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High turnover rate of free phospholipids in soil confirms the classic hypothesis of PLFA methodology

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ABSTRACT

Phospholipid fatty acid (PLFA) analysis has been widely used to study soil microbial community structure. Soil PLFAs are taken to be indicative of living organisms since phospholipids are assumed to rapidly degrade after cell death. However, the turnover rate of phospholipids has never been accurately quantified. For this purpose, a short incubation experiment was conducted by amending paddy soil with extracted phospholipids, using the ^{13}C labeling technique and PLFA analysis to quantify phospholipid degradation. Both bacterial (*Methylocystis* sp. and *Escherichia coli)* and fungal (*Simplicillium subtropicum*) phospholipids had high turnover rates. The half-life ($t_{½}$) for different phospholipids ranged from 14 to 27 h and the average $t_{½}$ for total phospholipids was about 20 h at 25 °C but nearly double that at 15 °C. However, phospholipids had a similar turnover rate in a soil with lower microbial biomass (84 mg C kg^{-1}) compared to a soil with higher microbial biomass (305 mg C kg^{-1}). Assimilation of ^{13}C into other phospholipids was very low but followed the same timescale. Overall, this provides for the first time direct evidence for high turnover rates in soil through the analysis of specific ^{13}C-labelled PLFAs and confirms the classic hypothesis that intact phospholipids represent living cells, necessary for the validity of the established PLFA methodology.

1. Introduction

Phospholipids are specific components of cell membranes and some characteristic phospholipid fatty acids (PLFAs) can be indicative of major taxonomic groups, e.g., methanotrophs, fungi and actinomycetes (Frostegård and Bååth, 1996; Bossio and Scow, 1998). PLFA analysis provides an effective non-culture-based measurement for fingerprinting soil microbial communities (Frostegård et al., 2011; Yao et al., 2015) and has been widely used to investigate the changes of soil microbial communities in different land uses or environmental conditions (Frostegård et al., 1996; Bossio et al., 1998; Adam et al., 2015; Long et al., 2018).

There are two main advantages of the PLFA method for assessing soil microbial community structure over the most popular DNA-based techniques used nowadays: PLFA analysis can provide accurate quantification of microbial biomass and can be more sensitive in detecting shifts in microbial community structure, especially in distinguishing microbial community members involved in a distinct substrate decomposition when combined with ^{13}C stable isotope probing (Ramsey et al., 2006; Yao et al., 2015). Another important advantage is that soil phospholipids are taken to be indicative of only living organisms since it is assumed they rapidly degrade after cell death (White et al., 1979; Pinkart et al., 2002; Frostegård et al., 2011), but some other biomarkers, e.g., soil intact DNA, can even survive several months or years. Phospholipids include two hydrophobic fatty acid tails and a hydrophilic glycerol 3-phosphate head. The phosphate group can be modified with some other organic moieties such as ethanolamine, serine and choline. Initial degradation is probably through the action of phospholipases and phosphodiesterases (Albright et al., 1973). The phosphate head is then easily separated by the action of soil

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phosphatases. Although a few studies have determined fatty acid turnover using whole cell labelling or lipid fraction analysis (Rethemeyer et al., 2004; Kindler et al., 2009), no systematic studies have focused on this classic hypothesis underpinning PLFA methodology and the turnover rates of free phospholipids in soil have never been accurately quantified.

To determine the degradation rates of specific phospholipids and whether different phospholipids have different turnover rates in soil, we selected two bacteria (*Escherichia coli* and *Methylocystis* sp.) and a fungus (*Simplicillium subtropicum*). Using ^{13}C-labelled carbon sources, the ^{13}C-labelled phospholipids were extracted, amended into soils, and their turnover rates calculated. A secondary aim was to determine how the turnover rates of phospholipid were affected by temperature and level of microbial biomass.

2. Materials and methods

2.1. Soil and ^{13}C-labelled phospholipids

Soil was collected from the surface (0–15 cm) of a paddy field at Changde, (N29°19′19.17″ E111°19′23.22″), Hunan province in December 2017 and the soil belongs to Gleyi-Stagnic Anthrosols according to CSTC (Chinese Soil Taxonomic Classification). Briefly, the soil was classified as a clay loam with total C and nitrogen (N) contents at 16.6 g kg^{-1} and 2.3 g kg^{-1}, respectively. Soil pH was 5.2 and acid phosphatase activity was 223 nmol g^{-1} h^{-1} using the fluorimetric protocol of DeForest (2009). Soil microbial biomass was 305 mg C kg^{-1} and total PLFA content was approximately 20.2 nmol g^{-1}. A second soil with low microbial biomass (84 mg C kg^{-1}, 5.2 nmol g^{-1} total PLFA) was collected from a paddy field at Taihu (N30°23′9.43″ E116°11′5.51″), Anhui province, which was also Gleyi-Stagnic Anthrosols group. This soil was a clay loam with total C and nitrogen (N) contents at 6.8 g kg^{-1} and 0.9 g kg^{-1}, respectively. Soil pH was 5.3 and acid phosphatase activity was 197 nmol g^{-1} h^{-1}. Both air dry soils were ground to pass a 0.15 mm sieve.

