A modified cell extraction method to access microbial community structure in soil samples by phospholipid fatty acid analysis

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The aim of this chapter is to present a modified cell extraction method that is effective in increasing biomass recovery, especially when biochemical techniques like Phospholipid Fatty Acid Analysis (PLFA) are use to determine biomarkers for soil community structure. Extremely low biomass is a limiting factor for obtaining accurate and representative prokaryotic and eukaryotic community structure and composition in soil matrices. In this study, a combination of an inorganic cell releasing buffer and surfactants along with a density gradient separation provides an effective method for cell elution from sediment over other approaches from low biomass soil samples by avoiding traditional problems with direct biochemical extraction methods, e.g. ionic adsorption, pH, and humic acid interferences. Loam soil (10⁶ cells/g of soil) and experimentally determined low biomass (10² cells/g of soil) samples were treated with modified cell extraction method to compare numbers of signature phospholipids biomarkers, their response (concentration), and microbial community structure in each tested soil. A higher biomass yield, measured in pico moles (pm) total lipid, and higher microbial diversity were obtained in both high and low biomass soils samples, but several important groups such as methane oxidizers, sulfate reducers, fungi and iron reducers, were only detected with the new method. Genomic DNA extractions were also successfully extracted from the PLFA aqueous phase, showing high molecular weight recovery suitable for further DNA finger-printing techniques.

Keywords: Nykodenz; low biomass; modified cell extraction; PLFA

1. Introduction

Microbial diversity studies are critical to our functional understanding of microbial community structure and function in soil and other ecosystems [1]. Soil microbial communities remain some of the most difficult communities to characterize, because of their immense phenotypic and genotypic diversity [2]. Soil is a complex and heterogeneous matrix containing many microhabitats enabling microorganisms to aggregate into heterogeneous assemblies and communities. A representative estimate of microbial diversity is a prerequisite for understanding the functional activity of microorganisms in all ecosystems [3]. To achieve this different types of soil may require, depending on the degree of particle and pore size, a more complete sample homogenization to improve penetration of solvents, extraction buffers or surfactants. Since less than 1% of soil microorganisms can be cultivated from their natural environments and direct counts do not provide anything other then the crudest level of identification or activity information, new and more sensitive approaches are required that provide better measures of community structure and function [4].

Techniques such as Polymerase Chain Reaction (PCR), Terminal Restriction Fragment Length Polymorphisms (TRFLP), and Denaturing Gradient Gel Electrophoresis (DGGE) are among the most useful for direct soil characterization [5]. These nucleic acid-based techniques can detect distinctive microbial populations from (rDNA) fingerprints which can be used to directly determine microbial community structure differences at different degrees of resolution. To complement the limited phenotypic characterization (nutritional and physiological), a biochemical approach is often applied using phospholipids as microbial community biomarkers. [6,7].These complex aliphatic macromolecules are the main component of cell membranes and cell walls and differentiate organisms in terms of functional groups, biomass, physiological status and stress, and in a few cases even species identification [43]. Phospholipid Fatty Acid Analysis (PLFA) not only detects changes in the population, but also physiological responses of cells to shifts in their microniche [8].

PLFA, together with nucleic acid-based molecular techniques, are a powerful combination for elucidating microbial interactions, but they are both limited by biomass concentration [7].Microbial biomass has to be sufficiently high enough for detection, and detection limits for many of these techniques are below biomass concentrations found in many samples, especially those from extreme environments. Methods for DNA and PLFA extraction are divided into two categories: those in which cells are lysed within the soil (direct extraction) and those in which cells are removed first from the soil matrix and then lysed (indirect cell extraction) [9-10]. The main objective of this study was to develop an improved soil biomass recovery technique which could be used with PLFA to provide a better indication of microbial community structure in soil, especially from low biomass soil samples. One of the strategies that have proven most useful for cell extraction is density centrifugation gradient involving Nykodenz (Fisher Scientific Inc.) (1.3 g/mL) Nykodenz serves to efficiently detach cells from soil particles and isolate them from denser soil aggregates without affecting functional integrity [14]. Studies have reported this technique's versatility for yielding high molecular weight

DNA, cell isolation for flow cytometry to define active microbial members in soil, and for purification of eosinophils and mononuclear cells [12].

