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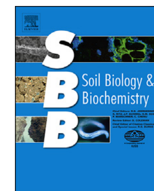

Can a metabolomics-based approach be used as alternative to analyse fatty acid methyl esters from soil microbial communities?

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ABSTRACT

Modern high-throughput approaches such as metabolomics, holds promise for investigating microbial communities. This study evaluated the quantitative and qualitative data generation potential of a metabolomics-based approach to characterise the fatty acid methyl esters (FAMEs) of soil microbial communities against that of traditional microbial lipid analyses, including fractionated phospholipid fatty acid (PLFA) and total lipid extract (TLE) analyses. The results showed that the extraction method and different derivatisation techniques had an effect on FAME concentrations and on repeatability between sample replicates. An assessment of the applicability of the different methods to distinguish between soil microbial communities exposed to various soil fumigant treatments in a greenhouse, showed that even though the metabolomics analysis gave higher FAME yields than PLFA analysis, its discrimination potential between treatments were much lower. Therefore, PLFA analysis was recommended for FAME characterisation in microbial communities. The untargeted metabolomics analysis has potential in differentiating between different treatments, despite representing the larger soil community and not microbial communities *per se*.

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1. Introduction

Phospholipid fatty acid (PLFA) analysis (Peacock and White, 2016) has been used extensively in soil studies throughout the past 40 years to characterise microbial community structure and it is still a valuable method. A search (September 2016) on Scopus for “soil AND plfa” in the title, abstract and keywords for the period 2013 to 2016 showed 584 hits. Of these, 273 were for articles published in 2015 and 2016. This clearly shows that the PLFA method is still widely used in soil investigations.

Traditional signature lipid biomarker (SLB) analyses, e.g. fatty acid methyl esters (FAMEs) from PLFA or total lipid extract (TLE) analyses are well-known for their capacity to characterise microbial communities in response to environmental disturbances (Chen et al., 2013; Drenovsky et al., 2004; Fichtner et al., 2014). SLB analysis from polar lipid (PL) fractions, aims to accurately characterise the PLFAs only, whereas TLE analysis includes all lipids in a sample. It should be noted that the term “TLE” is also referred to in some studies as “whole lipid extract” (Cescut et al., 2011; Donato et al., 2011). When using the PLFA method, the TLE is fractionated into various lipid classes and generally, only the PL fraction containing the phospholipids is used for further derivatisation and analysis (Guckert et al., 1985). This provides an indication of viable microbial biomass and signature fatty acid biomarkers representative of specific microbial groups. When using the TLE method there is no fractionation of the extract and the range of lipids detected include neutral lipids, glycolipids and PLs (Drenovsky et al., 2004; Kaur et al., 2005; White et al., 1979; Zelles, 1999). For both the PLFA and TLE methods, the lipids are converted to their respective FAMEs through methylation before being analysed by gas chromatography-mass spectrometry (GC-MS). However, different derivatisation procedures have been found to influence FAME recovery yield and quality (Basconcillo and McCarry, 2008; Chowdhury and Dick, 2012; Gómez-Brandón et al., 2010) and it is important to ascertain the most suitable combination of extraction and derivatisation techniques for optimal FAME characterisation in different sample matrices.

The PLFA method is well-known and often used; however, the considerable number of studies that suggested modifications to the

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original extraction, fractionation and derivatisation procedures has led to uncertainty about the most appropriate procedures. In addition, the literature is rife with inconsistent interpretations of PLFA data sets. Two recent reviews by Willers et al. (2015a; 2015b) examine these issues and they will not be discussed again here. These uncertainties about FAME analysis prompted the current investigation into an alternative by exploring a modernised high-throughput metabolomics approach. Considering that a metabolomics-based approach requires less sample clean-up and preparation (Garg et al., 2015; Wood, 2014), it may provide a higher sample throughput compared to traditional Bligh and Dyer (1959) extraction followed by fractionation and FAME analyses. Despite the increasing utilisation of metabolomics-based approaches in environmental studies, no clear comparison has been made between conventional SLB techniques and a metabolomics-based approach. For the latter, two types of analyses can be used, namely targeted or untargeted. With targeted analysis, a specific group of metabolites is quantified (Shulaev, 2006) while untargeted analysis provides a total profile of all the measurable metabolites in a sample (Dunn et al., 2013).

The aim of this investigation was to evaluate the targeted analysis of selected FAMEs as obtained from PLFA, TLE and metabolomics methods respectively, to characterise the microbial community structure of soil samples. Homogenised soil samples were characterised first and from these results, the PLFA and metabolomics methods were deemed the most appropriate. The resulting FAMEs obtained from the PLFAs and the FAMEs and metabolites obtained from the metabolomics analyses, were then compared for their ability to distinguish between soil microbial communities exposed to different fumigant treatments in a greenhouse experiment. The soil fumigants included the biofumigants canola (*Brassica napus*) and mustard (*Brassica juncea*), as well as the chemical fumigant metham sodium.