To prepare ^{13}C-labelled phospholipids, the two strains of bacteria (*E. coli* DH5α and type II *Methylocystis* sp. strain Rockwell ATCC 49242) were cultured in BYP (beef yeast extract peptone) medium (with added ^{13}C-labelled glucose) and AMS (Ammonium mineral salts) medium (with added ^{13}C-labelled methyl alcohol), respectively. The fungus (*S. subtropicum*), previously isolated from the paddy soil, was cultured in modified Martin medium using ^{13}C-labelled glucose as the carbon substrate. The culture media (60 ml) were added to 120 ml serum bottles and placed on an orbital shaker at 150 rpm. *E. coli* was incubated at 37 °C for 24 h, *Methylocystis* sp. at 28 °C for 5 days, and *S. subtropicum* at 30 °C for 3 days. After harvest, the bacterial media were centrifuged and the deposit washed three times with sterilized 0.9% NaCl. The fungal medium was filtered (0.45 μm) to harvest the hyphae and washed as above. All the ^{13}C substrates were added at 20 atom%. The same atom percent excess was achieved in microorganisms since the labelled C substrate was the sole carbon source. Harvested material was freeze-dried before phospholipid extraction using the same method as for soil PLFAs (Frostegård et al., 1993) but without the methyl esterification step. Briefly, lipids of the freeze-dried pure culture (the same as the soil) were extracted with Bligh and Dyer solution, and after a liquid (chloroform plus-lipids) liquid (methanol and water) separation, the phospholipids were separated from the neutral lipid and glycolipids on a silicic acid column (White et al., 1979). The phopholipid extracts were dissolved in 500 μl methyl alcohol and added to 40 g soil along with 10 ml sterilized water, mixed well, freeze-dried (Christ ALPHA 2–4 LD plus, Germany) and stored at −80 °C.

2.2. Incubation of phospholipids in soil

We set up three labelled treatments: PE (phospholipids of *E. coli*), PM (phospholipids of the methanotrophic bacterium) and PF (phospholipids of the fungus). The amount of phospholipids added to the soil in PE, PM, PF was 2.9, 1.0 and 3.3 nmol PLFA g^{-1}, respectively. A control was also set up without added ^{13}C to enable correction of the data for ^{13}C natural abundance. After preincubation for 48 h at 25 °C and 50% WHC (water holding capacity), the Changde soil was amended with 10% (w/w) of the phospholipid impregnated soil and mixed thoroughly. Then 5 g (dry weight) was distributed into different serum bottles (100 ml) with a total of 21 bottles set up for each treatment. All bottles were covered with needle-punctured aluminum foil to maintain a stable aerobic condition and incubated at 25 °C and 95% relative humidity. Three replicates were randomly chosen for measuring the PLFAs at seven time intervals: 0, 2, 6, 12, 24, 48 and 120 h.

2.3. Effects of temperature and soil biomass C on phospholipid turnover in soil

We investigated the effect of temperature and size of the soil microbial biomass on the turnover of phospholipids. The experiment was set up as above but with the soil amended with 17% (w/w) of the fungal phospholipid impregnated soil. Two sets of the high microbial biomass soil (Changde) were incubated at 15 °C and 25 °C, respectively. Similarly, the low microbial biomass soil (Taihu) was incubated at 25 °C. Samples were collected after 0, 6, 24, 48 and 120 h.

2.4. Soil PLFA extraction and ^{13}C-PLFA analysis

Soil PLFA was extracted from 3.0 g freeze-dried soil using a modified version of the Bligh and Dyer method (Frostegård et al., 1993). Briefly, soil samples were extracted twice with 22.8 ml one-phase chloroform-methanol-citrate buffer (0.15 M, pH 4.0); the volume ratio of the mixture was 1:2:0.8. The phospholipids were separated from the neutral lipid and glycolipids on a silicic acid column (Gehron and White, 1983) and were methylated using a mild alkaline methanolysis to derivatize them to their corresponding fatty acid methyl esters (FAMES) (White et al., 1979). Methyl nonadecanoate fatty acid (19:0) was added as an internal standard. Finally, the FAMEs were dissolved in 150 μl n-hexane.

The FAMES were identified on a gas chromatograph equipped with a flame ionization detector (GC-FID, Agilent 7890B, USA) using a HP-Ultra 2 5% Phenyl Methyl Silox GC column (25 m length, 0.2 mm i.d., 0.33 μm film thickness, Agilent Technologies, Inc. USA) with the injector and detector maintained at 250 and 300 °C, respectively. A single 2 μl split injection, with a 1:100 split, was analyzed at an initial temperature of 170 °C and then ramped up at a rate of 5 °C min^{-1} to 260 °C, followed by a ramp of 40 °C min^{-1} to 310 °C and held for 1.5 min, The H_2 carrier gas was held at a constant head pressure of 9 psi. Peaks were identified using bacterial fatty acid standards and MIDI peak identification software (MIDI, Inc., Newark, DE) (Bossio and Scow, 1998).

