of the 219 samples of the above project 47 samples were randomly selected for the comparative analysis of the classical and high throughput methods taking into account the constraint of the available number of PTFE centrifuge tubes and the necessity of performing the extractions in a single batch to avoid variability between separate extractions.

The general progression of sample preparation and analysis of soil fatty acids using the classical method and the high throughput method follows the same key phases (Figure 1). The lipids are extracted in a two-phase extraction process, using methanol, chloroform, and water [41].

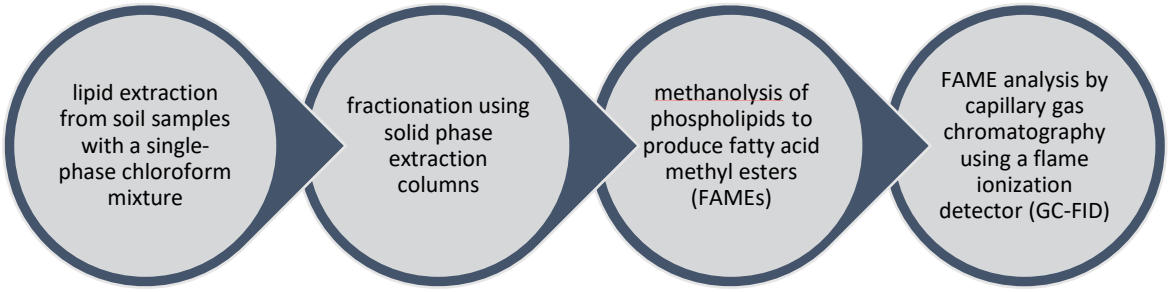

Figure 1. Overview of the method.

The lipid content of the soil samples was extracted using the Bligh and Dyer extractant, as previously described [40], into the chloroform mixture. The lipids extracted are fractionated into the respective fatty acid fractions according to the increasing polarity. Neutral lipid fatty acids (NLFA), glycolipids, and phospholipid fatty acids (PLFA) are eluted with chloroform, acetone, and methanol respectively using solid-phase extraction (SPE). The fatty acids are then derivatized by mild alkaline methanolysis (transesterification) to make them apt for gas chromatographic analysis. Samples were analyzed using GC-FID.

4.5. Solvent selection

Tests were performed on the substitution of n-hexane with a more appropriate solvent, that could last a GC analysis run time of approximately 5 days. For this, 100 µL of n-hexane, 2,2,4-trimethylpentane, toluene, octane, and dimethyl sulfoxide (DMSO) were transferred to separate 1.5 mL glass conical vials, saturated with 500 µL of the respective solvent, with GC glass inserts, in a 96 well multi-tier unit. The multi-tier unit was placed near a heat radiator to emulate approximate conditions the samples will be subjected to during the GC run. Solvents that showed promising evaporation characteristics were researched for the Kovats' retention index in the National Institute of Standards and Technology (NIST) [43] web page for the possible retention overlaps with the analytes of interest.

4.6. Analysis of fatty acids using Classical and High throughput methods

Attempts were done to follow the chemical logic of the classical method as much as possible during the method development for the high throughput method, taking the method described by Buyer and Sasser [2] as general methodological guidance. For the detailed description of reagents and solvent mixtures used, refer to Annex 2.

4.6.1. Lipid extraction

Taking into account the fact that soil is a highly heterogeneous matrix and to minimize sample-to-sample variability that could occur in the two methods, approximately 4 g of dry soil was weighed into clean and dry PTFE screw-cap centrifuge tubes. This represents the appropriate weight for the two methods combined [1,2]. To these 47 tubes and the blank tube, 30 mL of Bligh and Dyer extractant was added, representing excess extractant over the combined volumes of the two methods (15 mL for the classical method and 10 mL for the high throughput method). These tubes were then vortexed for 15 seconds and kept at room temperature, with intermittent vortexing every half an hour, for 2 hours. After centrifuging for 10 minutes at 3700 rpm the supernatant was pipetted to clean 16 mm x 150 mm screw-cap glass tubes in the respective volumes of the two methods (15 mL for the classical method and 10 mL for the high throughput method). The mass of the soil sample represented by the above volumes of extractant, out of the total volume of 30 mL, was back-calculated for the calculation of the concentrations of the fatty acid per gram of soil sample. The subsequent stages of extraction were done following the protocols of the respective methods.

Classical method: Chloroform and citrate buffer (4 mL each), was added to the glass tubes, vortexed for 1 min, and kept overnight for the phase separation. The lower phase (3 mL) was pipetted to clean 13 mm x 100 mm screw-cap glass tubes, using a glass Pasteur pipette connected by an adapter to the automatic pipetter. These extracts were evaporated to dryness under nitrogen at 40˚C. Samples were stored at -20˚C till the next phase.

High throughput method: Similarly, chloroform and citrate buffer (3 mL each), was added to the glass tubes, vortexed for 1 min, and kept overnight for the phase separation. The lower phase (3 mL) was pipetted to clean 13 mm x 100 mm screw-cap glass tubes and the procedure was followed as above. A single Bligh and Dyer extraction of 10 mL was opted for as described by Buyer and Sasser [2], as opposed to the two initial extractions performed in the classical method with a total

volume of 15 mL. The volumes of chloroform and citrate buffer were added in similar proportions to that of the classical method.

4.6.2. Lipid fractionation

Classical method: The samples were dissolved in 100 µL of chloroform, vortexed, and transferred to the SPE (500 mg Silica) syringe barrel columns, which were fixed to the vacuum manifold unit via spigots. The process was repeated for another 100 µL of chloroform and the NLFA fraction was collected to clean 13 mm x 100 mm screw-cap glass tubes by eluting each column with 1.5 mL of chloroform. The glycolipid fraction was eluted with 6 mL of acetone and was discarded. Finally, the PLFA fraction was collected to clean 13 mm x 100 mm screw-cap glass tubes by eluting each column with 1.5 mL of methanol. The tubes containing the NLFA and PLFA fractions were evaporated to dryness under nitrogen at 40˚C. Samples were stored at -20˚C until the next phase.

High throughput method: Similarly, the samples were dissolved in 1 mL of chloroform, vortexed, and transferred to the SPE (50 mg Silica) 96 well plate units, which were placed on the 96 well multi-tier units with 9 mm x 44 mm (1.5 mL) glass conical vials. The NLFA fraction was eluted using 0.5 mL of chloroform by centrifugation for 5 min at 3700 rpm. The glycolipid fraction was eluted, by centrifugation for 5 min at 3700 rpm, with 3 mL of acetone, and was discarded. Finally, the PLFA fraction was collected to clean 9 mm x 44 mm (1.5 mL) glass conical vials by eluting with 1.5 mL of methanol under the flow of gravity. The tubes containing the NLFA and PLFA fractions were evaporated to dryness under nitrogen at room temperature. Samples were stored at -20˚C until the next phase. Where applicable, the pipetting was done using multichannel pipettes and using a custom-built PTFE multi-channel reservoir (Annex 4). The volumes used in the elution of the NLFAs and the PLFAs remained the same as that of the classical method as it is the optimum volume applicable in the multi-tier system. The volume of acetone used was cut down in half because the method proposed by Buyer and Sasser [2] involved only 1 mL of acetone and since this volume is proportional to the volume of Bligh and Dyer extractant used in the same article.

4.6.3. Addition of internal standard

To the PLFA and NLFA tubes and vials of the two respective method extractions, 100 µL of the working solution (0.023 mg/mL) of the internal standard (methyl nonadecanoate) was added and dried under nitrogen at room temperature. Samples were stored at -20˚C until the next phase.

4.6.4. Mild alkaline methanolysis (transesterification)

Classical method: To the tubes, 1 mL of toluene: methanol mixture was added, vortexed for 5 seconds, and 1 mL of potassium hydroxide (0.2 M) in methanol was added. The tubes were incubated at 37˚C for 15 minutes in the heating module. After allowing the tubes to cool down for 20 minutes, 2 mL of hexane: chloroform mixture, 0.3 mL 1 M acetic acid solution, and 2 mL of distilled water were added. The tubes were vortexed for 1 min and centrifuged at 3000 rpm for 5 minutes. The upper layer was pipetted into fresh, clean 13 mm x 100 mm screw-cap glass tubes, using glass Pasteur pipettes connected by an adapter to the automatic pipetter. The tubes were evaporated to dryness under nitrogen at room temperature and were stored at -20˚C until the next phase.

High throughput method: To the vials, 0.2 mL of potassium hydroxide (1 M) in methanol: toluene mixture was added, covered with a PTFE/Silica mat, vortexed, and incubated at 37˚C for 15 minutes in a water bath, with intermittent agitation. The tubes were allowed to cool for 20 minutes and 0.4 mL of 0.75 M acetic acid solution was added. To this 0.4 ml of chloroform was added, vortexed briefly, and left to stand for the phase separation (approximately 1 minute). A 0.3 mL of the lower phase was pipetted to clean 9 mm x 30 mm (1.0 mL) glass conical vials. This was repeated to another 0.4 mL of chloroform and 0.4 mL of the lower phase was pipetted into the same vials. The vials were evaporated to dryness under nitrogen at room temperature and were stored at -20˚C until the next phase. The pipetting was done using multichannel pipettes and using a PTFE multi-channel reservoir. This phase emulated the method described by Buyer and Sasser [2] with the modification of the number of moles of potassium hydroxide and acetic acid to represent that of the classical method.

4.6.5. Gas chromatographic analysis

Classical method: The samples were dissolved in 100 µL of n-hexane, vortexed for few seconds, and were transferred to glass inserts (250 µL) with polymer feet inserted in GC vials, saturated with 500 µL of n-hexane. The vials were capped using screw caps with PTFE septa and analyzed using the gas chromatography instrument.

High throughput method: The samples were dissolved in 100 µL of toluene, vortexed for few seconds, and were transferred to glass inserts (250 µL) with polymer feet inserted in 9 mm x 44 mm (1.5 mL) glass conical vials, saturated with 500 µL of toluene. All pipetting was performed

using multichannel pipettes. The vials were covered with a PTFE/Silicone mat and run using the gas chromatography machine.

