Metabolising old soil carbon: Simply a matter of simple organic matter?

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ABSTRACT

Bare fallow soils that have been deprived of fresh carbon inputs for prolonged periods contain mostly old, stable organic carbon. In order to shed light on the nature of this carbon, the functional diversity profiles (MicroResp™, Biolog™ and enzyme activity spectra) of the microbial communities of long-term bare-fallow soils were analysed and compared with those of the microbial communities from their cultivated counterparts. It was assumed that the catabolic and enzymatic profiles would reflect the type of substrates available to the microbial communities. The catabolic profiles suggested that the microbial communities in the long-term bare-fallow soil were exposed to a less diverse range of substrates and that these substrates tended to be of simpler molecular forms. Both the catabolic and enzyme activity profiles suggested that the microbial communities from the long-term bare-fallow soils were less adapted to using polymers. These results do not fit with the traditional view of old, stable carbon being composed of complex, recalcitrant polymers. Microbial communities from the long-term bare-fallow soils tended to preferentially use substrates with higher nominal oxidation states of carbon relative to the substrates used by the microbial communities from the cultivated soils. This suggests that the microbial communities from the long-term bare-fallow soils were better adapted to using readily oxidizable, although energetically less rewarding, substrates. Microbial communities appear to adapt to the deprivation of fresh organic matter by using substrates that require little investment, such as enzyme production.

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1. Introduction

The mean residence time, i.e. the inverse of the decomposition rate, of soil organic carbon (SOC) is extremely variable, ranging from a few days to several centuries or even millennia (Trumbore, 1997; Jenkinson et al., 2008). These differences reflect a combination of the intrinsic decomposability of the C and the environmental constraints on decomposition (Schmidt et al., 2011). The old organic C in soil has generated much interest because it represents the majority of SOC and the stability of this C is uncertain, in particular when faced with external perturbations, such as climate change or changes in land management (e.g. Fang et al., 2005; Reichstein et al., 2005; Davidson and Janssens, 2006; Hartley and Ineson, 2008). Although this C is treated as one or two homogeneous pools in most C dynamics models, i.e. the old pool in ICBM (André and Kätterer, 1997), the slow and passive organic C in the Century model (Parton et al., 1987) or the humus and inert pools in the RothC model (Jenkinson and Rayner, 1977), it is likely that it is made up of a diverse range of organic compounds that are stabilised through a range of different mechanisms. The different organic compounds that constitute SOC have (i) different inherent kinetic properties (Davidson and Janssens, 2006), (ii) display differential adsorption to mineral surfaces (Kleber et al., 2007), (iii) are more or less accessible to enzymes catalysing their degradation (Ekschmitt et al., 2005) or (iv) their decomposition rates are constrained by the physiology of the decomposer populations (Ekschmitt et al., 2005; German et al., 2011). If accurate predictions of the response of SOC to external perturbations are to be obtained,
then a clear understanding of these mechanisms is required (Conant et al., 2011). However, progress in this area has been hampered by a poor understanding of the nature of the molecular compounds that constitute the old, stable organic C in soil.

Long-term bare fallow field trials are interesting with regard to the study of old SOC as much of the C with short residence times has been mineralised during the bare-fallow period. Previous studies on long-term bare fallow soils have shown that the C in soils that have been subjected to bare fallow management for more than 60 years is almost entirely in the form of stable C, i.e. C that is hundreds or thousands of years old (Barré et al., 2010): the old C has been, to a certain extent, isolated by microbial decomposition processes that occurred naturally during the bare-fallow period. The diversity of microbial communities from long-term bare-fallow soils is not believed to be reduced by the long-term deprivation of fresh organic matter (Paterson et al., 2011; Hirsch et al., 2009). However, the data related to the functional diversity of these communities is not consistent: some studies have indicated that the functional diversity is unaffected (Hirsch et al., 2009; Guenet et al., 2011), but others have shown that the capacity to degrade certain compounds is diminished (Paterson et al., 2011).