2. Materials and methods

2.1. Chemicals and glassware

The following high purity solvents (Honeywell Burdick & Jackson®) were obtained from Anatech Instruments (Pty) Ltd. (Olivedale, South Africa): acetone, chloroform, hexane, methanol and water. Potassium hydroxide (KOH) and dipotassium hydrogen phosphate (K_2HPO_4) from Merck Millipore (Modderfontein, South Africa). Glacial acetic acid; silicic acid; 2,2,4-trimethylpentane (isooctane); N,O-bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane (BSTFA + TMCS, 99:1), hereafter referred to as BSTFA-TMCS; methoxyamine hydrochloride and anhydrous pyridine for the methoxyamination solution, from Sigma-Aldrich (Pty) Ltd. (Johannesburg, South Africa). Internal standards were used for quantification and quality control purposes and also purchased from Sigma-Aldrich (Pty) Ltd. (Johannesburg, South Africa). The internal standard (IS) methyl nonadecanoate (Me-C19:0) consisted of C19:0 FAME dissolved in isooctane (50 pmol ml^{-1}), whereas the IS mix consisted of L-norleucine, 3-phenylbutyric acid, 2-acetamidophenol and nonadecanoic acid, dissolved in methanol (5 μg ml^{-1}). The IS mix was used for data alignment in the untargeted data analysis. Glassware as required was obtained from Lasec South Africa (Pty) Ltd. (Midrand, South Africa). All glassware used for the analytical procedures were washed with tap water and phosphate-free detergent (Liqui-Nox®, Alconox Inc., Separations, Johannesburg, South Africa), rinsed thoroughly with tap, deionised and nano-pure water, air-dried and heated in a muffle furnace at 450 °C for 4 h to remove any possible lipid contaminants. The Teflon-lined caps were washed with phosphate-free detergent, rinsed with tap, deionised and nano-pure water, air-dried and sonicated in acetone for 2 min.

2.2. Experimental design

The experiment consisted of a laboratory study followed by a greenhouse study. The laboratory study used homogeneous soil samples to evaluate the targeted analysis of selected FAMEs as obtained from the PLFA method (PL-FAMEs), TLE method (TL-FAMEs) and a metabolomics based approach. The latter entailed the separate analysis of the organic and aqueous phases after extraction (Chen and Chen, 2014), followed by targeted and untargeted data analysis. The most appropriate derivatisation was determined by applying the following procedures to the extracts obtained by all three methods: (i) methanolysis for targeted analysis of PL-FAMEs; (ii) methanolysis followed by silylation of PL-FAMEs and TL-FAMEs; (iii) methanolysis followed by oximation and silylation of PL-FAMEs and TL-FAMEs, and (iv) for the metabolomics method the TL-FAMEs and aqueous phases were oximated followed by silylation. All procedures were conducted on six replicates.

After consideration of the results from the laboratory study, a greenhouse study was conducted to assess the potential of the PLFA method against the metabolomics method for their ability to distinguish between soil microbial communities exposed to different soil fumigant treatments. The greenhouse was maintained at a temperature range between 26 °C (day) and 20 °C (night). A randomised block design was used to place 12 plastic pots filled with homogenously mixed agricultural soil in the greenhouse. The 12 pots consisted of four treatments of 3 replicates each, which included the treatments: (i) soil only (control), (ii) soil with canola (*B.napus*) green manure, (iii) soil with mustard (*B.juncea*) green manure and, (iv) soil fumigated with metham sodium. After soil fumigant applications, the pots were kept at 50% water holding capacity to ensure sufficient release of isothiocyanates (Morra and Kirkegaard, 2002). The experiment was conducted over 28 days. Canola (*B.napus*) and mustard (*B.juncea*) in the early flowering stage (~80 days of growth) (McCully et al., 2008) were used as biofumigants. The glucosinolate levels are the highest in *Brassica* roots during this growth stage (Sarwar and Kirkegaard, 1998). On day 0 of the experiment, chopped plant material (roots, shoots and leaves) were incorporated at a rate of 15 g kg^{-1} soil at a depth of 10–20 cm into the relevant treatment pots (Omirou et al., 2011). Soil treatments without added biofumigants were also mixed at a depth of 10–20 cm to ensure similar soil disruption (Potgieter et al., 2013). The commercial soil fumigant, metham sodium (HERBI-FUME®, 510 g L^{-1}, Nulandis®, Lilianton, South Africa) was diluted to an aqueous solution of 10 g L^{-1} and applied to the treatment pots at a recommended application dosage of 300 μg g^{-1} (Omirou et al., 2011). The soil was mixed at a depth of 10–20 cm to ensure the even distribution of the chemical fumigant.

2.3. Sample preparation

For the laboratory study, a composite soil sample was obtained from an agricultural site by taking individual samples from the top 0–15 cm soil layer. The soil was mixed thoroughly, homogenised by sieving through a 2 mm sieve to ensure the removal of all visible plant components and divided into subsamples of approximately 50 g each. Samples were frozen, lyophilised and stored at −80 °C until further use. The homogeneity of the replicate soil samples was confirmed by testing the repeatability with a standard PLFA analysis, to ensure that the samples were representative of a single composite soil sample providing similar results. A low percentage relative standard deviation (%RSD) indicates high repeatability among replicates. The %RSD was below 10% for total FAMEs and for

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