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Short communication

Comparison of fatty acid methyl ester methods for characterization of microbial communities in forest and arable soil: Phospholipid fraction (PLFA) versus total ester linked fatty acids (EL-FAME)

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ABSTRACT

Phospholipid fatty acid (PLFA) and ester-linked fatty acid methyl ester (EL-FAME) extraction methods have been commonly used for estimating microbial biomass and characterizing microbial community composition in soil. However, to our knowledge, there is currently no comparison of these two methods across different ecosystems. A regression analysis for 8 different soils showed that there were significant linear relationships between PLFA and EL-FAME extracts (r^2 = 0.97, 0.98 and 0.72, respectively) for determining the fungal (18:2ω6,9) and bacterial abundance and fungal-to-bacterial ratio (F:B ratio). However, regression lines of forest soils for fungal abundance and F:B ratio had a slight but significantly higher intercept than those of arable soils, i.e. higher EL-FAME:PLFA ratio for fungi in forests than those in arable soils. This might be due to that EL-FAME extracts may have additional factors such as physiological status of fungi and the inclusion of substantial amount of humic substances that affect quantitative determination of fungal biomass. Overall, EL-FAME method is simple and would produce similar results to PLFA method for bacteria in both quantitative and qualitative assessments when comparing different soils across ecosystems. However, for fungi, PLFA method would be more suitable than EL-FAME method.

Fatty acids are powerful indicators of soil microbial community composition (Bååth, 2003; Frostegård and Bååth, 1996; Willers et al., 2015a,b; Zelles, 1999). Phospholipid fatty acid (PLFA) extraction methods have been widely used to estimate microbial biomass and community structure in soil (Frostegård and Bååth, 1996; Frostegård et al., 2011; Willers et al., 2015a,b). A simpler method, the ester-linked fatty acid methyl ester (EL-FAME) method, has also been used to characterize soil microbial community composition (e.g. Mechri et al., 2010; Vallejo et al., 2012). The EL-FAME method uses the same mild alkaline reagent as the PLFA method, which extracts only ester-linked fatty acids from soil samples. However, the extraction procedure of EL-FAME is performed directly on soil samples, with no previous separation of lipid fractions; therefore, EL-FAME profile may originate from other lipids than phospholipids, such as glycolipids and neutral lipids (Zelles, 1999). EL-FAME have well characterized responses in soil microbial communities to environmental events, such as heavy metal pollution (Hinojosa et al., 2005), carbon (C) and nitrogen (N) supplementation (Hopkins et al., 2008), N deposition (Zechmeister-Boltenstern et al., 2010), grazing (Gass and Binkley, 2011) and hurricane disturbance (Cantrell et al., 2014). In addition, previous studies have reported that both PLFA and EL-FAME yielded similar microbial community compositions in agricultural (Drijber et al., 2000), mining (Hinojosa et al., 2005) and forest soils (Zechmeister-Boltenstern et al., 2010). However, to our knowledge, there is no information on whether consistent results between PLFA and EL-FAME methods can be obtained across ecosystem types. This prevents us from applying these methods to assess, for instance, soil microbes across different landscapes under environmental change. Thus, this study compared the PLFA and EL-FAME methods with forest and arable soils.

Soil samples were collected at eight sites (four forests and four croplands) in Japan. The soil properties of the sites are presented in Table 1. Soil 1 is a no-till soil with weeds used as living mulch without chemical fertilizer. Rice bran or oil cake are applied at the time of planting. Until 5 years ago, this soil was tilled with weed removal and

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Table 1
Characteristics of the eight study soils.

Sample No.	Ecosystem	Lat/long	Soil type	pH (H_2O)	Total C (%)	C/N
1	No-till farm	34.35/136.03	Gleysol	6.00	2.24	10.40
2	Till farm	36.01/140.21	Andosol	6.50	6.69	15.80
3	No-till farm	35.28/139.35	Andosol	6.10	5.07	15.52
4	Till farm	35.28/139.35	Andosol	6.14	5.22	13.84
5	Deciduous broad-leaved forest	43.29/143.50	Humic Cambisol	6.12	12.08	12.66
6	Deciduous broad-leaved forest	35.92/138.81	Humic Cambisol	4.92	13.16	14.88
7	Mixed broadleaf-conifer forest	33.14/132.91	Humic Cambisol	4.43	22.14	20.95
8	Evergreen broad-leaved forest	26.74/128.23	Helvic Acrisol	5.16	7.71	20.21

Data of total carbon (C) and carbon-to-nitrogen ratio (C/N) for soil 5–8 were provided by the Ministry of the Environment, Monitoring Sites 1000 Project (GBDataPackage2014ver1.zip, downloaded from http://www.biodic.go.jp/moni1000/findings/data/index.html).