Microbial communities adapt to the substrate that is available to them. There is a tight link between microbial communities, their immediate environment and the resources available to them: biogeographic studies have shown that microbial communities are mainly influenced by local environmental properties (e.g. Fierer and Jackson, 2006) and the experimental evolution of microorganisms has shown that they adapt rapidly and increase their fitness towards the available substrate in simple and complex environments (Lenski et al., 1991; Barrett et al., 2005). It has also been shown that bacteria do not lose the ability to grow on substrate to which they have not been exposed for many generations, but that they generally perform less well than lines evolved on the substrate (MacLean and Bell, 2002). Finally, relationships between the activity profiles of microbial communities and the composition of soil organic matter across a range of soils have been identified (Grandy et al., 2009).

Old C is difficult to isolate from soil as the C is not neatly compartmentalised. Traditionally, old, stable C has been viewed as being chemically recalcitrant, made up of large, complex macromolecules (Stevenson, 1994). This view of stable organic matter is sometimes referred to as the “polymer model” of soil organic matter. However, recent research suggests that the stability of old C in soil is the result of a combination of physicochemical and biological factors that reduce the rate at which old C is decomposed rather than the result of the intrinsic molecular properties of the C (Schmidt et al., 2011). For example, near edge X-Ray Absorption Fine Structure spectroscopy analyses have suggested that older C (measured as $^{14}$C age) can contain a greater proportion of easily metabolisable molecules than younger C (Kleber et al., 2011). Distinct differences in the relative abundances of various chemical groups between one of the long-term bare-fallow soils (Ultuna) included in this study and the same soil having received some form of organic input have also been identified (Gerzabek et al., 2006).

Based on these insights, it was hypothesised that the functioning (i.e. enzymatic spectra and catabolic capacities) of microbial communities from long-term bare-fallow soils would be adapted to using simple, labile substrates rather than complex substrates. The reasons for testing this hypothesis were twofold. The first was to search for corollary evidence that old SOC contains proportionally more simple, readily metabolisable, substrates than old SOC (Kleber et al., 2011) or that complex polymers in old C are simply not available to or used by soil microbial communities. In the present paper, we used the microbial communities as “biological in situ probes” of the organic matter. The second reason was to document how the functional diversity of microbial communities changed when deprived of fresh plant organic matter inputs for prolonged periods. The hypothesis was tested by comparing the catabolic profiles and enzymatic spectra of microbial communities from four long-term bare-fallow soils and from their cultivated counterparts. It was expected that catabolic profiles of the microbial communities from the long-term bare-fallow soils would display a more pronounced preference for simple substrates and would be less capable of using complex molecules than their counterparts from cultivated soils. However, the bare-fallow soils were not expected to completely lose the capacity to metabolise complex or plant derived compounds. It was also expected that the enzyme activity profiles of the long-term bare-fallow soils would contain relatively more enzymes targeting simple molecules.

2. Material and methods

2.1. Soils

In spring 2010, samples were collected from four long-term bare fallow sites (Versailles, France — 48°48’ N, 2°08’ E; Grignon, France — 48°51’ N; Ultuna, Sweden — 59°49’ N, 17°38’ E; Lanna, Sweden — 58°34’ N, 13°10’ E) and their cultivated counterparts and all samples were stored at 4°C until use. The bare-fallow plots at the sites had received no C inputs, except from occasional algae and weed growth despite repeated weeding, for between 82 and 14 years. Soil characteristics of the four sites are given in Table 1. Three replicate plots were sampled at all the sites. Prior to analysis, all soils were sieved <2 mm and incubated at 20°C for two weeks at 45% water holding capacity (WHC).

2.2. Catabolic profiles

Catabolic profiles were constructed for all the soils using Biolog EcoPlates™ (Biolog Inc., Hayward, CA, USA) and for three of the soils using the MicroResp™ system (Campbell et al., 2003). The Grignon soil contained carbonates and therefore reliable MicroResp profiles could not be established. The MicroResp profiles were established using 22 substrates belonging to 6 different molecular families (Table 2). Here, substrates were dissolved in deionised water (30 mg C per mL soil water) and dispensed at a rate of 30 μL solution per well, bringing the water content of the soils to 55%.