2. Methods

2.1.Phospholipid fatty acid extraction

All solvents used for PLFA were of high purity Gas Chromatography (GC) grade. The modified Bligh and Dyer protocol, [14] which is briefly described, was used for the total lipid extraction for all samples. The controls included a 12 g sample of loamy soil (10^6 cells/g of soil) untreated and treated with the cell extraction method. In addition, experimental samples were obtained by weighing 50 g of the low biomass soil (10^2 cells/g of soil), with and without the treatment. All samples were stored at -80°C until used and vacuum dried before starting a 24 h total lipid extraction using methanol, chloroform and phosphate buffer (50 mM, pH 7.4) in a 2:1:0.8 ratio (Sigma Aldrich). The extraction mixture was allowed to stand overnight in darkness at 4°C after a 2 min sonication. The single phase extract was separated from the solid material by centrifugation at 2000 rpm for 20 min and decanting into a separatory funnel. An additional 35 mL volume of chloroform was used to wash the solids, which were re-centrifuged with a final addition of chloroform to the extract. An additional 35mL volume of water was added to the extract to force the separation of the aqueous from the organic phase. After complete separation the aqueous phase was taken for molecular purposes. The total lipid extract was dried under a gentle stream of nitrogen and then dissolved in chloroform.

2.2.Phospholipid Fatty Acid Analysis (PLFA)

Total lipids were separated into different lipid classes using miniature silicic acid column chromatography. Neutral lipids, glycolipids, and phospholipids were obtained by eluting with chloroform, acetone and methanol, respectively. The phospholipid fraction was used for the profiling after being subjected to a mild alkaline transmethylation with KOH at 60°C for 30 min. Finally, the PLFAs were quantified using GC- Flame Ionization Detection (FID) (Hewlett-Packard model 5890 Series 2) comparing them with the Microbial Identification Index (MIDI) as a standard (Sassler, 1990). Method blanks were extracted with each set of samples and were assumed to be free of contamination if chromatograms of the blanks contained no peaks. Chromatograms signals were identified by the MIDI software and linked to specific bacterial groups as listed in Table 1. Biomass was estimated by the use of an external standard 11:0 Fatty Acid Methyl Ester (FAME) (Matreya, Pleasant Gap, PA) which converted GC response to picomole (pm) lipid. Cell density was calculated using the approximation of 2.5 x 10^5 cells/ pm FAME recovered (Tunlid, 1989)

2.3.Live and Dead Cell Method using Epifluorescence Microscopy

The detection of the microbial population in the soil samples was determined by using a Live/Dead Baclight Bacterial Viability kit (Invitrogen, Carlsbad, CA). Samples taken directly from soil were diluted $(10^{-3}, 10^{-4})$ in 50 mM K₂HPO₄ and 0.03% of sodium pyrophosphate (NaPPi). From the highest dilution, 500 µl were removed and mixed with 120 µl of fluorescent dyes (GFP/FITC; green for live cells, and Rhodamine red for dead cells), and incubated in the dark for 15 min. The dye was washed by adding 5 – 10 mL of K₂HPO₄, mixed gently by vortex, and filtered through a black polycarbonate membrane. The membranes were mounted on a glass slide, and after placing a cover slip, the fluorescent samples were observed using an epifluorescence microscope at 100X oil magnification.

2.4.Cell extraction method

Cell extraction was performed by adding soil samples (40 g) into sterile bottles containing 360 mL of cell releasing buffer (0.2M NaCl, 50 mM Tris-HCl (pH 8.0), 0.05M Nappi (pH 7.0) and 0.01% of Tween 80). Approximately 40-50% bottle free space was allowed for better extraction. The soil was mixed by inversion with the buffer for 2 h at room temperature. The tubes were then centrifuged at 1000 X g for 10 min to remove soil particles. Nykodenz (1.3 g mL⁻¹) was placed in a clean tube (Falcon tubes; 50mL), and the soil extract was poured without disrupting the Nykodenz phase (1:3 ratio of extract to Nykodenz). The Nykodenz and extract mixture was then centrifuged at 8,700 rpm (10,000 X g) for 30 min at 4°C. After centrifugation a white interlayer is formed between the soil extract and the Nykodenz which contains extracted cells. Careful removal of the whitish interphase was done using a glass Pasteur pipette. In cases where humic acids were present, interphase removal was done 2-3 cm above the Nykodenz phase. The cell-containing phase was transferred to a sterile tube and 3 volumes of 5 mM phosphate buffered saline (PBS pH 7.0) and 10 mM NaPPi (pH 7.0) were added. Cells were pelleted down by centrifugation at 10,000 X g for 20 min. The cell pellets were washed with 1mLof 5mM NaPPi prior to PLFA analysis.