fertilized with chemical fertilizers for more than 30 years. Soil 2 has been tilled with weed removal and fertilized with organic manure since 2009. The sites of Soils 3 and 4 were kept as grasslands for more than 30 years before the agricultural experiment site was established in 2010. While both soils were fertilized with chemical fertilizer, Soil 3 is no-till with weeds used as living mulch and Soil 4 is tilled with weed removal. The site information of forest soils (Soil 5–8) can be found in Ishihara et al. (2011). Three soil samples per site were collected using a 100 cm^3 corer to a depth of 5 cm. Immediately after the sampling, soils were sieved (< 2 mm), and roots and coarse litter fragments were removed. The three samples of each site were mixed thoroughly and frozen at −20 °C immediately after sieving. Three subsamples of each soil sample were extracted for both the PLFA and EL-FAME methods. The PLFA analysis was conducted with a procedure based on those of White et al. (1979) and Niwa et al. (2008). The EL-FAME analysis was conducted using the method of Schutter and Dick (2000). Both methods employ a mild alkaline methanolysis to convert from lipids to fatty acid methyl esters (FAMEs), which extracts only ester-linked fatty acids. FAMEs were analyzed by gas chromatography using an Agilent 7890A gas chromatograph with an HP-ULTRA 2 capillary column (internal diameter, 0.2 mm; film thickness, 0.33 μm) with a 5% phenyl phase (Agilent Technologies, Santa Clara, CA) under an automated MIDI system (MIDI, Newark, DE, USA) based on GC-FID separation. Fatty acids were identified by the Sherlock® microbial identification system (version 6.2) using the PLFAD1 peak library (MIDI, Newark, DE, USA). Concentrations of single FAMEs were calculated using the internal standard (19:0) peak as a reference. Fungal biomass was quantified based on 18:2ω6,9 content, and bacterial biomass was quantified as the sum of 15:0, 17:0, 15:0iso, 15:0anteiso, 16:0iso, 17:0iso, 17:0anteiso, 16:1ω7, 17:0cyclo, 18:1ω7 and 19:0cycloω8. The F:B ratio was determined from the ratio of 18:2ω6,9 to the sum of bacterial markers. The fatty acids 16:0 10-methyl, 17:0 10-methyl and 18:0 10-methyl were used to indicate Actinomycetes (Actinobacteria) (Willers et al., 2015a,b). The fatty acid 16:1ω5 was used as a marker for arbuscular mycorrhizal (AM) fungi (Olsson, 1999). Total lipid abundance was calculated as the sum of these markers and 18:1ω9. We did not use 18:1ω9 as a specific microbial indicator due to the possibility of being contained not only in fungi but also in bacteria (Frostegård et al., 2011). The fatty acid 16:0 was excluded from the analysis because this lipid is ubiquitous and dominant in plants and microorganisms (Klamer and Bååth, 1998).

All statistical analyses were performed using R 3.1.2 (R Development Core Team, 2014). A regression analysis was performed to clarify whether there are linear relationships between EL-FAME and PLFA microbial groups. To compare the intercepts of the regressions between EL-FAME and PLFA microbial groups in forest and arable soils, an analysis of covariance (ANCOVA) was used. The values of fungal biomass and F:B ratio were log-transformed for the regression analysis and ANCOVA. To determine differences in the microbial lipid profiles across different soils in each method, a principal component analysis (PCA) was performed. For the PCA analysis, lipid abundance was converted to mole percent values (each lipid abundance/total lipid abundance) and these values were transformed in the negative arcsine of the square root.

Across all samples, the mean concentration of EL-FAME 18:2ω6,9 (41 nmol g^{-1} soil) was approximately 3.3-fold greater than that of PLFA (12 nmol g^{-1} soil), whereas the total bacteria of EL-FAME (327 nmol g^{-1} soil) was approximately 1.3-fold greater than that of PLFA (251 nmol g^{-1} soil). Results of the regression analysis showed that fungal, bacterial and actinomycetal abundance had significant linear relationships between PLFA and EL-FAME extracts (Fig. 1A and D, Fig. S1A). However, regressions of fungal (18:2ω6,9) abundance, F:B ratio and fungal:total FAME ratio showed that the lines of forest soils had higher intercepts than those of arable soils (Fig. 1A–C). According to the ANCOVA, these differences of the regression lines between forest and arable soils were significant ($P < 0.05$). Namely, the EL-FAME:PLFA ratio for fungal markers was higher in forest than in arable soils; forest soils had a 1.19-fold greater mean value of this ratio than arable soils. This indicated that ecosystem type was related to differences in fungi between extraction methods. In addition, regardless of ecosystem type, relative content of fungal markers of EL-FAME was about 2-fold greater than that of PLFA (Table S1). These variances could be associated with the neutral lipid fatty acids (NLFA) and glycolipid fatty acids that can be extracted by the EL-FAME but not by the PLFA method. Bååth (2003) indicated that the NLFA:PLFA ratio changes depending on the nutrient status or physiological state of soil fungi. According to this author, when fungal growth is limited by nutrients, excess C would be stored in neutral lipids. Therefore, the higher EL-FAME:PLFA ratio for fungi in forests compared to arable soils might be due to fewer available nutrients per available C content for fungal growth. Besides, the lipid 18:2ω6,9 has been found in plants (Frostegård et al., 2011). Neutral lipids are decomposed much more slowly than phospholipids (Kuzyakov et al., 2014), while phospholipids are rapidly degraded following cell death (White et al., 1979; Zelles, 1999). Therefore, fatty acids derived from dead plant materials would have much more impact on an EL-FAME profile than on a PLFA profile. Since there is a huge amount of humus derived from plants in the forest floor, the higher plant-derived fatty acids may also possibly increase the 18:2ω6,9 EL-FAME in forest soils. Altogether, although we cannot specify why forest soils had higher EL-FAME:PLFA ratio for fungi than arable soils, possible reasons might be due to 1) the difference in physiological status of fungi and/or 2) different content of plant-derived fatty acids in soils. Furthermore, we used GC-FID with MIDI software that identifies peaks based on retention time, but some peaks derived from organic substances can overlap with peaks of microbial FAMEs (Fernandes et al., 2013). Given that EL-FAME extracts contain aliphatic compounds derived from organic substances, this organic matrix can interfere with the quantification of EL-FAMEs by GC-FID and may an additional factor accounting for the differences detected between the two methods. Therefore, it is desirable to confirm the identification of peaks with GC–MS.

The sum of bacterial markers from forest soils had a lower intercept