Table 1

<table>
<thead>
<tr>
<th>Soil</th>
<th>Soil C (%)</th>
<th>Soil N (%)</th>
<th>C-to-N ratio</th>
<th>pH (H2O)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Versailles&lt;sup&gt;a&lt;/sup&gt; fallow</td>
<td>0.56</td>
<td>0.06</td>
<td>9.4</td>
<td>4.4</td>
</tr>
<tr>
<td>Versailles&lt;sup&gt;a&lt;/sup&gt; cultivated</td>
<td>1.45</td>
<td>1.20</td>
<td>11.9</td>
<td>6.8</td>
</tr>
<tr>
<td>Grignon&lt;sup&gt;a&lt;/sup&gt; fallow</td>
<td>0.86</td>
<td>0.09</td>
<td>9.6</td>
<td>8.3</td>
</tr>
<tr>
<td>Grignon&lt;sup&gt;a&lt;/sup&gt; cultivated</td>
<td>1.11</td>
<td>0.11</td>
<td>9.8</td>
<td>8.3</td>
</tr>
<tr>
<td>Ultuna&lt;sup&gt;a&lt;/sup&gt; fallow</td>
<td>0.97</td>
<td>0.10</td>
<td>9.7</td>
<td>6.1</td>
</tr>
<tr>
<td>Ultuna&lt;sup&gt;a&lt;/sup&gt; cultivated</td>
<td>1.12</td>
<td>0.11</td>
<td>10.2</td>
<td>6.2</td>
</tr>
<tr>
<td>Lanna&lt;sup&gt;a&lt;/sup&gt; fallow</td>
<td>1.89</td>
<td>0.14</td>
<td>13.5</td>
<td>6.4</td>
</tr>
<tr>
<td>Lanna&lt;sup&gt;a&lt;/sup&gt; cultivated</td>
<td>1.96</td>
<td>0.15</td>
<td>13.1</td>
<td>6.4</td>
</tr>
</tbody>
</table>

<sup>a</sup> Field site established in 1928.
<sup>b</sup> Field site established in 1956.
<sup>c</sup> Field site established in 1996.
addition only were used as controls. The soils were then incubated for 6 h at 25 °C (Campbell et al., 2003). The CO2 evolved was determined by reading the gel detector plate in a plate reader at 570 nm (Multiskan RC, Labsystem Finland). The CO2 evolved was measured with a BioTek EL800 Universal plate reader (Bio-Tek Instruments, Winooski, VT). Enzyme activity is expressed as the quantity of p-nitrophenol (μg) released min⁻¹ g⁻¹ dry soil equivalent.

2.3. Enzyme assays

Eight enzyme activities involved in the decomposition of simple (β-glucosidase, α-glucosidase and β-xylosidase, N-acetylglucosaminidase) and more complex (cellulase, glucose oxidase, amylase and xylanase) substrates were assayed colorimetrically. The principal function of β-Glucosidase is to catalyse the hydrolysis of cellubiose to glucose and that of α-glucosidase is to cleave off glucose residues from the non-reducing end of glucose polymers containing α-(1→4) glucosidic bonds. β-XYlosidase hydrolyzes 1,4 linked β-D-xylose residues from β-D-xylosides. Cellulase catalyses the degradation of cellulose and polysaccharides built up of β-1,4-linked glucose units to release glucose, cellubiose and oligosaccharides (Deng and Tabatabai, 1994). Amylase plays a significant role in the breakdown of starch by releasing glucose or oligosaccharides from starch (Das and Varma, 2011). Glucose oxidase is indirectly involved in the oxidation of lignin and humic material through the production of hydrogen peroxide which is required by peroxidases to catalyse the reaction (Gallo et al., 2004). Xylanase is involved in the decomposition of hemicellulose and disrupts hemicellulose–lignin associations (Dungait et al., 2012). N-acetylglucosaminidase plays a role in the degradation of chitin and other β-1,4-linked glucosamine polymers (Sinsabaugh, 2005). N-acetylglucosaminidase, β-xylosidase, β-glucosidase and α-glucosidase activities were measured using p-nitrophenol based substrates. Microplate wells were loaded with 50 μl of a 1:10 soil-distilled water solution, 25 μl phosphate buffer and 50 μl of the appropriate substrate (71.9 mmol l⁻¹). Microplates were incubated for 1 h at 37 °C. At the end of the incubation the microplates were centrifuged (14,000 g for 5 min) and 50 μl of the supernatant transferred to a microplate containing 250 μl 2% Na2CO3 to stop the enzymatic reaction. Well absorbance (410 nm) was measured with a BioTec EL800 Universal plate reader (Bio-Tek Instruments, Winooski, VT). Enzyme activity is expressed as the quantity of p-nitrophenol (μg) released min⁻¹ g⁻¹ dry soil equivalent.