The ^{13}C/^{12}C ratios of individual PLFAs were determined by GC-C-IRMS using a Trace GC 1310 gas chromatograph (Thermo Fisher Scientific , USA) with combustion column attached via a GC IsoLink II and then ConFlo IV to a Delta V Advantage isotope ratio mass spectrometer (all Thermo Fisher Scientific , USA). The Trace GC 1310 contained a HP-5 column (50 m length, 0.2 mm i.d., 0.33 μm film thickness; Agilent Technologies, Inc. USA). The qualitative FAMEs samples were concentrated to 50 μl and 1 μl were injected in a splitless mode via an inlet held at 250 °C, and the He carrier gas was maintained at a constant flow rate of 1.0 mL min^{-1}. The GC oven was initially set at 100 °C, held for 1 min, ramped at 20 °C min^{-1} to 190 °C, then at 1.5 °C min^{-1} to 235 °C, and finally at 20 °C min^{-1} to 295 °C; where the temperature was held for 15 min (Thornton et al., 2011). The absolute ^{13}C/^{12}C ratios was quantified by calibrated CO_2 as the reference gas in IRMS. Otherwise, the state of IRMS was checked by known ^{13}C- methanol first when measuring the highly labelled PLFA samples. Qualitative analysis of FAMEs in IRMS was through comparing the sequencing and relative length of peaks determined by GC-FID and GC-C-

IRMS. After amendment with a known amount of labelled PLFA, soil PLFA was extracted and the recovery rate was calculated. Overall, recovery was calculated as 95% of that added. The results are presented in terms of PLFAs, recognizing that individual PLFA turnover represents the turnover of the phospholipid from which they are derived.

2.5. Statistical analysis

^{13}C-labelled PLFA data was processed as in previous studies (Wang et al., 2014) and analyzed using SPSS 20 (SPSS Inc., Chicago). Namely, the δ^{13}C values of FAMEs were determined using Eq. (1):

$$\delta^{13}C(‰) = (R_{sample} - R_{standard})/R_{standard} \times 1000 \quad (1)$$

where R is the ratio of the heavy to light isotopes in the individual FAMEs and standard (V-PDB, R = 0.0111802). The working standard was CO_2 (δ^{13}C value of −20.11‰).

The δ^{13}C value of each fatty acid molecule was corrected for the C added during derivatization using a mass balance Eq. (2):

$$n_{cd}\delta^{13}C_{cd} = n_c\delta^{13}C_c + \delta^{13}C_d \quad (2)$$

where n is the number of C atoms, c represent the fatty acid, d is the derivatizing agent (methanol and the δ^{13}C value was −29.33‰), and cd is the corresponding derivatized compound of fatty acid.

Nonlinear curve fitting (the asymptotic model) was applied to the changes over time; plots were drawn with Origin 9.0. The half-life time and residual amount of each phospholipid or set of phospholipids was calculated from the curve fit parameters using Excel 2007. Student's t-test was also performed using Excel 2007.

3. Results

3.1. PLFA profiles of microorganisms and soil

In the preparation of the ^{13}C-labelled phospholipids using pure cultures, a range of PLFA compounds were obtained. *E. coli* contained mainly 16:0, cy17:0ω7c, 18:1ω7c and cy19:0ω7c (Fig. S1A), as found previously (Kindler et al., 2009; Zelles, 1997). The *Methylocystis* sp. contained mainly 18:1ω8c and 18:1ω7c (Fig. S1B) as is typical for *Methylocystis* species (Bodelier et al., 2009). The fungus, *S. subtropicum*, contained mainly 16:0, 18:2ω6,9c, 18:1ω9c and 18:0 (Fig. S1C), as is common in fungi (Zelles, 1997). The total PLFA profile of the Changde paddy soil showed a typical composition dominated by i15:0, 16:1ω7c, 16:0, 10Me16:0, 18:1ω9c and 18:1ω7c (Fig. S2). Hence, the set of ^{13}C-labelled phospholipidss contains most of the major soil PLFAs apart from i15:0 and 10Me16:0 though 16:1ω7c was only found in trace amounts in the two bacteria.

3.2. Characterization of phospholipid degradation

Although the characteristic ^{13}C-labelled PLFAs were very different between the PE and PM treatment, and the initial ^{13}C-PLFA content in the PE treatment was 3.5 times more than in the PM treatment, however, the ^{13}C-PLFA in the three treatments changed with a similar pattern (Figs. 1–3). The total ^{13}C-labelled PLFA content followed the asymptotic model in all the three treatments and declined to a residual amount of about one-fifth. The half-life time ($t_{1/2}$) of total ^{13}C- PLFA in the PE, PM and PF treatments were not significantly different with 20, 20 and 21 h, respectively (Table 1) and the ratios of residual PLFAs to applied PLFAs were similar in each case (Figs. 1A–3A).

