Changes in Functional Response of Soil Microbial Community along Chronosequence of Spontaneous Succession on Post Mining Forest Sites Evaluated by Biolog and SIR Methods

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Abstract: Soil formation in post-mining sites is crucial for restoring ecosystem function, and soil formation depend on the accumulation of soil organic matter and the development of an active microbial community. In this study, we used substrate-induced respiration (SIR) and Biolog plates to characterize microbial catabolic profiles in a chronosequence of soil samples from 15 unreclaimed post-mining sites in Sokolov, Czech Republic. The sites had been undergoing spontaneous succession for 3 to 45 years. Biolog ECO plates included 31 substrates. Of substrates used for SIR (glucose, chitin, cellulose, Tween 80, phenylethylamine, N-acetyl-D-glucosamine, L-asparagine, D-mannitol, D-galacturonic acid, α-cyclodextrin, and 4-hydroxy benzoic acid), eight were also used for the Biolog plates. Soil respiration, total bacteria number, and culturable bacteria number were also measured. The total and culturable number of bacteria increased with site age ($p < 0.01$ and $p < 0.05$, respectively). The percentage of culturable bacteria decreased with site age ($p < 0.01$). Biolog analysis indicated that average well-color development (AWCD), evenness, and richness increased with site age. SIR data indicated that only average activities tended to increase with site age ($p = 0.06$). According to redundancy analysis (RDA), the eight substrates, which were commonly used in both methods (SIR and BIOLOG) explained 74.4% of the variation of data from all Biolog substrates.

Keywords: Community-level physiological profiling; soil carbon content; spontaneous succession; tertiary clay material

1. Introduction

During open-cast coal mining, large amounts of spoil material are excavated and deposited in heaps. This material typically contains low amounts of recent organic matter (even if the content
of fossil geogenic carbon is high in some cases) and low biological activity [1]. Soil formation in these post-mining sites is crucial for restoring ecosystem function [2], and soil formation depends on the accumulation of soil organic matter and the development of an active microbial community [3].

A forestation of post-mining sites is a common and effective way to speed up soil formation and whole ecosystem recovery [1,2]. Soil microorganisms are involved in decomposition and transformation of large array of organic compounds [4,5] which in turn affect many key ecosystem processes such as carbon cycling, release of greenhouse gases, organic matter accumulation in soil and decontamination [6–8]. Individual soils may vary in their ability to process individual organic compounds which in turn will affect key ecological processes in particular soils. This variability is given by variation in soil properties, which modify microbial activity as well as by composition of the microbial community [4,5]. The ability of microbial community to process organic substrates can be measured by several ways, such as analysis of enzymatic activity of the soil [9], by study of the fate of labeled model substances in soil [10], or by study the rate in which microorganisms process set of organic substances added to soil commonly known as microbial catabolic profiles [11]. Despite molecular methods based on soil DNA analysis are useful for understanding gene profile, it is still necessary to measure the real microbial catabolic activity in soil processes and ecosystem functions recovery.

A microbial catabolic profile can be measured by several methods. Among these methods, Biolog plates and substrate-induced respiration (SIR) are widely used [12,13]. The Biolog method uses multowell culture plates, each well of which contains a specific substrate and dye. In the SIR method, substrates are added as a solution to the soil, and the respiration response is measured [14]. In the Biolog method, only culturable microbe in substrate responds to the substrate, on the other hand in SIR whole microbial community responds to the substrate. Although these two methods detect different microbial community responses to substrate, variation of the microbial catabolic profile would be detected along chronosequence in two methods.

A microbial catabolic profile would change in the soil process. Soil carbon content and microbial biomass increased and microbial metabolic coefficient (specific respiration) decreased with succession in post-mining ecosystems [1,3,15,16]. It is generally expected that the microbial community shifts from bacterial to fungal dominance during succession [17,18], although this increase may not be gradual and may display some oscillation [9]. The microbial community seems to be more adapted to the utilization of more complex C sources during succession [9,11].

The aim of this study was to explore the development of the functional response of the soil microbial community during primary succession. We tested the hypothesis that microbial activity will increase and the catabolic profile in Biolog and SIR will become more diverse in chronosequence. We also expect the community will become more adapted to utilize more complex resources than the easily decomposing substrate and the proportion of cultivable bacteria in the community will decrease.