4.7. GC parameters

The samples were run in an Agilent 7890A Gas Chromatography System equipped with an Agilent GC Sampler 80 autosampler. The classical method used an autosampler tray holder with tray type VT98 and the high throughput method used a tray holder modified to tray type DW96 to accommodate the 96 well multi-tier unit. A sample volume of 1 µL was injected using a 10 µL GC Liq4 V2 syringe (using 6 filling strokes) after three pre-clean solvent washes (filling to a volume of 50%) followed by a pre-clean sample wash of volume 1 µL. The inlet was on splitless mode with purge flow to split vent set at 0.5 minutes to 40 mL/min. The heater temperature was maintained at 300˚C and the carrier gas pressure was set at 3.5 psi. The septum purge flow was set to 3mL/min. The NLFAs and the PLFAs were separated using a J&W 125-5532 DB-5ms column (30 m x 530 µm x 1.5 µm) with helium as the carrier gas at a flow of 4 mL/min at 3.5 psi and an average velocity of 32 cm/s. The initial oven temperature was set at 80˚C for 1 min, then ramped to 160˚C at a rate of 20˚C/min, after which it was ramped at a rate of 5˚C/min till it reached 270˚C, bringing to a total run time of 37 minutes. The flame ionization detector (FID) was used at a temperature of 300˚C, an airflow of 400 mL/min, a hydrogen fuel flow of 30 mL/min, and a makeup nitrogen flow of 25 ml/min. The system was controlled by OpenLAB CDS ChemStation Edition for GC Systems (Agilent Technologies).

4.8. Data analysis

The concentration of the analytes of interest (ng/g of soil) was calculated with respect to the internal standard (methyl nonadecanoate) according to equation 1.

$$C_{analyte\ (\frac{ng}{g})} = \frac{\frac{A_{analyte}}{A_{int.std.}} * Int.std. * \frac{V_{extraction}}{V_{analysis}}}{W_{soil}}, \quad (1)$$

where $C_{analyte\ (\frac{ng}{g})}$ is the amount of analyte in a gram of soil (ng/g), $A_{analyte}$ is the peak area of the analyte of interest, $A_{int.std.}$ is the peak area of the internal standard, $Int.std.$ is the amount of internal standard added to each tube (2300 ng), $V_{extraction}$ is the volume of chloroform used during the initial extraction (7.95 mL in the classical method and 5.63 mL in the high throughput

method), $V_{analysis}$ is the volume of chloroform used for analysis, and W_{soil} is the dry weight of the soil sample.

The final concentration of the analyte of interest is calculated relative to the molar mass of the methyl ester of the internal standard (methyl nonadecanoate) using equation 2.

$$C_{analyte\ (\frac{nmoles}{g})} = \frac{C_{analyte\ (\frac{ng}{g})}}{M_{int.std.}}, \quad (2)$$

where $C_{analyte\ (\frac{nmoles}{g})}$ is the amount of analyte in a gram of soil (nmoles/g), $C_{analyte\ (\frac{ng}{g})}$ is the amount of analyte in a gram of soil (ng/g), and $M_{int.std.}$ is the molar mass of the methyl ester of the internal standard methyl nonadecanoate (M = 312 g/mol).

4.8.1. Fatty acid profiling in the evaluation of microbial biomass

The fatty acids were identified taking into account the relative retention times, with respect to the internal standard, and comparing with the relative retention times of confirmed peak identities on independent studies performed on sample analysis using GC-MS. The fatty acid biomarkers were used as indicators for soil microbial groups [1]. One fatty acid from the NLFA fraction and 21 fatty acids from the PLFA fraction were identified and used in the statistical analysis. The complete list of fatty acids analyzed is given in Annex 3. All the above calculations were performed in Microsoft Excel (Microsoft 365).

For consistency, lipid profiles of the samples that had 10 ml of supernatant for the high throughput method and 15 ml of supernatant for the classical method, in the initial Bligh and Dyer extraction phase, were used for the statistical analysis. The proceeding statistical analysis was performed using R [44] (version 4.0.4) in Rstudio (version 1.4.1103) using packages tydyverse (version 1.3.0; for data wrangling and graphical representations) and vegan (version 2.5-7; the community ecology package with applications in ecological data statistics).

The Fungi: Bacteria ratio is calculated by dividing the summed concentrations of the biomarkers representing the fungi (18:2ω6,9 in the PLFA fraction and 16:1ω5 in the NLFA fraction) by the summed concentration of the biomarkers indicative of bacteria (i15:0, a15:0, i16:0, a17:0, 17:0, 10Me17:0, 10Me18:0, and cy19:0 in the PLFA fraction) in each sample. The results were used to compare the microbial biomasses of the samples obtained by the analysis using the two methods

and Welch two-sample t-test was performed, using R, to find the presence of a significant difference between them.

Linear correlation studies were performed between the amount of 16:1ω5 in the NLFA fraction and the number of reads of the small sub-unit region (SSU) to assess the abundance of arbuscular mycorrhiza (AM) fungal species. The correlation between the amount of NLFA 16:1ω5 and the number of taxa identified by SSU studies was analyzed to assess the number of AM fungal species. Similarly, linear correlation studies were performed between the amount of 18:2ω6,9 and 16:1ω5 in the PLFA fraction and the number of reads of the internal transcribed spacer region (ITS) to assess the abundance of general fungal species. The correlation between the amount of PLFAs 18:2ω6,9 and 16:1ω5 and the number of taxa identified by ITS studies was used to assess the number of general fungal species. The SSU and ITS data were taken from the studies done on the same set of samples carried out in an independent project (EIC Environmental program project SLTOM19092 [42]).

4.8.2. Fatty acid profiling in the evaluation of microbial community profile

The non-metric multidimensional scaling (NMDS) analysis was performed on all the biomarker data collected from the two methods, using the metaMDS function of the vegan package in R. This was performed to visualize the possible differences between the two soil microbial communities with respect to their fatty acid profiles. The significance of the observed pattern in NMDS was assessed by PERMANOVA analysis using the adonis function in the vegan package with 999 permutations.

5. RESULTS AND DISCUSSION

5.1. Solvent selection

Preliminary tests conducted on the high throughput method applicability in the current laboratory settings showed that n-hexane, during the stage of GC analysis, evaporated completely within a day, at that volume and conditions of application. This was due to the inferior sealing capabilities of the glass vials by the prescored PTFE/Silicone mats used in the 96 well plate multi-tier systems. A crude experiment performed on the evaporation of n-hexane, 2,2,4-trimethylpentane, toluene, octane, and dimethyl sulfoxide (DMSO) highlighted toluene, octane, and dimethyl sulfoxide (DMSO) as potential candidates for sample dissolution solvents. These three solvents were observed to be present in substantial volume, to be sufficient for the GC needle to extract the sample for injection, after placing the multi-tier unit for 5 days (Figure 2).

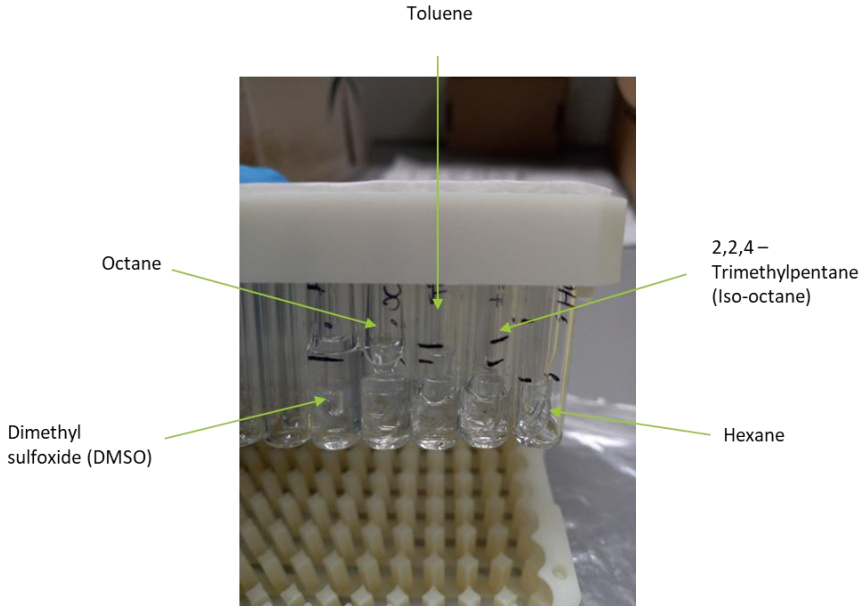

Figure 2. The solvent evaporation experiment. The solvents toluene, octane, and dimethyl sulfoxide (DMSO) were observed to be in substantial amounts after 5 days in the multi-tier setup.

The Kovats' retention indices were obtained from the National Institute of Standards and Technology (NIST) web page. These retention indices were compared with Kovats' retention index of the first analyte of interest, methyl ester of 14:0 (methyl tetradecanoate), and they were found to be much lower than that of methyl tetradecanoate (Kovats' retention index = 1710) (Table

2). This retention index provides a system-independent comparison for the retention times of the solvent and the analytes. Taking into account the suitability for the current setup, with respect to the evaporation characteristics and the Kovats' retention index, and the immediate availability in the Plant Ecology Laboratory, toluene was chosen as the solvent for the sample dissolution for GC analysis.

Table 2. Kovats' retention indices of the solvents and other information. Kovats' retention indices of the solvents under study were taken from the National Institute of Standards and Technology (NIST) [43] website and the approximate values are presented.

Solvent	**Density (g / mL)**	**Melting point (˚C)**	**Boiling point (˚C)**	**Kovats' retention index in nonpolar columns (Approximate)**
n-hexane	0.66	-96 to -94	68.5 to 69.1	600
2,2,4-trimethylpentane	0.69	-107.4	99.3	690
toluene	0.87	-95	111	780
octane	0.70	-57.1 to -56.6	125.1 to 126.1	800
dimethyl sulfoxide (DMSO)	1.10	19	189	780

5.2. Overall method performance

The novel high throughput method was carried out following the Bligh and Dyer extraction chemistry as closely as possible to the classical method routinely followed in the Plant Ecology Laboratory [1]. The author proposes that the solvents and reagents be prepared fresh, before each phase of analysis, at required volumes. The phases of soil fatty acid extraction, silica SPE fractionation, mild alkaline transesterification, and the GC-FID analysis were performed by scaling down the method to suit the 96 well multi-tier system.