The characteristic PLFAs from *E. coli, Methylocystis* sp. and *S. subtropicum* were used to compare the degradation process of the different specific PLFAs in the soil. All the PLFAs showed a fast degradation, and the $t_{1/2}$ for different PLFAs ranged from 14 to 27 h. The turnover rate of cy19:0ω7c was the highest, while 18:2ω6,9c had the lowest. There was some evidence (*t*-test significant at p = 0.016) that the turnover of saturated PLFAs (mean $t_{1/2}$ 15.7 h) was faster than the monoenoic PLFAs (mean $t_{1/2}$ 19.8 h) with the polyunsaturated 18:2ω6,9c being even slower. The $t_{1/2}$ of 16:0 in the PE treatment was not significantly different from that in the PF treatment (Figs. 1B–3B). Similarly, the origin (*Escherichia coli* and *Methylocystis* sp.) and amendment amount (6.0 and 5.0 ng C g^{-1}) did not have a significant effect on the degradation rate of 18:1ω7c (Figs. 1D–2B). The residual amounts for the individual PLFAs showed a trend for less monoenoic PLFAs (14%) to be retained than saturated PLFAs (19%) but this was not statistically significant (p = 0.13).

3.3. Effect of temperature and microbial biomass on phospholipid degradation

The degradation rate was much slower at 15 °C than at 25 °C (Fig. 4). According to the degradation curve, the $t_{1/2}$ of total PM-derived PLFAs was 22 h at 25 °C but 39 h at 15 °C (Fig. 4), giving a Q_{10} of 1.8.

The size of the soil microbial biomass had little effect on the PLFA turnover rates in the two soils (Fig. 4). Although microbial biomass C and total PLFA content in the Changde soil were nearly 4 times those in the Taihu soil, the $t_{1/2}$ was 22 and 23 h, respectively.

3.4. Secondary assimilation

Increased labelled C contents were immediately found in some specific PLFAs, e.g., 14:0, i15:0 and 16:1ω5c, suggesting that secondary assimilation may be occurring during the incubation. Based on the dynamic change of ^{13}C-labelled i15:0, the final ^{13}C assimilation content in the PE, PM and PF treatments was 0.03, 0.02 and 0.04 ng C g^{-1}, respectively (Fig. 5). The amount of ^{13}C assimilation into i15:0 was less than 1% of the original i15:0 content, indicating that any secondary assimilation effect can be largely ignored in the labelling experiment. The dynamic changes of ^{13}C labelled 16:1ω5c and 14:0 were similar to the trend of i15:0 (Fig. S3). However, the final ^{13}C assimilation contents were much smaller (approximately 0.01 ng C g^{-1}), suggesting that secondary assimilation is related to soil microbial community structure and PLFA profile. The more abundant a PLFA is in natural soil, the more it will show uptake of ^{13}C by secondary assimilation.

4. Discussion

4.1. Phospholipid turnover rates

Our results have shown a rapid disappearance of free phospholipid from soil. It is important to recognize that the breakdown of phospholipid will consist of, first, cleavage of the various components by soil enzymes and, second, utilization of the free fatty acids, glycerol and other organic moieties as a carbon source. In this analysis only the first step is measured, and we cannot speculate on the rate of the second process. However, the appearance of label in the PLFA i15:0 indicates that even after only 6 h there might be some reuse of the added phospholipid fatty acid carbon backbone. Moreover, intact uptake of entire fatty acids cannot be ruled out as a reason for the appearance of labelled phospholipids, but in the case of the iso and anteiso fatty acids this is unlikely, as their biosynthesis starts from a different (branched) precursor and the classical biosynthesis pathway does not go via post-methylation of the linear (in this case intact taken up) fatty acid chain.

Different from the previous studies which have determined phospholipid degradation using ^{13}C-labelled microbial cells (Klamer and Baath, 1998; Kindler et al., 2009), the labelled phospholipids were added in a free form in this study. White et al. (1979) labelled phospholipids *in situ* using ^{32}P-phosphate and followed their turnover in sediment; they recorded an initial half life of 2 days under aerobic conditions but 12 days under anaerobic conditions. Harvey et al. (1986), also using sediment, showed ^{14}C-labelled phospholipid to