2.5.DNA extraction from the aqueous phase obtained by PLFA

To determine the possibility of extracting DNA out the aqueous phase of the PLFA after performing the modified cell extraction method, the aqueous phase of the PLFA extraction was initially processed by adding 1 volume of isopropanol, and 10% of 3 M sodium acetate buffer (pH 7.0). After precipitation of the DNA by incubation on ice for 2 h, the samples were centrifuged at 10,000 X g for 30 min at 4°C. The pellets were washed twice with 80% ethanol, centrifuged at 10,000 X g for 15 min in between washes and air dried for 3 min. The DNA samples were then resuspended in 400 μ L of TE (Tris EDTA) buffer. The partially purified DNA samples were cleaned up further by extracting twice with equal volumes of chloroform, centrifuged at 10,000 X g for 5 min, and precipitating the collected supernatant for 30 min on ice after adding 0.6 volume of isopropanol. Finally, the alcohol precipitated samples were centrifuged at 14,000 X g for 10 min, the pellet washed with 80% ethanol, and after the final centrifugation (as described above), the pellet was air dried for 3 min and resuspended in 100 μ L TE buffer. The presence of DNA in samples was confirmed by electrophoresis in a 1% agarose using 1X TAE (Tris-acetate EDTA) as buffer.

3. Results

3.1.Biomass determination of soil samples by epifluorescent microscopy and Gas Chromatography

The live/dead cell count by fluorescent microscopy for the loam soil and the low biomass samples showed cell counts of 10^6 and 10^2 cells/g of soil respectively. The chromatograms obtained from GC analysis of the samples after PLFA confirmed high recovery of lipids indicating higher cell density (loam soil; Fig. 1A) and barely-detectable signatures in low biomass samples (Fig. 1B-D) without the modified cell extraction method.



Fig. 1. Loam soil samples (A) shows higher number of PLFAs peaks on soil processed with only 12 g compared with 50g of low biomass soil in samples B-D. A (Control; loam soil), others represent experimental soil depth from 11.4 m (A), 15.2 m (B) and 18.1m (C) respectively. Each peak represents a different phospholipid detected.

3.2.Biomass and microbial community structure comparison using the modified cell extraction method

The highest value in microbial biomass detected using the traditional PLFA technique without performing the modified method was 0.1 pm on loam soil, and none detected in the low biomass soil. On the other hand, when the PLFA was performed on samples treated with the modified cell extraction method, increase in detection was observed for all soil samples. These values increased from 0.1 pm to 0.9 pm in loam soil, and from being undetectable to up to 0.7 pm (2.5 x 10^5 cells of PLFAs extracted/ g of sample) (Fig 2).



Fig.2. When compared the soils untreated (UT) and treated (T) with the modified cell extraction method (L: loam T: top-core = clay, M: Middle-core = clay, and Bottom-core = sandy). The T soil samples, allowed the extraction of a higher biomass and the detection of a more diverse group of microbes. The biomass measurement calculated as picomols and every sample was normalized in mole percent, and expressed in picomols (2.5×10^5 cells of PLFAs extracted/g of sample). The microbial diversity was higher for treated soils for both soil types (Table 1). The number of functional groups found in the loam soil increased from 5 to 9, while the treated low biomass soils increased from 1 to 8 different microbial groups (Fig 2).

Table 1. Lipid markers detected by Gas Chromatography (GC) to represent taxonomic groups of microorganisms.