The Bligh and Dyer extraction was performed in the classical two-step extraction procedure where the first stage involves the solid/liquid extraction and the subsequent stage of liquid/liquid extraction [41]. Citrate buffer enhances lipid extraction by improving the contact of chloroform and methanol with the microbial cell membrane [33]. This buffer was first proposed by Frostegård *et al* [30] and is reported to give a higher yield compared to other buffers used in other similar

protocols (phosphate and acetate buffer) [3]. If an end-over-end shaker is not at your disposal, the author recommends incubating the samples with intermittent brief vortexing for efficient extraction during this phase. A sample batch of 48 tubes (the blank included) could be conveniently processed at a given moment which involves 3 sets of centrifugations, using the standard rotors with 16 wells, for the set of large polytetrafluoroethylene (PTFE) tubes and two such batches can be pooled together for the subsequent stages of the method, to use 96 well multi-tier system. The hydrophobic lipids will be partitioned to the organic solvent-rich ternary fluid phase, which would be later processed for analysis, while the water-rich phase will contain the unwanted compounds partitioned to it.

The lipid fractionation and the latter phases were performed using a custom-made PTFE trough and multi-channel pipettes for all pipetting purposes along with the multi-tier unit. The use of a PTFE trough minimizes the effect of possible extraneous chemicals that could encounter from the use of commercially available plastic troughs, due to plasticizers and slipping agents that are used in their manufacturing process. This effect requires further investigation, in future studies, to assess how significant of an issue this would be for method reproducibility. It was observed that the centrifugations done during this stage introduces the possibility of solvent leakage owing to the poor sealing properties of the PTFE/Silicone mats and the 96 well multi-tier unit (MTP System ABS Plate). This led to the mild dissolution of the acrylic butyl styrene base plate and running the risk of losing its structural integrity. It could be recommended that the centrifugation be omitted altogether and performing the elution stages under the flow of normal gravity or invest in a commercially available 96 well plate vacuum manifold unit. The in-house manufactured 96 well plate nitrogen evaporator (Annex 4) performed satisfactorily, with minimal addition of contaminant as seen from the blank runs, but has the drawback of being not that efficient and the limitation of application to ambient temperatures. It could be proposed that a commercially available nitrogen evaporator would drastically overcome these issues and would be a great investment for a laboratory intending to implement this method.

Mild alkaline methanolysis (transesterification) was done resulting in the derivatization of the fatty acids specifically from the phospholipid and neutral lipid origin of the microbes. Acetic acid was used to neutralize the solutions [45]. The analysis stage was done in the multi-tier unit using an Agilent (Agilent Technologies, Inc. USA) 7890A Gas Chromatography System equipped with an updated Agilent GC Sampler 80 autosampler, to accommodate the 96 well plates. The instrument

parameters which were already optimized and routinely used in the classical method were opted for also in the high throughput method.

The author aimed at developing a high throughput method that was significantly faster, more convenient, and cost-effective, while being comparable to the classical protocol in ecological studies. The classical method could accommodate the processing of 16 samples at a given time while the high throughput method has the convenience of processing 96 to 192 samples per round, including blanks, depending on the practical expertise of the analyst. This dramatically cuts down on the overall processing time, the use of solvents, and the costs per sample. The investment for a few extra laboratory materials such as the 96 well nitrogen evaporator and 96 well vacuum manifold along with other paraphernalia could considerably improve the method performance, making it a useful upgrade for a laboratory that requires analysis of a large number of samples quickly, especially on temporal and spacial research work. The author recommends the procurement of at least 6 multi-tier units to make the fractionation stage much more convenient.

The current study successfully managed to identify and quantify 23 biomarker fatty acids, including the internal standard (19:0), which include 16:1ω5 from the NLFA fraction and i14:0**,** 14:0, i15:0, a15:0, 15:0, i16:0, 16:1ω7, 16:1ω5, 16:0, 10Me16:0, a17:0, 17:1ω8, 17:0, 10Me17:0**,** 18:2ω6,9, 18:1ω9, 18:1ω7, 18:0, 10Me18:0, cy19:0, and 20:0 from the PLFA fraction (as seen in the chromatograms Figures 3 through 6). The NLFA 16:1ω5 is used as a biomarker indicative of arbuscular mycorrhiza (AM) and the PLFA 18:2ω6,9 is indicative of the biomass of the general fungal population [4]. The total list of biomarkers and their structures is provided in Annex 3. The chemical identity of the peaks was confirmed by the analysis of two samples, that provided most of the analyte peaks, using gas chromatography with mass spectrometric detection.

It could be noted that the high throughput method could provide cleaner chromatograms compared to the classical method (seen in the PLFA chromatograms; Figures 3 and 4). The author has noted that the use of a fresh PTFE/Silicone mat prior to the sample analysis in the GC provides cleaner chromatograms, primarily due to the reduction of sample carry-over effect observed on a mat used throughout the sample preparation stage. This effect was observed in the NLFA fraction in the current study (Figures 5 and 6), which was carried out with the same mat being used throughout the analysis while the PLFA fraction was analyzed with the improvement of a fresh mat being used at the final stage.

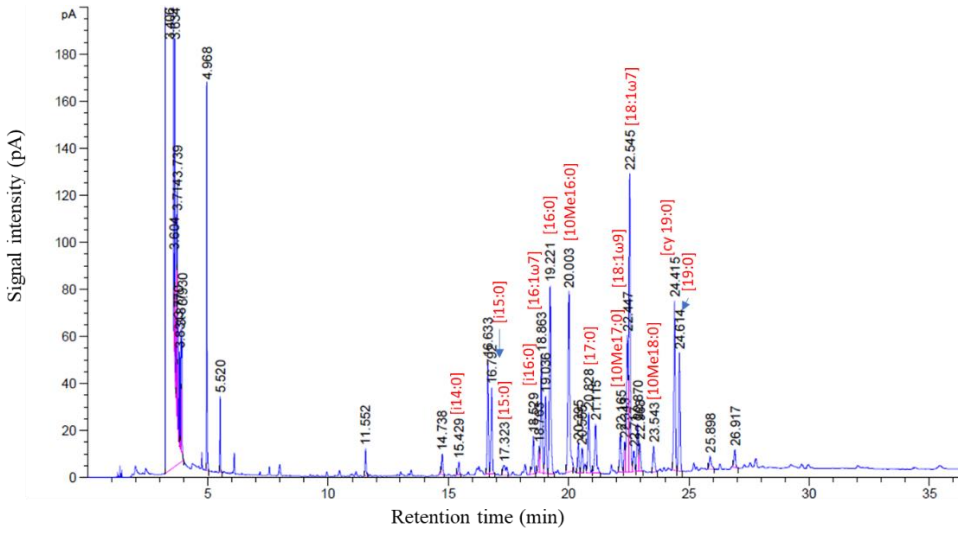

Figure 3. The chromatogram of the PLFA fraction of a sample analyzed using the high throughput method. Multiple biomarkers were detected along with the internal standard (19:0). Some of the fatty acid methyl ester peaks are labeled above.

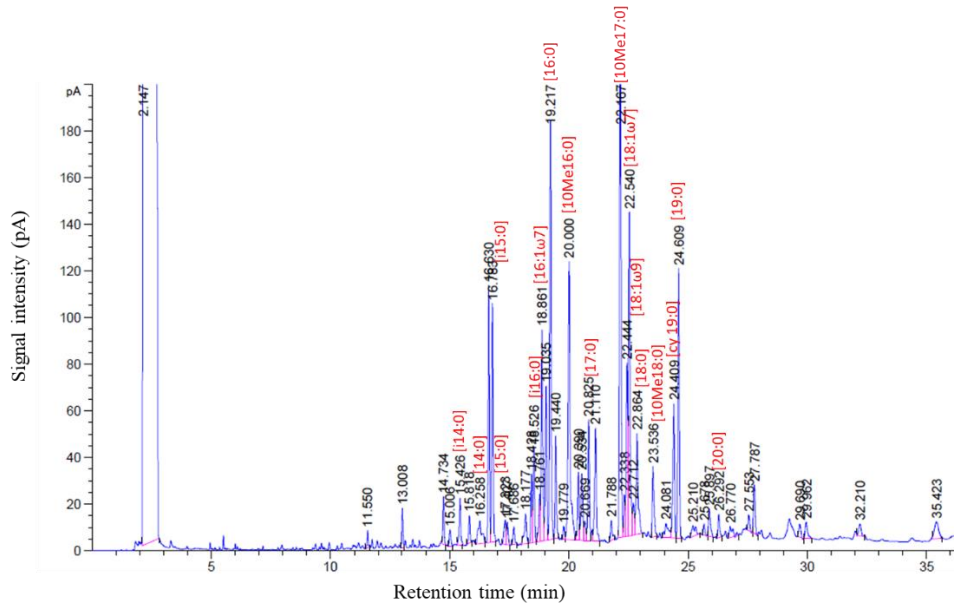

Figure 4. The chromatogram of the PLFA fraction of a sample analyzed using the classical method. Multiple biomarkers were detected along with the internal standard (19:0). Some of the fatty acid methyl ester peaks are labeled above.

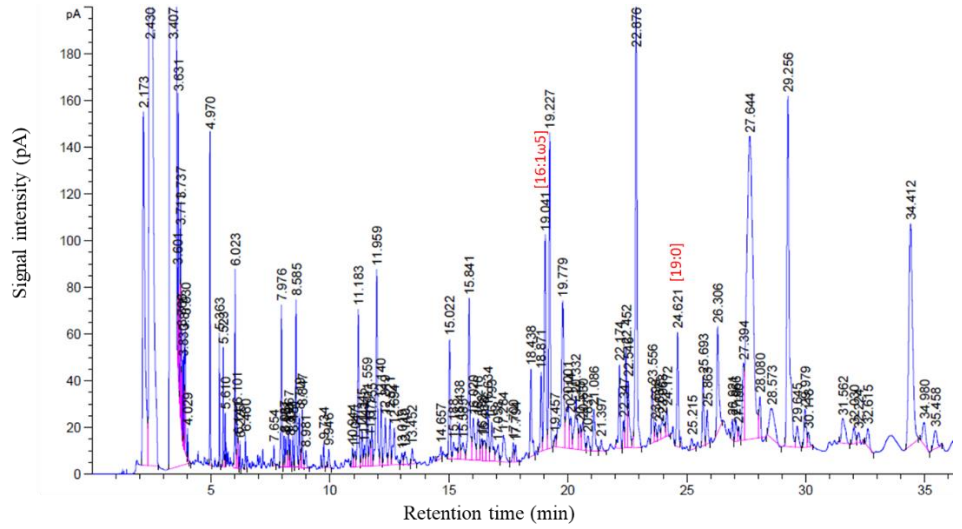

Figure 5. The chromatogram of the NLFA fraction of a sample analyzed using the high throughput method. The biomarker 16:1ω5 was detected along with the internal standard (19:0).