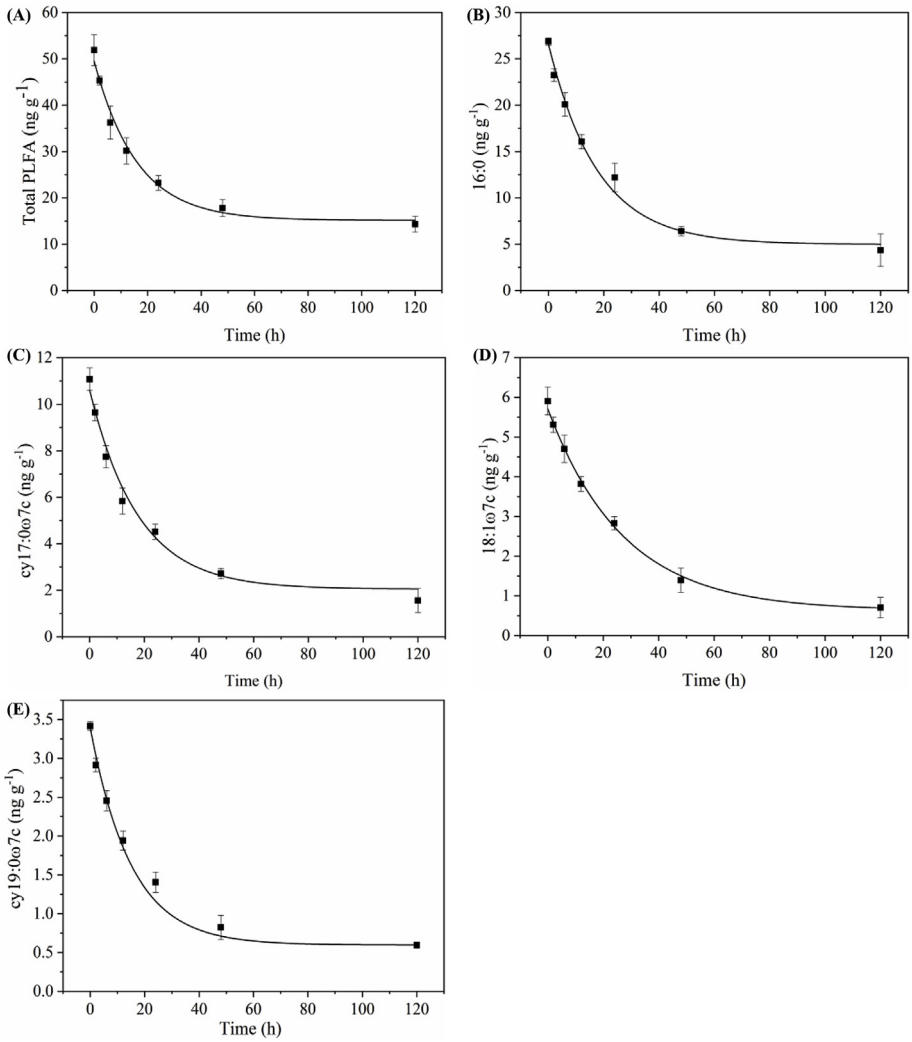

Fig. 1. Changes in total and individual ^{13}C-labelled PLFAs (ng C g^{-1}) from *Escherichia coli* (PE treatment) during incubation in soil. Bars indicate standard deviations of three replicates.

hydrolyse rapidly with a half life of 12 h, while $^{14}C–CO_2$ did not appear until 24 h. Klamer and Baath (1998) estimated the half life of killed phospholipid in compost to be 1–7 days. Kindler et al. (2009) followed the fate of ^{13}C-labelled *E. coli* phospholipids in soil but adding whole cells as a dying population; they estimated that half the population was intact upon addition to the soil. They recorded an initial half life of 66 h for the added phospholipids, followed by a much longer period of slow decay, though this period was characterized by the recycling of ^{13}C into other microorganisms. This indicates a slower decay of phospholipid when added as whole cells though the exact physiological state of their cells was unclear and the first sampling was only after seven days. de Vries et al. (2009) followed the fate of fungal phospholipid under conditions where they observed a significant decrease in fungal biomass over two weeks. They were surprised to find little change in fungal phospholipids, which they ascribed either to the low incubation temperature (12 °C) or to the increase in mean hyphal diameter. They postulated that the bulk of the phospholipid was in thick hyphae such that the observed loss of thin hyphae did little to reduce total phospholipid. Nevertheless, it could indicate some preservation of phospholipid in fungal residues. Following the addition of ^{13}C glucose, Gunina et al. (2017) estimated the mean turnover time of phospholipid in an agricultural soil to be 44 days, i.e. a half life of 30 days. Hence, there is a difference in the turnover rate of phospholipids within the living cell, within cells that have died but the phospholipids remain intact within the cell membrane, and dead cells that have lysed and released their phospholipids into the soil.