The xylanase, amylase and cellulase activities were assayed by quantifying the production of reducing sugars in samples in the presence of carboxymethyl-cellulose, xylan or starch (0.1 g 10 ml⁻¹ distilled water). A soil-distilled water suspension (1:10; 0.5 ml) was added to 0.25 ml phosphate buffer and 0.5 ml substrate solution. After incubation at 37 °C for 1 h, the samples were centrifuged at 14,000g for 5 min and 250 μl of the supernatant used to determine the concentration of reducing sugars (Nelson, 1944; Somogyi, 1945). The absorbance was measured at 630 nm and the enzymatic activity was defined as the quantity in μg of reducing sugars released h⁻¹ g⁻¹ dry soil equivalent.
Glucose oxidase activity in a soil suspension (1:10) was measured using the coupled O-dianisidine–peroxidase reaction method. A soil-distilled water suspension (50 μl) was added to 25 μl citrate buffer and 50 μl β-D-glucose (0.1 M). After incubation at 37 °C for 1 h in the dark, the samples were centrifuged at 14,000g for 5 min and 50 μl of the supernatant were transferred to a microplate containing 150 μl of the reactive solution (O-dianisidine 0.1 M and horseradish peroxidase 50 units/ml) and incubated for 20 min at 37 °C in the dark. The H₂O₂ produced resulted in the oxidation of O-dianisidine by horseradish peroxidase and the colour development was monitored spectrophotometrically at 500 nm. Enzyme activity is expressed as the quantity of O-dianisidine oxidized (μg) h⁻¹ g⁻¹ dry soil equivalent.

All measurements were made at ambient soil pH. Wells with soil and buffer but without substrate were used as blanks. All the assays were carried out in triplicate thus ensuring the reproducibility of the laboratory analyses.

2.4. Quantification of microbial communities by real-time PCR

In order to provide some explanation for the results obtained, we also determined the bacterial to fungal ratios of the microbial communities, because this ratio is believed to affect the carbon-use efficiency of microbial communities (Sakamoto and Oba, 1994). Were there to be significant changes in the bacterial to fungal ratio, then one might expect changes in the catabolic profiles measured by MicroResp that are independent of the capacity to use the substrates. Total genomic DNA was extracted from 250 mg of fresh soil using the Power Soil DNA Isolation Kit (MoBio, USA), following the manufacturer instructions. The quality of DNA extractions was checked by electrophoresis on 1% agarose gels stained with GelRed (Molecular Probes, USA) using a Gel Doc image analyser (BioRad, USA). The DNA concentration was quantified using Quan-tITM dsDNA High-Sensitivity Assay Kit (Invitrogen, Canada). All DNA extracts were then diluted to 0.5 ng μl⁻¹ for subsequent analysis. The quantification of soil microbial communities was performed by real-time PCR amplification targeting the 16S rRNA gene for bacteria and the internal transcribed spacer (ITS) gene for fungi on a StepOne™ Real-Time PCR (Applied BioSystem, USA) as following. Each reaction was carried out using previously described primers 341F 5’-CCTACGGGAGGCAGCAG-3’ and 534R 5’-ATTACCGCGCTGCTGCA-3’ for 16S rDNA and ITS3 5’-GATACCGTCAAGAACGCAGC-3’ and ITS4 5’-TTCCTCCGCTTATGATATGC-3’ for ITS. Each reaction contained 1 ng of DNA template, 7.5 μl of Power SYBR® Green PCR Master Mix (Applied Biosystem, USA) and 0.1 μM of each primer in a total reaction volume of 20 μl. The PCR program included an initial denaturation step at 94 °C for 4 min, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C and 55 °C (respectively for 16sDNA and ITS) for 30 s, extension at 72 °C for 10 s, and 1 cycle and 30 s at 80 °C. The melting curve was obtained by slow heating with a 0.1 °C/s increment from 65 °C to 95 °C, with fluorescence acquisition at 0.1 °C intervals. PGEM-T easy (Promega) containing 16S rRNA genes from *Pseudomonas aeruginosa (strain 01)* linearized by Sall were used to generate copy number standard curves. The results were analyzed using stepOne V 2.2.2 software.