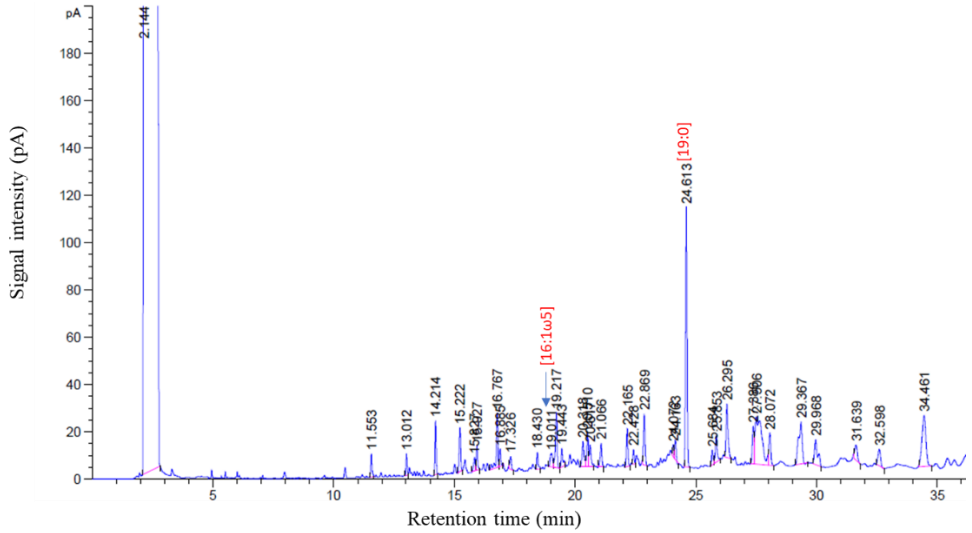

Figure 6. The chromatogram of the NLFA fraction of a sample analyzed using the classical method. The biomarker 16:1ω5 was detected along with the internal standard (19:0).

Furthermore, the high throughput method has a higher risk of having extraneous peaks due to the practical difficulty of pipette needles traversing the phase boundary during the final phase of extraction of methanolysed fatty acids from the lower organic (chloroform) layer. This issue could

be easily rectified by the use of narrower pipette needles, letting the phase separation take place for a longer time (allowing for 1 or 2 minutes), and by improving the pipetting skills of the analyst.

Thus, it could be observed that the two methods performed in a comparable manner. This is further validated by the fact that the total sample profiles of the PLFAs i15:0, 16:0, i16:0, 16:1ω5, 16:1ω7, 10Me16:0, 17:0, a17:0, 17:1ω8, 10Me18:0, 18:0, 18:1ω7, 18:1ω9, 18:2ω6,9, and cy19:0 (15 biomarkers) providing comparable patterns (refer Annex 5). It could be also noted that the extraction capability of the high throughput method is higher for many fatty acid biomarkers as observed from higher amounts of the individual biomarkers detected in the comparative analysis of the two methods for the same samples (Annex 5). This could be due to the use of the bigger volume of chloroform (1 mL), compared to the 2 x 100 µL used in the classical method, during the sample dissolution for the sample fractionation stage and/or due to the temperature effect of sample drying under nitrogen at ambient temperature (as opposed to the 40˚C used in many stages of the classical method) [46].

5.3. Classical and High throughput methods in evaluation of microbial biomass

The alteration of the soil microbial population in an ecosystem could function as a proxy for ongoing changes in the soil [27]. It has been reported that there is a high correlation between the membrane PLFA and NLFA biomarkers amount and the microbial biomass [6,27]. The microbial biomass could be assessed by summing up the fatty acid biomarker concentrations with the assumption that greater microbial biomass is indicative of healthier soil [47]. An important indicator of the soil nutrient levels and perturbation due to abiotic stresses is fungal: bacteria biomass ratio, which could be calculated from the ratio of the sum of amounts of the biomarkers representing fungi (PLFA 18:2ω6,9 indicative of general fungal load and the NLFA 16:1ω5 indicative of arbuscular mycorrhiza) to the sum of the amounts of the bacterial biomarkers (i15:0, a15:0, i16:0, a17:0, 17:0, 10Me17:0, 10Me18:0 and cy19:0). It was observed that the two methods provide a different interpretation of the soil health, the high throughput method showing a more fungal dominant community compared to the classical method (Figure 7). The high throughput method indicate a soil environment where there is a lower rate of decomposition and growth rate with low nutrient availability [9]. Alternatively, it could also suggest that the soil environment is more acidic as fungi have been observed to be more acid-tolerant [25]. Generally, a higher fungal: bacteria ratio is indicative of a more sustainable soil environment [47].

Further analysis of the fungal: bacteria ratio using the fatty acids with comparable fatty acid profiles (PLFAs 18:2ω6,9, i15:0, i16:0, a17:0, 17:0, 10Me18:0 and cy19:0) revealed a comparable pattern of ratios (Figure 7) but still the high throughput method showed a more fungal dominant soil community.

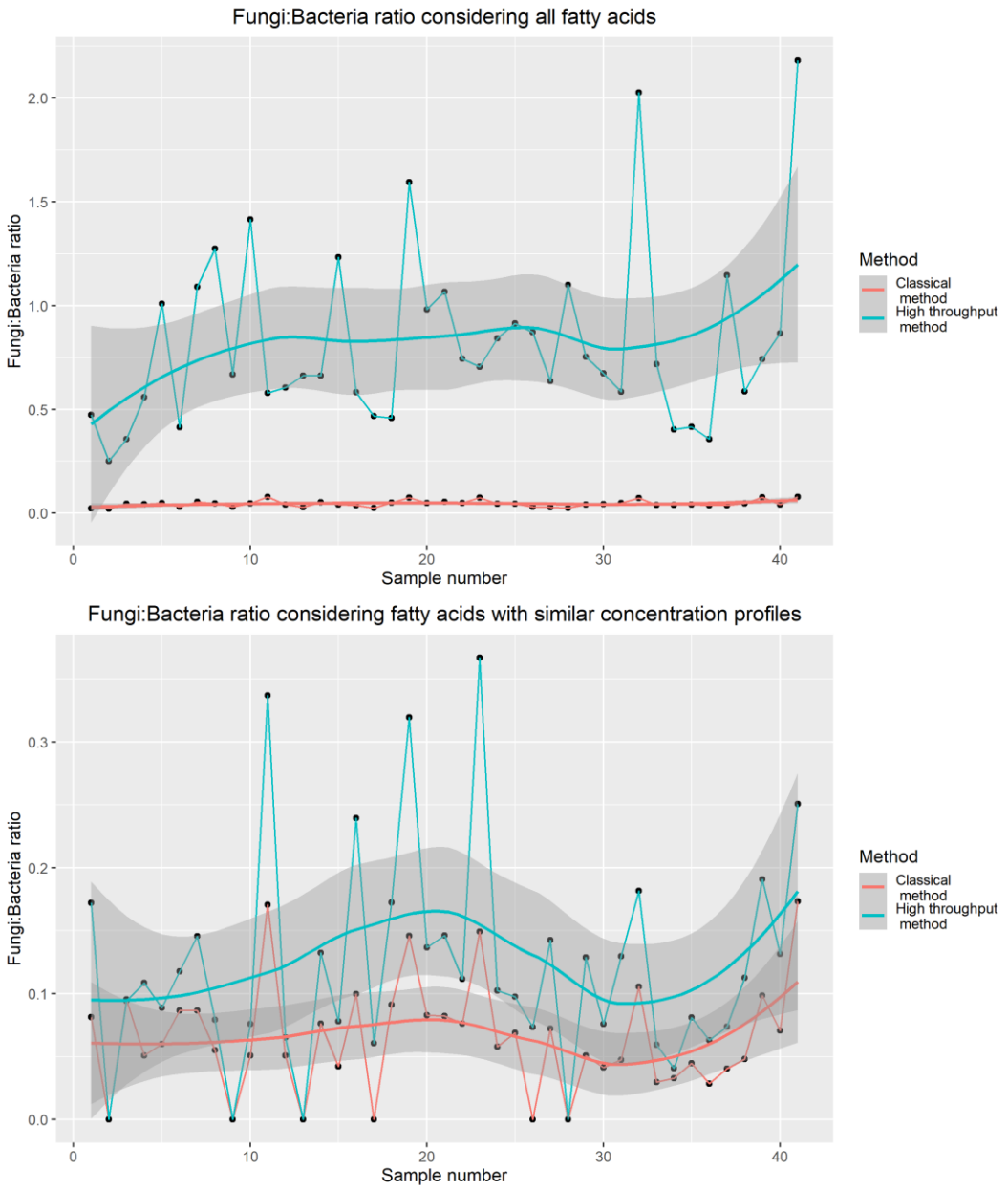

Figure 7. The comparison of Fungi: Bacteria ratios of the samples for the two methods. The fungi: bacteria ratio was first analyzed for all fatty acid biomarkers applicable in the analysis and next was performed for the fatty acids that gave similar fatty acid profiles.

The Welch two-sample t-test gave a p-value of 0.0003 with a 95% confidence interval of -0.087 to -0.027 confirming the presence of a significant difference between the ratios of the two methods. These results reiterate the previous research conclusions that the results obtained would vary with the method followed [24,47]. Thus the interpretation of results obtained from different methods should be done with caution.

A correlation analysis was done to elucidate the efficacy of the method by comparing the NLFA 16:1ω5 and PLFA 16:1ω5, which is indicative of arbuscular mycorrhizal fungi, with the arbuscular mycorrhizal fungal taxa count and their number of reads enumerated by the analysis of the small subunit (SSU) rRNA gene by an independent study. Similarly, the correlation analysis was done for the PLFA 18:2ω6,9, indicative of general fungal load, and the general fungal taxa and reads enumerated via the internal transcribed spacer (ITS) marker region analysis by an independent study.

The NLFA 16:1ω5 showed a positive correlation between the arbuscular mycorrhiza taxa (AM taxa) and read (AM reads) depicting a strong relationship between the NLFA 16:1ω5 and the number of AM fungal species and their abundance respectively, in both the methods (Figure 8 and 9), with a stronger correlation observed in the high throughput method compared to the classical method. A similar positive correlation is seen with respect to the PLFA 16:1ω5 with the high throughput method showing a much higher correlation (Figure 9). It is reported that the NLFA 16:1ω5 to be the more sensitive indicator of AM fungi compared to PLFA 16:1ω5 [4] but the ratio of these two biomarkers could provide a more robust indicator of these fungi.