For each phospholipid there was a residual amount which did not appear to be susceptible to degradation, at least within the timeframe of the experiment. This amount was still clearly present as phospholipid but still extractable by the modified Bligh and Dyer extraction procedure (Frostegård et al., 1993). We interpret this residual amount as phospholipid adsorbed to the soil, possibly to clay minerals or to a combination of organic material and clay minerals, in such a way that soil enzymes are sterically hindered from activity. There was no real difference between the two soils studied in this respect even though their carbon contents were quite different. However, they were both clay loams. Further experimentation with soils of varying mineral, as well as organic matter content, would be required to test this theory. Since the recovery was ca. 95% it implied that there wasn't a significant amount of PLFA that was bound and not extractable. Except for adsorbtion, another possible mechanism for the constant proportion of residual phospholipids is the reconstruction of phospholipid from intact uptake of entire fatty acids (Dippold and Kuzyakov, 2016).

4.2. Influencing factors

The observed Q_{10} (1.8) is similar to that obtained in a meta-analysis of soil acid phosphatase values (1.77) by Hui et al. (2013) and the

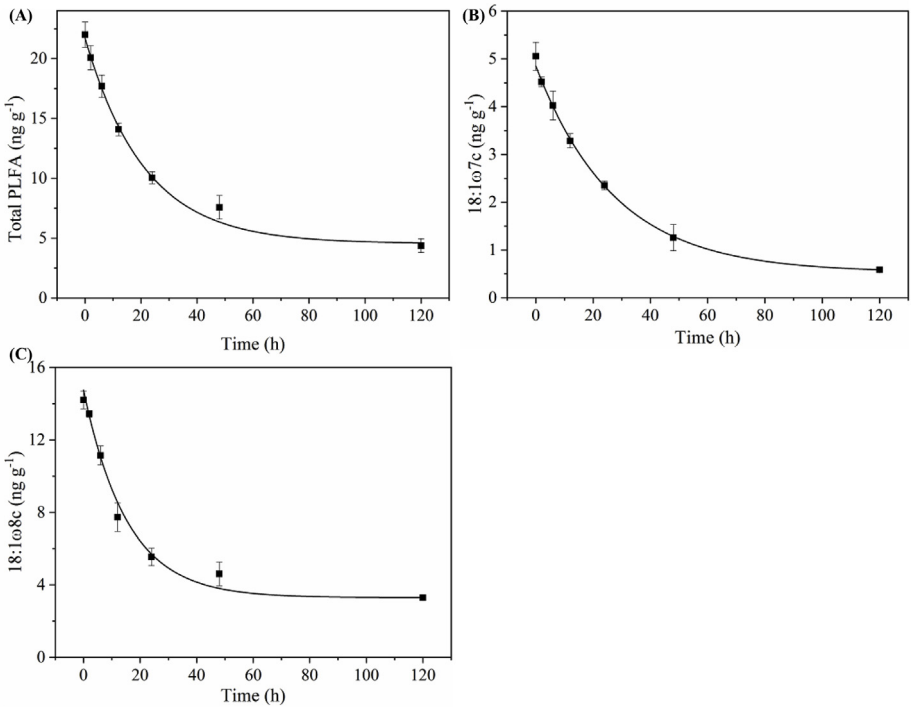

Fig. 2. Changes in total and individual ^{13}C-labelled PLFAs (ng C g^{-1}) from *Methylocystis* sp. (PM treatment) during incubation in soil. Bars indicate standard deviations of three replicates.

observed value for the decomposition of soil organic matter (Kirschbaum, 2006). Extrapolation to a soil temperature of 5 °C would give a mean $t_{1/2}$ of 70 h, assuming a constant Q_{10}.

Soil microorganisms can produce and release large amounts of extracellular enzymes which are main drivers for organic matter decomposition and nutrient transformation in soil (Anderson, 1984; Tripathi et al., 2007; Li et al., 2018; Zi et al., 2018). However, the degradation rates of organic matter and exogenous organic compounds are not directly related to the size of biomass, and substrate availability and enzyme activities are more important factors to determine the degradation (Shan-Min et al., 1987). In this study, the size of microbial community was not correlated with phosphatase activity (taken as representative of overall soil enzyme activity) and not the limiting factor for phospholipid decomposition in these soils. The results may suggest that much enzyme activity can be associated with soil extracelluar enzymes rather than intracelluar enzymes (Rao et al., 2000; Yao et al.,