2.5. Degradation energetics

It has been suggested that old, stable C does not yield sufficient energy to make investment in enzymes worthwhile for microbial communities (Fontaine et al., 2007). If this were the case, microbial communities adapted to using old C should be adapted to using substrates with a lower bioenergetic yield. The average bioenergetic potential of compounds preferentially used by microbial communities from either long-term bare-fallow or cultivated soils was determined using an energetic description of compounds which is based on the nominal oxidation state of carbon (NOSC). The NOSC itself is inversely related to the Gibbs energies of half reactions describing the complete mineralization of the compounds (LaRowe and Van Cappellen, 2011). The NOSC in the compounds used in the Biolog and MicroResp was calculated according to LaRowe and Van Cappellen (2011).

2.6. Statistical analyses

Principal components analysis was used to reduce the dimensionality of the catabolic and the enzymatic profiles using the Vegan package with R software (version 2.12.0 R Development Core Team 2008; R: a language and environment for statistical computing). The catabolic diversity of microbial communities was calculated using the Simpson Diversity Index and catabolic evenness (Kennedy and Smith, 1995). Significant differences between sites and treatments along the first two ordination axes, in the catabolic diversity and for individual enzyme activities were determined by two-way (site x management) ANOVA. The difference between the average bioenergetics potential of the substrate compounds preferentially used by the long-term bare-fallow or cultivated soils was determined by ANOVA. The preferential use of substrates by microbial communities was defined as the proportion of the total substrate use accounted for by a given substrate (i.e. in the case of the Biolog measurements, this was the individual well colour development divided by the sum of all well colour developments and in the case of the MicroResp measurements, this was the individual CO₂ production divided by the total CO₂ production). It was necessary to analyse the substrate preference in this way because substrate use is proportional to the microbial biomass present and differences in biomass would have masked the preferences of the microbial communities: indeed, the substrate-induced respiration measurement of the soil microbial biomass (Stenström et al., 1998) is based on the relationship between microbial biomass and substrate use.

The preference that microbial communities have for a given substrate is therefore defined relative to use of all the other substrates tested. It does not refer to the use other microbial communities might have for this substrate. For example, if microbial communities rely heavily on a single substrate and do not mineralise the other substrates to the same extent, then their preference for this substrate is high, regardless of the actual amount of the substrate mineralised. Microbial communities that mineralise this same substrate to a greater extent will have a lower preference for this substrate if they also mineralise other substrates equally. Each substrate was assigned to the long-term bare-fallow or cultivated group on the basis of the ANOVA.

3. Results

All three measures of microbial functional diversity (MicroResp, Biolog and enzyme activities) showed significant differences between the bare-fallow and cultivated treatments (Figs. 1–3). The differences between bare-fallow and cultivated plots were significant (P < 0.01) along both of the first two ordination axes of all the PCA. The eigenvalues of the first two principal components accounted for 90%, 59% and 89% of the total variance in the MicroResp, Biolog and enzyme data, respectively (Figs. 1–3). The differences between the bare-fallow treatments and their cultivated counterparts tended to increase as a function of the duration of the bare-fallow treatment in all three profiling methods, although the differences were more pronounced and more significant (P < 0.001) in the MicroResp profiles (Fig. 1) than in the Biolog
or the enzyme profiles (Figs. 2 and 3). The PCA loadings of the Biolog and MicroResp profiles indicated that the microbial communities from the bare-fallow soils were generally less adapted to using the polymers (tween 40, tween 80, glycogen, xylan) and better able to use simple molecules (organic acids, disaccharides; Figs. 1 and 2; Table 2). The pattern of enzyme activities was explained by β-glucosidase, N-acetylglucosaminidase and glucose oxidase activities (Fig. 3), all of which were significantly higher in the cultivated soils than in the bare-fallow soils ($P < 0.01$).

The enzyme activity patterns tended to be more similar in the bare-fallow soils than in the cultivated soils; i.e. there was a convergence in the enzyme activity patterns (Fig. 3). However, the opposite was true for the Biolog and MicroResp data (Figs. 1 and 2).