The correlation studies done on the PLFA 18:2ω6,9 and the general fungal taxa (FUN taxa) and reads (FUN reads) showed a negative correlation in both the methods (Figure 8 and 9), with a slightly stronger negative effect observed in the classical method (Figure 8). These findings pose a contradictory notion to the already established assumption of the fatty acid profiles and the genetic studies performed in soil microbial community studies to be correlated and comparable [9]. Further studies need to be done in this regard to elucidating the mechanisms underpinning this finding.

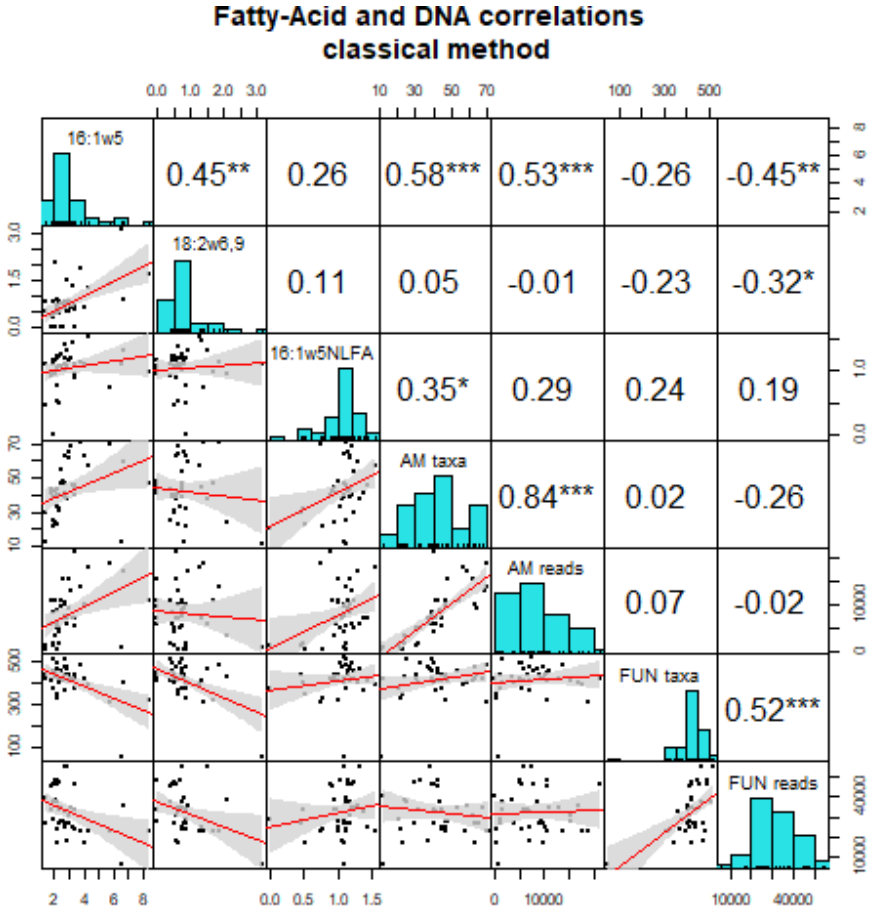

Figure 8. The correlation matrix showing coefficients and regression models between the PLFAs 16:1ω5, 18:2ω6,8 and NLFA 16:1ω5 and the arbuscular mycorrhiza fungi and general fungi read count and the taxa count analyzed using the data collected from the classical method, analysis of the small subunit (SSU) rRNA gene and internal transcribed spacer (ITS) marker region analysis. The correlation between NLFA 16:1ω5 and the number of AM fungal species (AM taxa) was 0.35 and AM fungal abundance (AM reads) was 0.29. The correlation between PLFA 16:1ω5 and the number of AM fungal species (AM taxa) was 0.58 and AM fungal abundance (AM reads) was 0.53. The correlation between PLFA 18:2ω6,9 and the number of general fungal species (FUN taxa) was -0.23 and general fungal abundance (FUN reads) was -0.32.

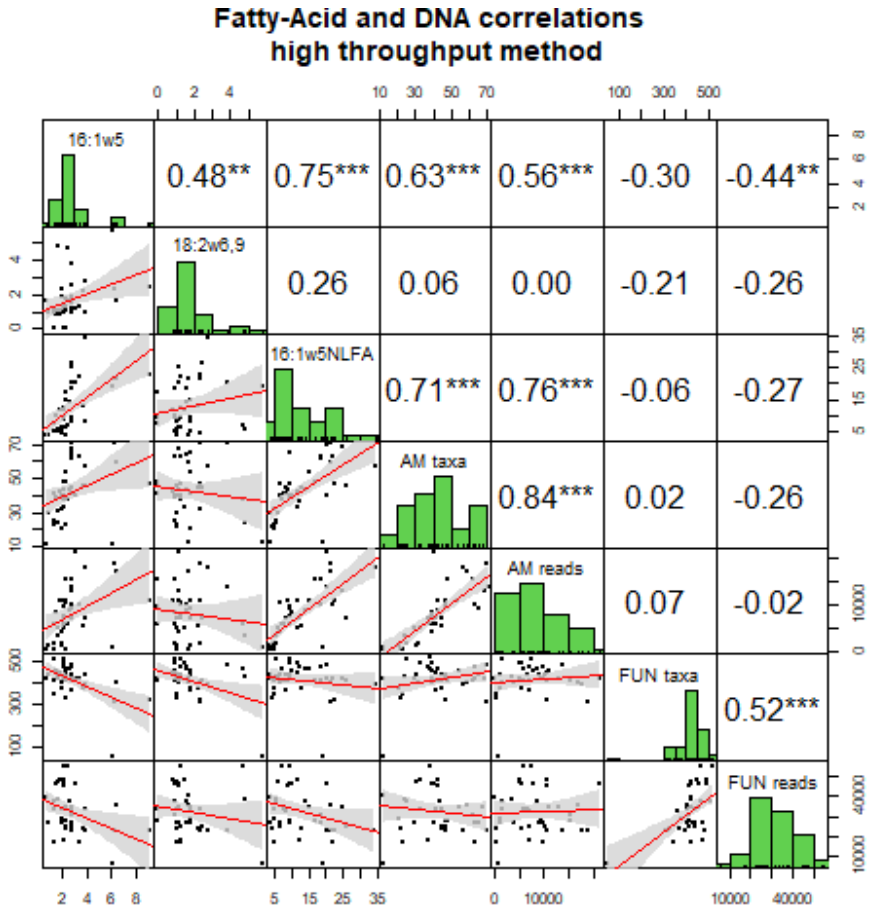

Figure 9. The correlation matrix showing coefficients and regression models between the PLFAs 16:1ω5, 18:2ω6,8 and NLFA 16:1ω5 and the arbuscular mycorrhiza fungi and general fungi read count and the taxa count analyzed using the data collected from the high throughput method, analysis of the small subunit (SSU) rRNA gene and internal transcribed spacer (ITS) marker region analysis. The correlation between NLFA 16:1ω5 and the number of AM fungal species (AM taxa) was 0.71 and AM fungal abundance (AM reads) was 0.76. The correlation between PLFA 16:1ω5 and the number of AM fungal species (AM taxa) was 0.63 and AM fungal abundance (AM reads) was 0.56. The correlation between PLFA 18:2ω6,9 and the number of general fungal species (FUN taxa) was -0.21 and general fungal abundance (FUN reads) was -0.26.

It has been warned about the shortcomings of calculating the diversity based on PLFA data, especially with regards to fungi owing to the lack of sufficient PLFAs representing fungal species [14]. Nevertheless, PLFA studies have the advantage of providing better phenotypical and

microbial activity-related information compared to rRNA analysis [30]. This would be a potential future study direction that could be explored using more planned complementary studies using PLFA analysis and rRNA analysis to provide a better understanding of soil microbial ecology.

5.4. Classical and High throughput methods in evaluation of microbial community profile

Various ordination techniques are being used for the analysis of soil microbial communities with respect to the PLFA and NLFA analysis. In the current research work, non-metric multidimensional scaling (NMDS) was performed for the total fatty acid marker profiles using the metaMDS function in the vegan R package. This revealed that the two soil communities from which the samples were taken, grassland and arable land, have different soil fatty acid communities. Both the methods showed comparable capabilities in distinguishing the two soil sources (Figure 4.9). Furthermore, permutational multivariate analysis of variances (PERMANOVA) analysis with 999 permutations using the adonis function in the vegan R package, on the relative abundances, provided a test for the relative ability of the methods to distinguish between the two sources of samples. This revealed that the high throughput method and the classical method were both capable of distinguishing the two sources in a comparable manner with the high throughput method describing the variability to slightly a lesser extent than the classical method (shown by their R^2 values 0.14 and 0.32 respectively) (Figure 10). Nevertheless, both methods show the similar potential of distinguishing different community structures.

This further drives home the importance of comparing results of studies done using the same method [24] and care must be taken in interpreting such results. Ordination studies in elucidating the soil communities are perhaps where PLFA and NLFA analysis is appropriately used [30], providing a reliable tool in such studies.