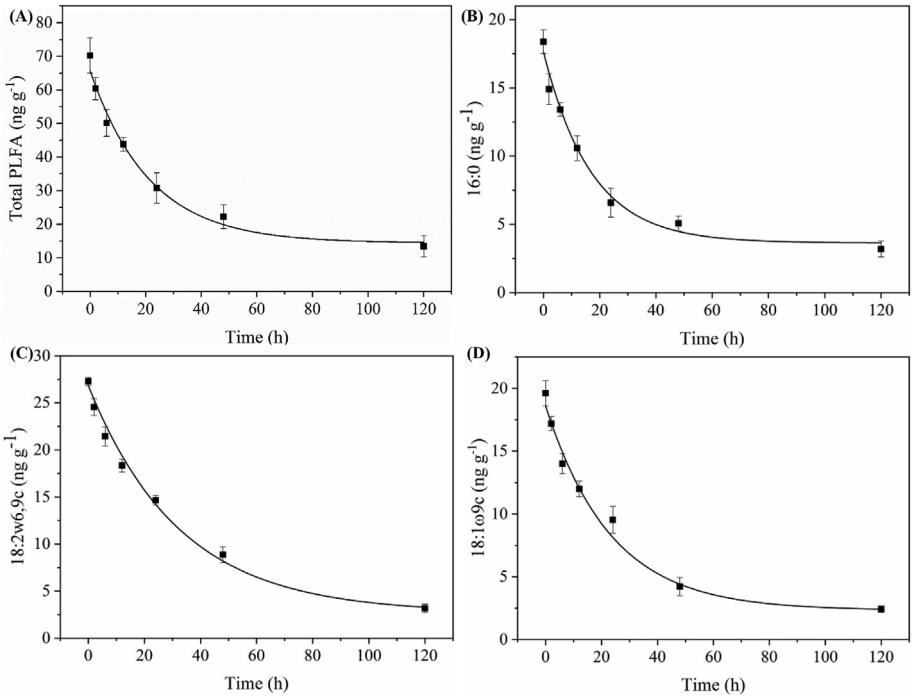

Fig. 3. Changes in total and individual ^{13}C-labelled PLFAs (ng C g^{-1}) from *Simplicillium subtropicum* (PF treatment) during incubation in soil. Bars indicate standard deviations of three replicates.

Table 1
Curve fit parameters for the asymptotic model of decay of individual PLFAs from *Escherichia coli* (PE), *Methylocystis*sp. (PM) and *Simplicillium subtropicum* (PF), and total (PF) PLFA under the high/low biomass and temperature treatments.

Treatments	Peak name	Equation(Asymptotic model)			R^2	$t_{1/2}$
		y = a+b*c^x				
		a	b	c		
PE	i15:0	0.032	−0.032	0.943	0.998	
	16:0	4.98	21.6	0.940	0.979	15 ± 4.1
	cy17:0ω7c	2.05	8.56	0.945	0.987	17 ± 2.7
	18:1ω7c	0.63	5.07	0.961	0.996	20 ± 1.9
	cy19:0ω7c	0.60	2.80	0.935	0.999	14 ± 1.7
	Total PLFA	10.37	38.7	0.952	0.994	20 ± 2.8
PM	i15:0	0.02	−0.017	0.900	0.899	
	18:1ω7c	0.53	4.32	0.963	0.999	21 ± 0.5
	18:1ω8c	3.30	11.45	0.941	0.998	17 ± 1.8
	Total PLFA	4.54	17.15	0.951	0.993	20 ± 1.8
PF	i15:0	0.040	−0.04	0.949	0.990	
	16:0	3.64	14.00	0.941	0.985	16 ± 2.9
	18:2ω6,9c	2.69	24.14	0.97	0.974	27 ± 1.8
	18:1ω9c	2.33	16.26	0.958	0.994	20 ± 4.8
	Total PLFA	14.4	51.22	0.954	0.984	21 ± 3.1
Low biomass25 °C	Total PLFA	22.67	89.72	0.958	0.986	23 ± 4.7
High biomass25 °C	Total PLFA	20.49	94.05	0.958	0.974	22 ± 5.1
High biomass15 °C	Total PLFA	33.31	80.51	0.969	0.983	39 ± 7.3

Notes: ± indicates standard deviation; for all $P < 0.01$.

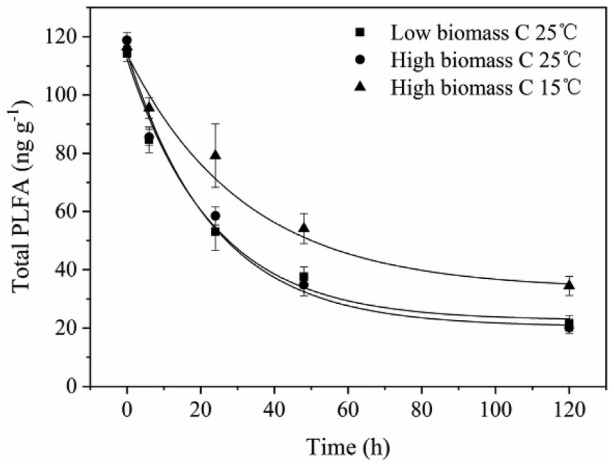

Fig. 4. Changes in total ^{13}C-labelled PLFA (ng C g^{-1}) during incubation in Changde (high biomass) soil at 15 °C and 25 °C and in Taihu (low biomass) soil at 25 °C. Bars indicate standard deviations of three replicates.

2009), which may be the case with the Taihu soil, particularly as it is a clay loam. Moreover, the greater biomass in the Changde soil may result in more fresh necromass compared to the Taihu soil, which can reduce the difference in phospholipid turnover rates.