There were no significant differences among soils or treatments in the catabolic diversity of microbial communities measured by Biolog (Table 3). There was, however, a significant reduction in catabolic diversity ($P < 0.01$; Table 3) of the long-term bare-fallow soils relative to their cultivated counterparts in the MicroResp profiles. The reduction in catabolic diversity increased with the age of the bare falls (Table 3). The reduction in catabolic diversity was not due to a reduction in catabolic richness, since each of the soils was able to use all the substrates, but was due to a reduction in evenness (Table 3).

The substrates that were significantly ($P < 0.05$) preferentially used by the long-term bare-fallow communities and their cultivated counterparts (i.e. the substrates responsible for the differences between management systems in Figs. 1 and 2) and the associated NOSC values are shown in Table 2. In the MicroResp approach, the average NOSC of the substrates preferentially used by the long-term bare-fallow communities was 1.37. This was
significantly higher \((P < 0.05)\) than that of the substrates preferentially used by the microbial communities from the cultivated soils (average of NOSC = −0.01; Table 2). Microbial communities from the long-term bare-fallow soils preferentially used organic acids, C substrates that are readily oxidized resulting in positive NOSC values. In comparison, microbial communities in cultivated soils preferentially used C substrates across all 6 molecular families. The average NOSC of the Biolog substrates used by the long-term bare-fallow communities (NOSC = 0.12) was also higher than that of the substrates used by the communities from the cultivated soils (NOSC = −0.48), although these differences were not significant (Table 2).

The bare-fallow treatment resulted in a significant overall decrease \((P < 0.01)\) of both 16S and 18S copy numbers, suggesting that both bacterial and fungal biomass was lower in the bare-fallow soils (Fig. 4). However, the higher variability associated with the qPCR data meant that only the difference in 18S copy numbers between the bare-fallow and cultivated soils from the oldest experiment in Versailles were significant. Furthermore, the bacterial to fungal ratio did not vary between treatments (Fig. 4).

4. Discussion

4.1. Substrate use as biological in situ probes of old C

The microbial communities from the long-term bare-fallow soils tended to use simple substrates and, in particular, molecules or their derivatives from the Krebs cycle (i.e. \(\alpha\)-ketoglutaric acid, citric acid or ascorbic acid in the MicroResp profiles, Fig. 1), suggesting that the recycling of microbial metabolites was a primary source of substrate for these communities. Microbial communities from the bare-fallow soils were also less able to use polymers (Figs. 1 and 2; Table 2), possibly due to the fact that they were less exposed to polymers. A corollary of this result is that old C contains less polymerised material than does the young C found in cultivated soils, assuming that microbial communities are adapted to their environment. Similar catabolic profiles were obtained for subsoil microbial communities (Salome et al., 2010) where the organic C is also old (Moni et al., 2010). The use of microbial community catabolic profiles to probe the old soil C yielded the same results as those obtained by Kleber and Johnson (2010) using more traditional analytical approaches. Furthermore, recent information gathered using spectroscopic, microscopic, pyrolysis and soft ionization techniques is also not consistent with the polymer description of old, stable C, i.e. a pool of intrinsically recalcitrant due to the formation of humic polymers (Schmidt et al., 2011). This is an important result as many SOC models assume that depolymerisation processes are rate limiting in the decomposition of old C (Conant et al., 2011). The mechanistic basis of these models may therefore be flawed, meaning that the models are unlikely to accurately predict the decomposition of old C under different conditions.

It should be noted however, that the polymers used in this study were mainly representative of plant inputs (e.g. glycogen, xylan, cyclodextrin). The molecular structure of organic matter of plant origin has little in common with microbially processed organic C, which is dominated by polymerised material.