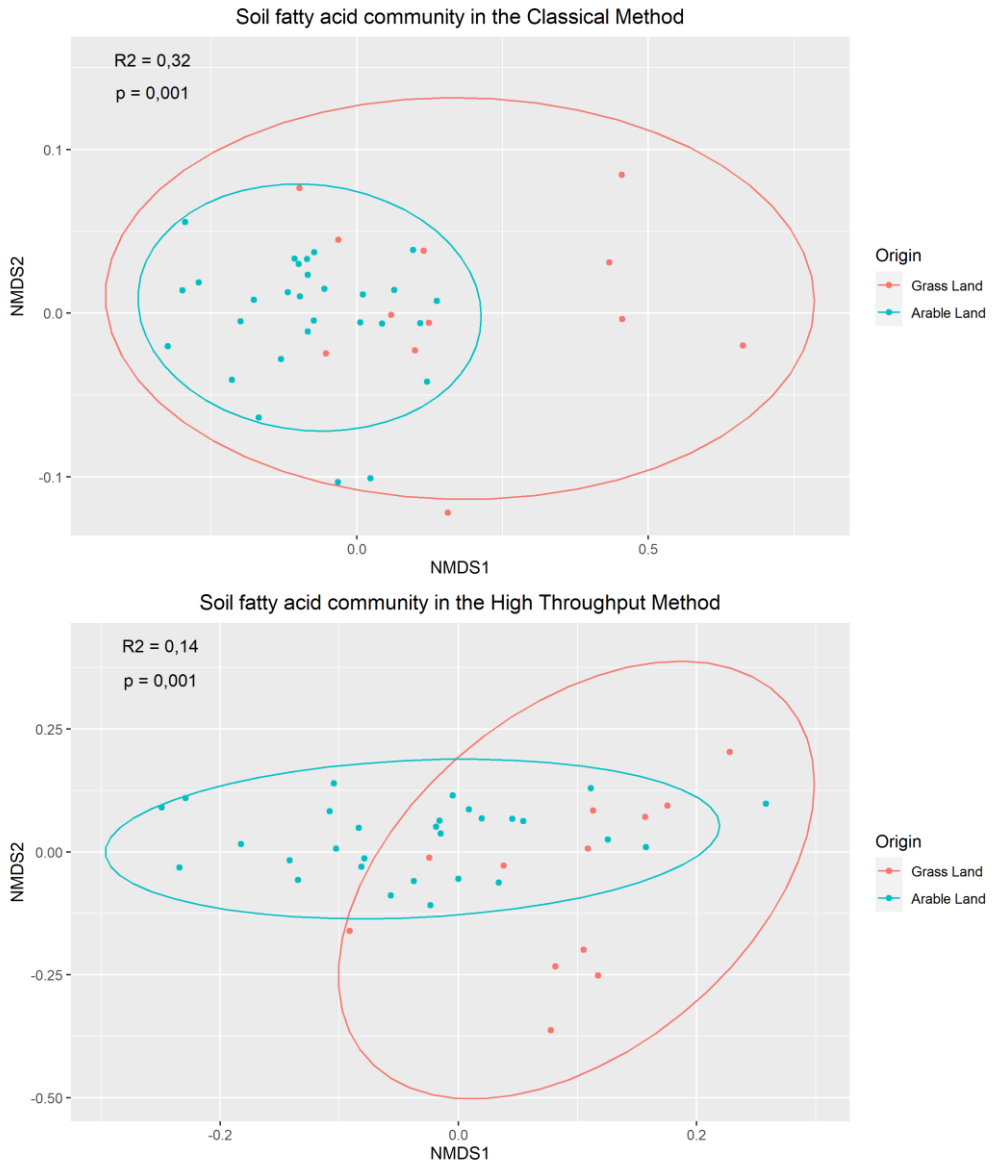

Figure 10. The analysis of soil microbial community by NMDS and PERMANOVA analysis using the vegan package in R. NMDS was carried out using the metaMDS function in the vegan R package and PERMANOVA was carried out using the adonis function in the vegan package in R. Both the classical method and the novel high throughput method showed similar abilities in distinguishing the two sample sources with R^2 of PERMANOVA analysis (of 999 permutations) for classical method and the high throughput method to be 0.32 and 0.14 respectively.

6. SUMMARY

The author aimed at establishing a novel high throughput method for the soil microbial PLFA and NLFA analysis, to be used in the Tartu University - Plant Ecology Laboratory. A novel high throughput method was successfully established using the protocol proposed by Buyer and Sasser [2] as a primary reference, with modifications to emulate extraction chemistry of the well-established classical method followed in the Plant Ecology Laboratory [1]. The novel method addressed the issues of the need to process large amounts of samples and at the same time, cutting down the amounts of solvents used, the costs of processing one sample, and the total processing time.

The microbial biomass studies performed using ecological matrices such as fungi: bacteria ratio and the studies done on the microbial community profile using NMDS and PERMANOVA revealed that the two methods provide very comparable ecological conclusions. This confirms the potential of the novel method as a substitute for the classical method. But the research findings demonstrate a general difference between the two methods with respect to the fatty acid profiles and their amounts in the soil, driving home the importance of formulating conclusions from data collected via the same method.

The author is aware of the practical issues and difficulties encountered during the method development stage and recommends improving the method by investing in equipment such as 96 well plate nitrogen evaporator and 96 well plate vacuum manifold system and tweaking the method in future applications. With the improvements made, this method would serve as a workhorse in soil fatty acid analysis in ecology laboratories that perform large studies of temporal and spatial variations.

Further studies need to be done on the disparity of the soil community results of soil fatty acid analysis using this method and that of genetic analysis. This could be a potential research avenue in elucidating the soil microbial community and ecology. Another research potential for the future includes studies into the resolving capabilities of the method of very similar soil communities. The repeatability and the reproducibility of the method need to be confirmed on further analysis. This could be achieved with the study of pure fungal and bacterial cultures along with interlaboratory comparisons.

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9. ANNEXES

ANNEX 1: CHEMICALS

Solvent / Chemical	Manufacturer	Grade	CAS No.	Mol Wt	Density	Melting point	Boiling point
				[g/mol]	[g/cm^3] at 20˚C	[˚C]	[˚C]
chloroform	Fischer Scientific UK	Distol-Pesticide residue grade, 99.8+%	67-66-3	119.38	1.564	-63.5	61.15
methanol	Honeywell, Germany	CHROMASOLV™, for HPLC, ≥99.9%	67-56-1	32.04	0.79	-98	64 - 65
methanol	Fischer Scientific UK	Distol-Pesticide residue grade	67-56-1	32.04	0.79	-98	64 - 65
acetone	Merck KgaA, Germany	ENSURE^R, ACS, ISO, Reag. Ph Eur	67-64-1	58.08	1.3580 – 1.3600	-94	56
acetone	Honeywell, Germany	puriss. p.a., ACS reagent, reag, ISO, reag. Ph Eur., ≥99.5% (GC)	67-64-1	58.08	1.3580 – 1.3600	-94	56
toluene	Merck KGaA	SupraSolv^R, For gas chromatography ECD and FID	108-88-3	92.14	0.87	-95	111
n-hexane	Honeywell, Germany	CHROMASOLV™, for HPLC, ≥97.0%	110-54-3	86.18	0.660 – 0.668	-95	66 - 69
citric acid	Merck KGaA	Anhydrous for synthesis, ≥99%	77-92-9	192.12	1.665	156	310
acetic acid	Merck KGaA	EMSURE^R, ACS, ISO, Reag. Ph Eur 100%	64-19-7	60.05	1.05	16 - 17	118 - 119
sodium hydroxide	Merck KGaA	EMSURE^R, pellets for analysis	1310-73-2	40,00	2.13	323	1388

potassium hydroxide	Merck KGaA	EMSURE^{R}, pellets for analysis	1310-58-3	56.11	2.044	360	1327
2,2,4-trimethylpentane	Lachner, Slovakia Republic		540-84-1	114.232	0.692	-107.38	99.3
toluene	Sigma-Aldrich, USA	CHROMASOLV^{R} Plus, for HPLC, ≥99.9%	108-88-3	92.141	0.87	-95	111
octane	PEAXNM, Russia		111-65-9	114.232	0.703	-57.1 - -56.6	125.1 – 126.1
dimethyl sulfoxide	Sigma chemical company, USA		67-68-5	78.13	1.1004	19	189

ANNEX 2: REAGENTS AND SOLVENT MIXTURE PREPARATION.

The volumes of the reagents and solvent mixtures were prepared fresh as needed.

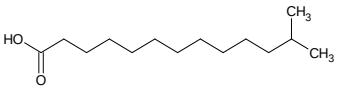
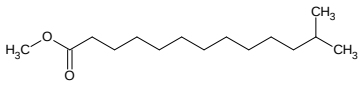
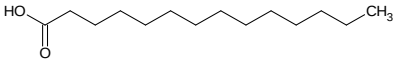
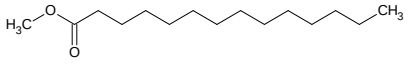
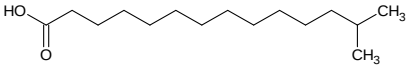
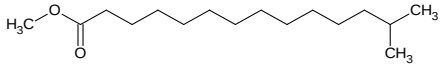
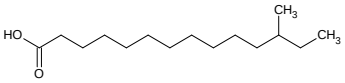
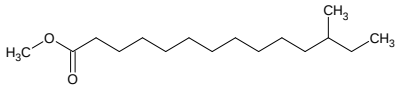
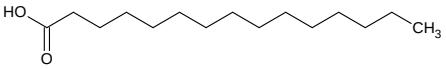
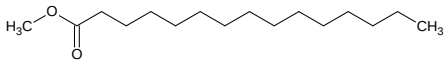
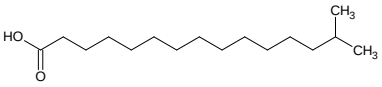
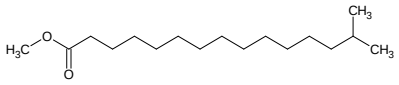
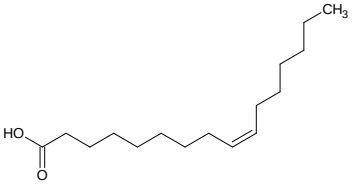
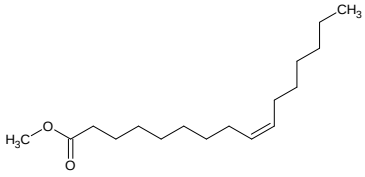
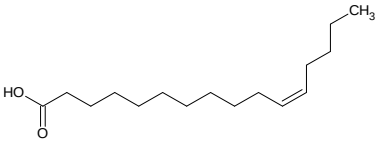
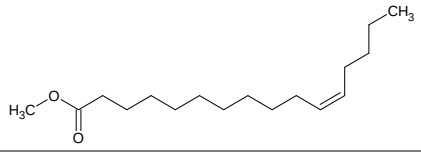
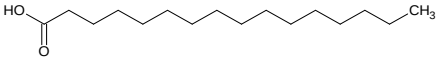
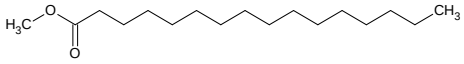
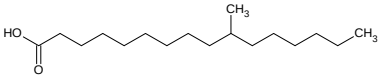
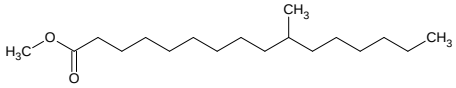
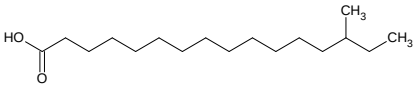
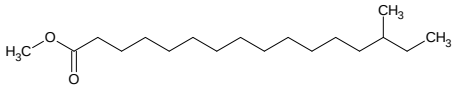
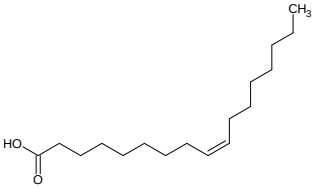
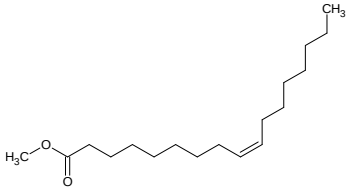
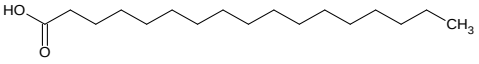
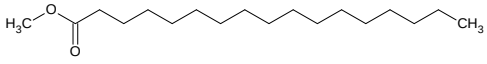
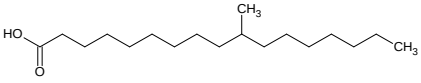
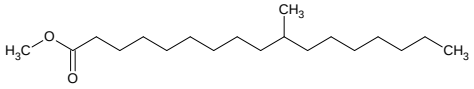
Reagents and solvent mixture used in the classical method