4.3. Secondary assimilation

Secondary assimilation is a universal complication of the stable isotope probe technique. During the labelling period, soil microorganisms can use the labelled C by utilizing the metabolites produced from primary consumers, or the dead primary utilizers (Uhlik et al., 2009; Yao et al., 2015). Recently, intact uptake of entire fatty acids is also regarded as an important mechanism for phospholipid synthesis (Dippold and Kuzyakov, 2016). The process has been proven to contribute to the membrane lipid recycling of both bacteria and archaea (Takano et al., 2010; Bore et al., 2017). The non-amended PLFAs (i15:0, 16:1ω5c and 14:0) are good biomarkers for detecting secondary assimilation in this study, since they were not included in the ^{13}C-labelled PLFAs but had relatively high abundances in the tested paddy soil (Fig. S2). The small assimilation amounts (0.02–0.04 ng C g^{-1}) of ^{13}C into i15:0 demonstrated that secondary assimilation was less than 1% of the original i15:0 content and had no significant effect on our quantitative results for the phospholipid degradation. Also, the observed increases in i15:0 and 16:1ω5c suggest both Gram positive and negative bacteria take part in secondary assimilation (Zelles, 1997). The rate of uptake might suggest direct incorporation of virtually untransformed phospholipid. Dippold and Kuzyakov (2016) added ^{13}C 16:0 as the free fatty acid, and found direct incorporation into phospholipids at three days, i.e. at least part of the fatty acid carbon backbone was taken up intact as opposed to being metabolized first to acetyl-CoA. Moreover, some low molecular weight compounds derived from the cleavage of phospholipid, e.g., the glycerol and the modified phosphate-linked groups, can be potential precursors for phospholipid biosynthesis. While the added ^{13}C phospholipid would seem to be the most likely source, we cannot exclude some possible contamination of low molecular weight compounds which were co-extracted with the PLFA material. The fact that the incorporation was very minor and appeared to be exhausted after 1–2 days (before complete hydrolysis of the added phospholipid) tends to support this.

5. Conclusion

Our results give strong support to the assumption that phospholipids released into the soil following microbial death are rapidly hydrolysed. This is essential for asserting that the commonly applied PLFA analysis measures only the microbial biomass but not microbial necromass, i.e. it represents the living soil community, whether bacteria, fungi or, indeed, other soil organisms. The only caveats are that (1), up to 20% of the released phospholipids may become adsorbed to the soil and some phospholipids can be reconstructed by intact recycling; and (2), the process is temperature sensitive and hence soil phospholipids might be expected to survive longer time in the frigid zone than in the temperate zone.

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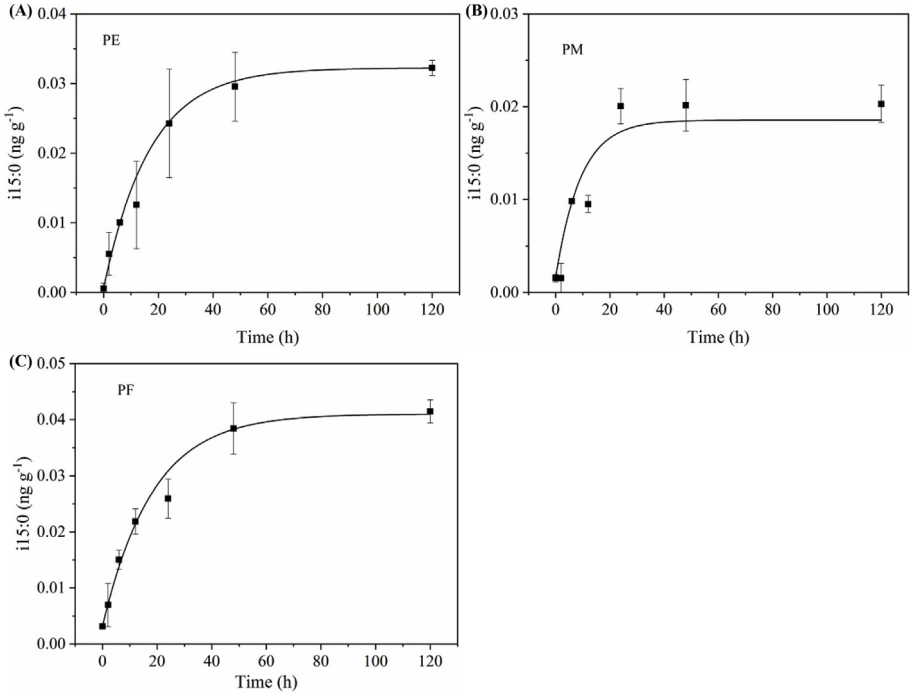

Fig. 5. Appearance of the ^{13}C-labelled PLFA i15:0 during incubation with ^{13}C-labelled PLFAs (ng C g^{-1}) from *Escherichia coli* (PE), *Methylocystis* sp. (PM) and *Simplicillium subtropicum* (PF). Bars indicate standard deviations of three replicates.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2019.05.023.

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