### Table 3
Average Simpson Diversity Index and catabolic evenness in long-term bare-fallow and cultivated soils.

<table>
<thead>
<tr>
<th>Biolog</th>
<th>Simpson’s diversity</th>
<th>Evenness</th>
<th>MicroResp</th>
<th>Simpson’s diversity</th>
<th>Evenness</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LTBF</td>
<td>Cultivated</td>
<td>LTBF</td>
<td>Cultivated</td>
<td>LTBF</td>
</tr>
<tr>
<td>Versailles</td>
<td>0.95&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.94&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.80&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.83&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.84&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Grignon</td>
<td>0.94&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.94&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.79&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.82&lt;sup&gt;a&lt;/sup&gt;</td>
<td>n.d.</td>
</tr>
<tr>
<td>Ultuna</td>
<td>0.94&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.95&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.84&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.80&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.87&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lanna</td>
<td>0.94&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.94&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.81&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.81&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.90&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

n.d.: not determined; different letters in exponent indicate significant differences \((P < 0.05)\) within a diversity measurement.
organic material (Grandy and Neff, 2008). It could therefore be argued that it is not surprising that the bare-fallow soils did not use these polymers as readily as the cultivated soils. If polymers that were more representative of microbially processed material had been used in the assays, then a different result might have been obtained. However, the fact that tween 40 was preferentially used by communities from cultivated soil (Table 2) tends to reinforce the idea that microbial communities from the long-term bare-fallow soils were less exposed to polymers as this molecule does not resemble plant derived polymers. Tween 40 has a lipid structure that can be mineralised more readily (i.e. a higher NOSC) than communities from cultivated treatments. Although these molecules (primarily organic acids; Table 2) are readily mineralised, they also tend to release less energy than molecules with lower NOSC (LaRowe and Van Cappellen, 2011). The microbial communities from the long-term bare-fallow soils appear to have adapted to the deprivation of fresh organic matter by using substrates that require little investment, but from which the energetic return is relatively low. This can be interpreted in three ways: (i) the microbial communities exposed to old C “ignore” this C, possibly because the energetic return cannot justify the investment required, and concentrate on using simple, reactive molecules that are present; (ii) the microbial communities were not exposed to complex molecules that require a heavy enzymatic investment to be degraded because old C is not composed of such molecular structures or (iii) although these molecular structures were present in the soil, they were not physically available to the microbial communities because of occlusion within aggregates, adsorption or spatial separation. It has recently been suggested that a finer understanding of the relationship between microbial community composition and functioning can be obtained using a microbial energetics approach (Herrmann et al., 2014). It may also be interesting to characterise the microbial community resource availability using NOSC in order to obtain a better understanding of C flows in soil.

Four bare-fallow sites were used in this study, each with different site-specific properties (e.g. soil properties, previous management history and climate). We were interested in determining whether there was a convergence of the functioning of the microbial communities in the bare-fallow sites. Do microbial catabolic profiles and enzymatic spectra evolve in the same way regardless of soil? If yes then there may be a convergence in the composition of old soil organic matter across soils, i.e. site specific properties and history have little influence on the composition of old C. Here the data were inconclusive: the enzyme profiles did appear to converge (Fig. 3), but the catabolic profiles did not (Figs. 1 and 2). This suggests that the molecular forms of the old C depend on the environment in which it has developed and provides some corroboration for the view that the persistence of C in soil is an ecosystem property (Schmidt et al., 2011).

4.2. Enzyme profiles

The enzyme activities did not provide as clear a picture of the functional differences between the microbial communities from the two treatments as did the catabolic profiles, with only three enzyme activities (glucose oxidase, β-glucosidase and N-acetylglucosaminidase) showing significant differences. The higher glucose oxidase levels suggest that peroxidase activity was greater in the cultivated soil, presumably because of the presence of lignin. The better usage of polymers by the microbial communities in the cultivated soils may be explained by a higher oxidase activity than in the long-term bare-fallow soils (Fig. 3). The higher levels of β-glucosidase probably reflect the presence of cellulose in the cultivated soils, although, surprisingly, neither cellulase nor xylanase activities were stimulated. This may possibly be because these substrates had already been depolymerised prior to sampling and during the incubation period before the assays. Despite the lack of difference in cellulase or xylanase activities between the treatments, the cultivated soils were better adapted to using xylan as a substrate in the MicroResp assay (Fig. 1). Chitin is a source of nitrogen and the microbial communities in the cultivated soils may have increased their N-acetylglucosaminidase activity to satisfy increased nitrogen requirements due to the presence of fresh plant C: the microbial communities may have sought to maintain their