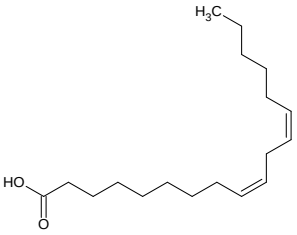
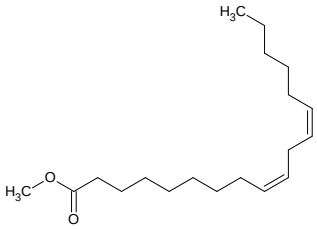
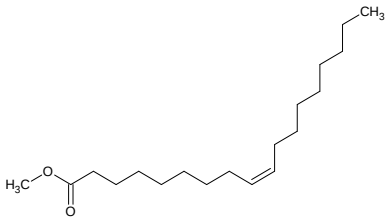
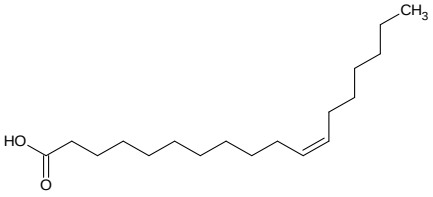
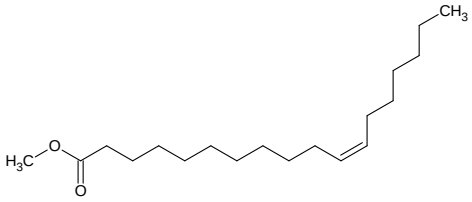
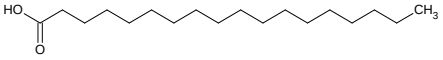
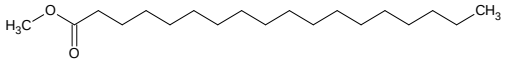
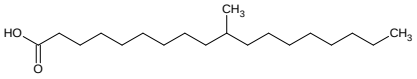
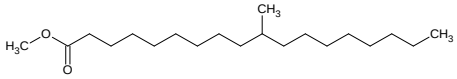
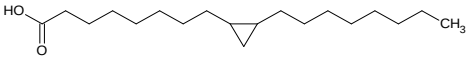
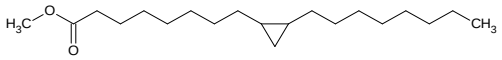
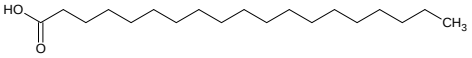
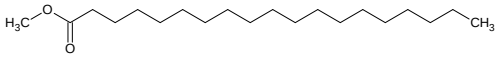
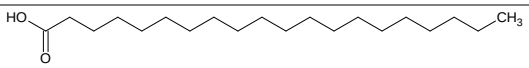
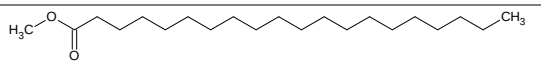
Reagent / Solvent mixture	Preparation
Citrate buffer (0,15 M / pH 4,0)	Dissolve 28.818 g of citric acid in 1 L of distilled water. The pH is adjusted to 4.0 using sodium hydroxide.
Bligh and Dyer extractant (B & D)	For the preparation of B & D of volume 600 mL 158 mL of chloroform, 316 mL of methanol, and 126 mL of citrate buffer are mixed in a glass bottle giving a volume ratio of chloroform: methanol: citrate buffer of 1: 2: 0.8
Toluene: Methanol mixture	Toluene: Methanol mixture of ratio 1:1 (v/v) is prepared by mixing equal parts toluene and methanol in a glass bottle.
Potassium hydroxide (0,2 M) in Methanol	Dissolve 11.222 g of potassium hydroxide in 1 L of methanol.
Hexane: Chloroform mixture	Hexane: Chloroform mixture of ratio 4:1 (v/v) is prepared by mixing 4 parts hexane and 1 part methanol in a glass bottle.
Acetic acid (1 M)	Prepare a 1 M acetic acid solution by adding 57.19 mL of glacial acetic acid to a 1 L standard flask half prefilled with distilled water. Make up the volume to 1 L with distilled water.
Internal standard (19:0 FAME) 0,023 mg/mL	A stock solution of 2.3 mg/mL of methyl nonadecanoate is prepared in 5 mL chloroform by weighing accurately 11.5 mg of the standard substance using a 5 digit analytical balance. The working solution is prepared by adding 100 µL of the stock into a 10 mL standard flask and making up the volume with chloroform.

Reagents and solvents used in the high throughput method

Reagent / Solvent mixture	Preparation
Citrate buffer (0,15 M / pH 4,0)	Dissolve 28.818 g of citric acid in 1 L of distilled water. The pH is adjusted to 4.0 using sodium hydroxide.
Bligh and Dyer extractant (B & D)	For the preparation of B & D of volume 600 mL 158 mL of chloroform, 316 mL of methanol, and 126 mL of citrate buffer are mixed in a glass bottle giving a volume ratio of chloroform: methanol: citrate buffer of 1: 2: 0.8
Potassium hydroxide (1 M) in Methanol: Toluene mixture	Methanol: Toluene mixture of 3: 1 is prepared by adding 3 parts methanol and 1 part toluene in a glass bottle. In 1 L of this mixture 56,11 g of potassium hydroxide is dissolved.
Acetic acid (0,75 M)	Prepare a 0.75 M acetic acid solution by adding 42.89 mL of glacial acetic acid to a 1 L standard flask half prefilled with distilled water. Make up the volume to 1 L with distilled water.
Internal standard (19:0 FAME) 0,023 mg/mL	A stock solution of 2.3 mg/mL of methyl nonadecanoate is prepared in 5 mL chloroform by weighing accurately 11.5 mg of the standard substance using a 5 digit analytical balance. The working solution is prepared by adding 100 µL of the stock into a 10 mL standard flask and making up the volume with chloroform.

ANNEX 3: STRUCTURE OF FATTY ACIDS AND FATTY ACID METHYL ESTERS.

Fatty acid	Structure	Fatty acid methyl ester (FAME)
i14:0		
14:0		
i15:0		
a15:0		
15:0		
i16:0		
16:1ω7		
16:1ω5		
16:0		
10Me16:0		
a17:0		
17:1ω8		
17:0		
10Me17:0		