Lipid marker	Microorganism	References
17:1w7c, 10me16:0, 17:1w6, 15:1, i17:1w7c, 10me16:0, cy18:0(w7,8), i15:1w7c, i19:1w7c, 17:1w6c, 15:1	Sulfate reducers	[19-44]
14:1w7, i17:1w8, 3-OH15:0, 9-OH16:0, 10-OH16:0, 11-OH16:0, 3OH 17:0	Geobacter sp	[1]
i15:0, a15:0, i17:0, a17:0	Bacillus sp.or Arthrobacter sp	[2-3]
16:1w5c, 16:1w8c	Type 1 methane Oxidizers	[16]
10Me18:0, 10Me17:0, 10Me16:0	Actinomycetes	[20]
18:2 w 6, 18:3w6, 18:3w3, 18:2w6,9c	Fungi	[3-4]
i14:0, a14:0, i15:0, a15:0, i16:0, i17:0, a17:0 (Branched PLFAs)	Gram-positive	[44]
i15:0 3OH, 15:1iso , 16:1 2OH, 16:1iso , 16:1w5c, 16:1w7c, 16:1w9c, i17:1, et al., 2003. 17:1w8c, 18:1w5c, 18:1w7c, 18:1w9c,	Gram-negative	[45]
cy17:0, cy19:0 (Monounsaturated PLFAs) 14:0, 15:0, 16:0, 17:0, 18:0 (biomass indicator)	General bacteria	[44]

4. Discussion

The aim of this work was to develop a modified biomass recovery technique for PLFA analyses that would provide better estimate of cell community structure in low biomass soil samples. The method described here emphasizes two principal experimental difficulties associated with indirect cell extraction methods: soil aggregate dispersion and separation of cells from soil particles by centrifugation [16]. The first aspect is carried out by a combined approach consisting of homogenizing the soil using an inorganic cell releasing buffer supplemented with two surfactants and reciprocal shaking as a cell detachment method. In order to maximize biomass recovery from soil samples, optimum concentrations of Tween 80 and sodium pyrophosphate (NaPPi) were determined by quantifying extracted cells through live/dead epifluorescent microscopy (data not shown). The second approach of the modified method after soil dispersion was to separate cells from soil particles using differential gradient centrifugation to enhance recovery of total lipids. In this step, soil particles with higher density than the extracted cells were removed there by increasing the efficiency of subsequent DNA and PLFA extractions. This type of approach could be used as a primary step in the determination of difficult and important assays, such as community metagenomic analysis of subsurface environments [10].

The modified cell extraction method was useful to acquire community composition data. This method provided almost a ten fold increase (from 0.1 to 0.9) of treated samples that originally had no detectable biomass. On experimental samples where previously only 0.1 pm lipid recovery was obtained, with the use of the modified method the total biomass detected increased around ten fold. While microbial groups were detected with all biomass levels, specific members of those groups were only detected using the modified cell extraction method. On loam soil samples up to 4 additional specific microbial groups were detected and defined due to the increase in recovery of PLFAs; sulfate reducers, actinomycetes, methane oxidizers and even members of the genus *Geobacter* and *Arthrobacter* (soil bacteria). On low biomass soil groups detected that were more representative of a total community, with a total of 8 different microbial groups including methane reducers (type 1) and sulfate reducers. Although PLFA that belong to fungi were detected without the enhanced extraction protocol, the protocol had a 20 fold increment in lipid recovery.

With this method, biomass recovery was increased substantially on the different types of soil samples tested. In addition, different approaches to isolate genomic DNA from PLFAs aqueous phase were intended such as total precipitation, which weren't successful, indicating that a modified approach was required. High molecular weight bands were only detected after removing the cells from the soil matrix. Therefore, traditional problems such as coextraction of inhibitors, sheared DNA and non accessed signature lipid biomarkers were reduced. These results therefore suggest that the yield of DNA per gram of soil depends on the method used and on the properties of the soil considered [33] Due to the concomitant high molecular weight DNA obtained, a combinatorial approach can be established in future work with DNA based tools to confirmed microbial groups detected and by this way enhancing additional techniques other than PLFA. In addition, the proposed method reduces the underestimation of cell number regardless the soil type, bringing a better representative sample of the microbes present in a given environment. An error of most direct extraction methods is the assumption that with greater DNA recovery should reflect a more representative or diverse sample, while DNA yield is not the best way to estimate diversity. New tools to rapidly compare and estimate microbial community structure are needed [34]. This article presents data suggesting the combined approach of PLFA with any other molecular and biochemical technique to confirm and analysis microbial communities in samples containing different soil types.

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