Fig. 4. Abundance of bacteria (number of 16S copies), fungi (number of ITS copies) and bacterial to fungal ratio measured by qPCR in long-term bare-fallow soils (light grey) and in cultivated soils (dark grey). The number of 16S and ITS copy numbers were significantly ($P < 0.05$) lower overall in the bare-fallow treatment. However, due to the high variability, the only significant difference ($P < 0.05$) observed at an individual site was between ITS copy numbers in Versailles. The bacterial to fungal ratio showed no significant difference between management systems. The bare-fallow in Versailles was established in 1928, the Ultuna and Grignon bare-fallows in 1956 and the Lanna bare-fallow in 1996.
stoichiometric balance through increased nitrogen foraging (Sinsabaugh et al., 2009). Although glycochen (a polymer containing α-(1–4) glucosidic bonds) was more readily used by microbial communities from the cultivated soils, neither amylase nor α-glucosidase were significantly higher in the cultivated soils. α-Glucosidase has been shown to be poorly related to the concentration of substrate (starch) present (German et al., 2011) and the same may be true for amylase.

Minerals are known to stabilise enzymes in soil whilst humic acids can either inhibit or stabilise enzyme activities (Allison, 2006; Burns et al., 2013). The bare-fallow soils likely had more mineral binding sites to stabilise extracellular enzyme activities and this may explain the relatively high activity levels in these soils despite the lower biomass (Fig. 4) and lower respiration rates (data not shown). The stabilisation of enzymes on soil surfaces means that enzyme production and total potential activity are not always strictly linked which may explain the discrepancies between the enzyme profiles and the MicroResp and Biolog profiles.

Enzymes are not produced equally by all microorganisms. For example, xylanase is mainly produced by fungi (Kandeler et al., 1999). In order to determine whether the effects of the bare-fallow treatment could be explained by differences in the abundance of bacteria and fungi, we determined the bacterial to fungal ratios of the long-term bare-fallow and cultivated soils. Although there was a decrease in the abundance of both bacteria and fungi, corroborating the results obtained by others (Paterson et al., 2011), no difference in the bacterial to fungal ratio was observed (Fig. 4). Therefore, changes in the relative amounts of bacteria or fungi can explain neither the differences in enzyme profiles nor differences in MicroResp profiles, through different C-use efficiencies (Sakamoto and Oha, 1994).

4.3. Functional diversity of microbial communities

The lack of significant differences among soils or treatments in catabolic diversity of microbial communities measured by Biolog (Table 3) confirmed previously obtained results from long-term bare fallow studies (Hirsch et al., 2009; Guenet et al., 2012). This result suggests that long-term bare fallow microbial communities had not lost much of their catabolic capacity during the bare fallow period. However, the reduction in catabolic diversity in the MicroResp profiles suggested the contrary. The reduction in catabolic diversity, due to a reduction in the evenness of substrate use, suggests that the long-term bare fallow soils were exposed to and readily used a narrower range of substrates than their cultivated counterparts (Table 2). The MicroResp system is known to be a more sensitive measure of the catabolic profiles of soil microbial communities and therefore better able to differentiate between microbial communities than is the Biolog system (Campbell et al., 2003). Although a number of studies have indicated that the structure of microbial communities and their functional diversity is relatively unaffected by long-term bare-fallow periods (Hirsch et al., 2009; Paterson et al., 2011; Börjesson et al., 2012), it would appear that community metabolism is affected. Furthermore, the bare-fallow effect on microbial community functioning is increased with the length of the bare-fallow period (Fig. 2). This divergence between microbial community diversity and functioning goes somewhat contrary to the generally held view that microbial communities show functional redundancy (Nannipieri et al., 2003). Functional redundancy in microbial communities suggests that reductions in microbial diversity are not reflected by changes in functioning because different species can carry out the same functions as those carried out by species that are lost. However, in the bare-fallow soils there was a loss of functioning despite the fact microbial diversity is not believed to have been reduced by the bare-fallow period (Hirsch et al., 2009; Paterson et al., 2011; Börjesson et al., 2012).

In conclusion, the use of microbial communities as biological “in situ probes” of the microbial environment tends to disprove certain long-held beliefs on the nature of old C in soil based on the “polymer model” of humic substances and suggests that the nature of this C merits further investigation. The hypothesis made that the microbial communities from long-term bare-fallow sites would be better able to use simple substrates was accepted. The data also suggested that there was a loss of functional diversity during prolonged deprivation of fresh organic matter.

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