18:2ω6,9		
18:1ω9		
18:1ω7		
18:0		
10Me18:0		
cy19:0		
19:0		
20:0		

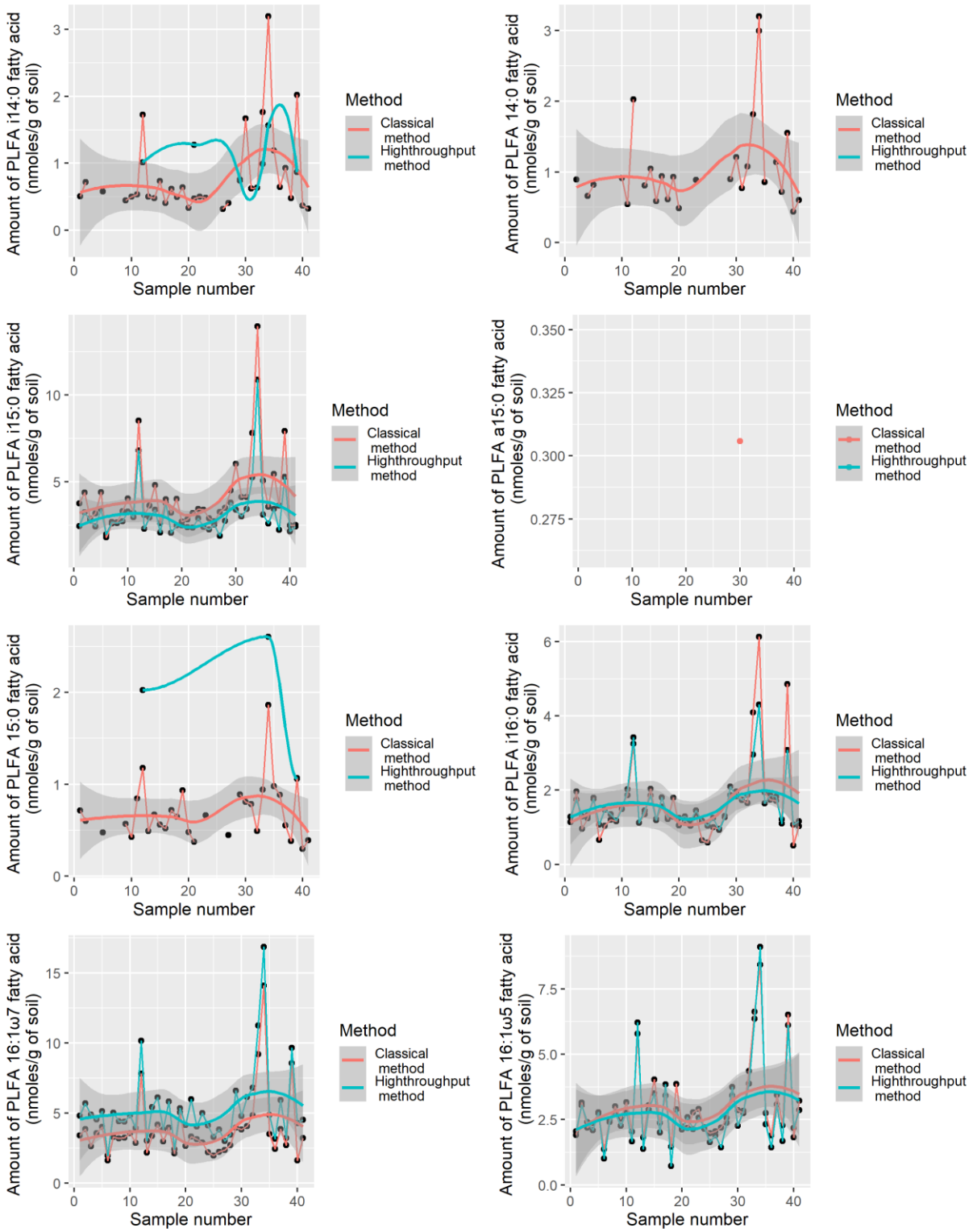
ANNEX 4: MATERIALS BUILT IN-HOUSE

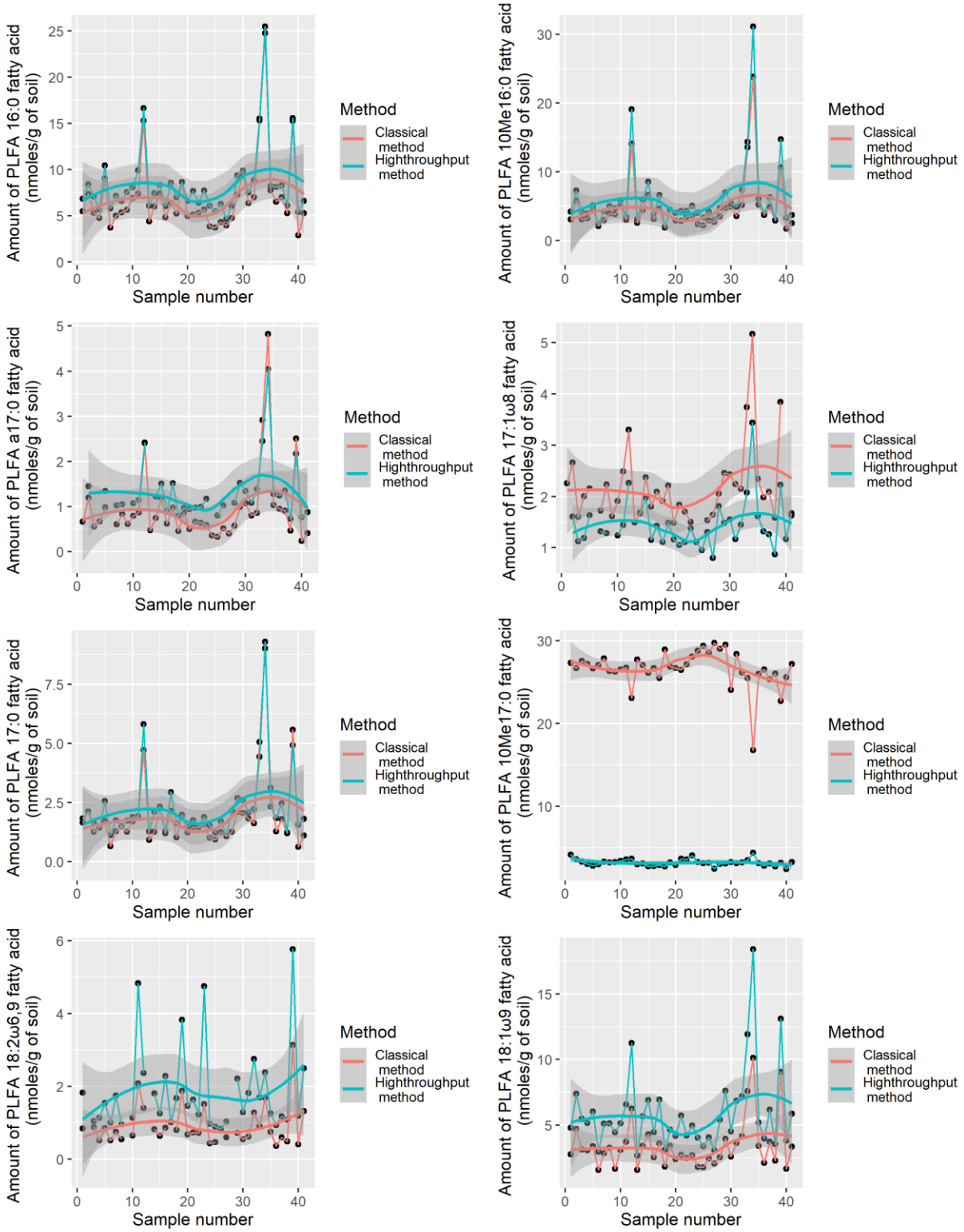
The 96 well plate nitrogen evaporator

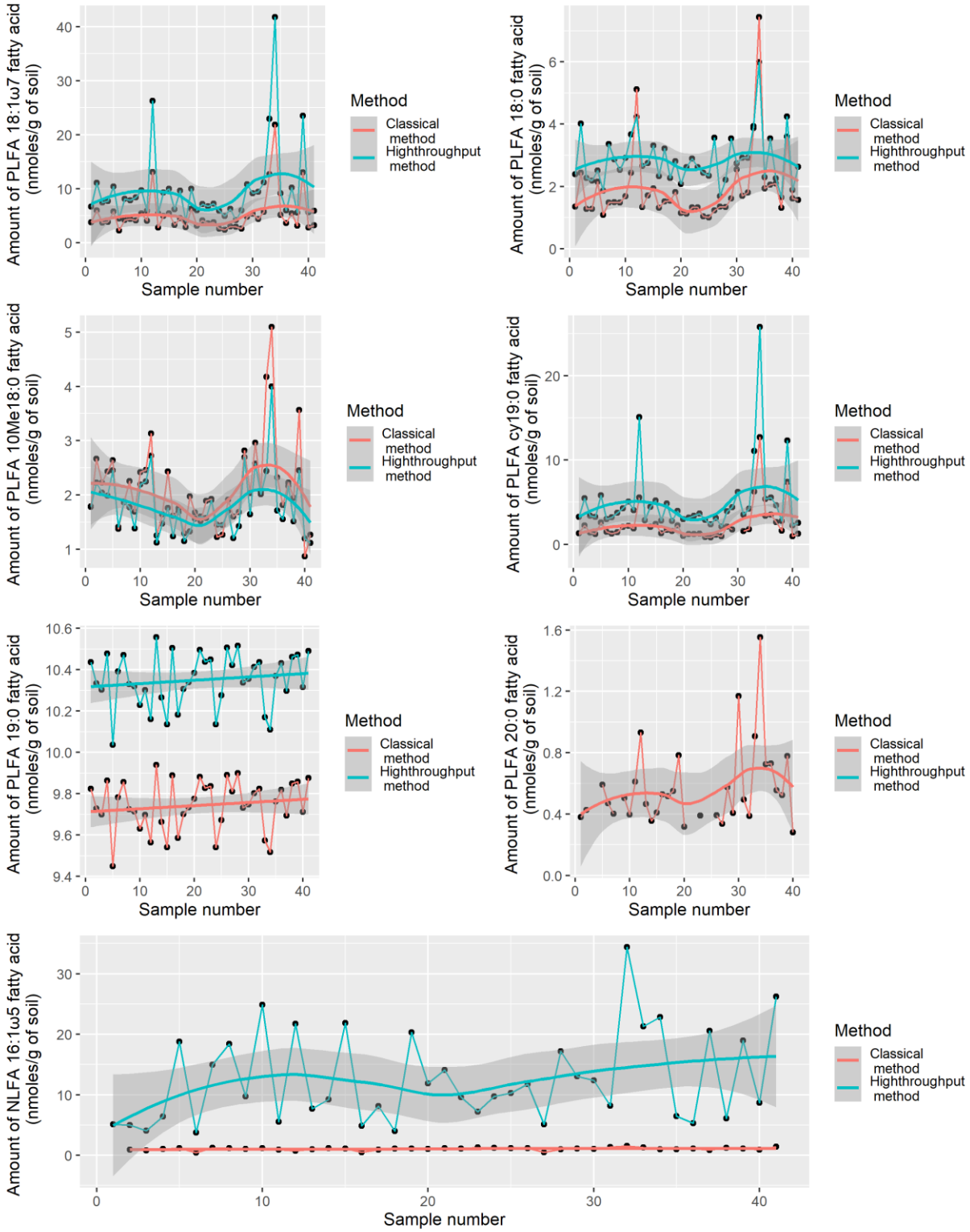
The PTFE multichannel pipette reservoir/trough

ANNEX 5: FATTY ACID AMOUNTS

Comparison of fatty acid amounts in each sample, analyzed in parallel, using the two methods.




Amount of PLFA 16:0 fatty acid (nmoles/g of soil)
Amount of PLFA 10Me16:0 fatty acid (nmoles/g of soil)
Amount of PLFA a17:0 fatty acid (nmoles/g of soil)
Amount of PLFA 17:1ω8 fatty acid (nmoles/g of soil)
Amount of PLFA 17:0 fatty acid (nmoles/g of soil)
Amount of PLFA 10Me17:0 fatty acid (nmoles/g of soil)
Amount of PLFA 18:2ω6,9 fatty acid (nmoles/g of soil)
Amount of PLFA 18:1ω9 fatty acid (nmoles/g of soil)
Sample number
Method
Classical method
Highthroughput method


Amount of PLFA 18:1ω7 fatty acid (nmoles/g of soil)
Sample number
Method
Classical method
Highthroughput method
Amount of PLFA 18:0 fatty acid (nmoles/g of soil)
Amount of PLFA 10Me18:0 fatty acid (nmoles/g of soil)
Amount of PLFA cy19:0 fatty acid (nmoles/g of soil)
Amount of PLFA 19:0 fatty acid (nmoles/g of soil)
Amount of PLFA 20:0 fatty acid (nmoles/g of soil)
Amount of NLFA 16:1ω5 fatty acid (nmoles/g of soil)

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25.05.2021