2. Methods

2.1. Study Sites

The study used a chronosequence of 15 post-mining sites that were 3 to 45 years old and that were covered with a spontaneously developing forest community. The sites were located in the Sokolov coal mining district (Czech Republic) (50.243069N, 12.6778072E). The spoil dumps were formed by tertiary clay material. The material has a pH of about 8, and the prevailing minerals are kaolinite, illite, calcium carbonate, and quartz. The surface of the plots is characterized by longitudinal depressions and elevations created by the heaping process. We sampled only soil from the depressions, where soil development is faster [3]. Herbs and grasses (Tussilago farfara and Calamagrostis epigeios) formed sparse vegetation on the 3- to 14-year-old sites. Shrubs (Salix caprea), herbs, and grasses covered the 15- to 25-year-old sites. Shrubs shaded nearly the entire soil surface, resulting in a weak herb and grass cover. In sites older than 35–40 years the shrubs are replaced by poplar birch forest (Populus tremuloides and Betula spp.), herb and grass cover become dense. The development of vegetation in
these sites is described in detail in Frouz et al. [19]. As a response to vegetation development the litter starts to accumulate in pioneer sites which resulted in formation of thick Oe fermentation horizon in shrub-dominated sites, later on the 25–45-year-old sites, when the sites get colonized by earthworms, rapid development of A layer follows [3,20,21].

2.2. Sampling

Soils were sampled from the top 0 to 5 cm of soil. The soil samples were passed through a 2-mm sieve and stored at 4 °C. Activity of the microbial community can be measured either as in situ characteristics, which are closely depending on actual configuration of various field conditions at a given moment such as moisture or nutrient availability, or as inherent soil characteristics, i.e., those that are characteristic for a given soil and can be easily measured in samples brought into the lab [22]. In our study, we focused on the second option. That is why we standardized moisture, temperature and also try to minimize variation in organic matter availability. Since sampling and sieving disturb the soil and may release additional carbon sources that would affect the catabolic profiles, we minimized these effects by storing the moist soil samples at 4 °C for 4 months before the samples were analyzed, this ensured that liable carbon released by the disturbance was consumed before the analysis [23,24]. Between the fifth and sixth months of this storage, all soil samples were analyzed.

2.3. SIR Method

SIR was measured after the separate addition of 11 organic substrates to the soil samples as described by Degens and Harris [25]. The substrates were glucose, chitin, cellulose, Tween 80, phenylethylamine, N-acetyl-D-glucosamine, L-asparagine, D-mannitol, D-galacturonic acid, α-cyclodextrin, and 4-hydroxy benzoic acid. Substrate concentrations were 15 mM for amino acid and amines, 60 mM for alcohols, 15 mM for aromatic chemicals, 75 mM for carbohydrate compounds, 190 mM for carboxylic acids, and 30 mM for the polymers [25]. Moist soil equivalent to 2 g of dry weight was placed in a 250-mL Erlenmeyer flask, which was sealed with Parafilm and incubated for 24 h at 25 °C. A substrate solution was then added to the flask at a 1:2 ratio of soil to solution. At the same time, a similar flask was prepared but was treated with deionized water rather than with a substrate solution. After addition of solutions or water, flasks were sealed and kept at 25 °C for 4 h before CO₂ concentration in the headspaces was measured with an open flow system using IRGA, S151 CO₂ analyzer (Qubit Systems, Ontario, Canada). SIR was calculated by subtracting the quantity of CO₂ evolved without substrate from the quantity evolved with substrate. Soil respiration was expressed as CO₂ µL h⁻¹ g⁻¹ dry soil. Each combination of soil sample and substrate was represented by three replicate flasks.

2.4. Biolog Method

Biolog microplate™ is used to measure microbial functional diversity as indicated by the microbial Utilization of available C [26,27]. This technique is based on the cultivation of soil suspension with given substrate and tetrazolium dye which is reduced by microbial respiration and serves as an indicator of microbial activity when the given sole-carbon-source has been utilized. Eco Microplates (Biolog Inc., Hayward, California, USA) containing 31 carbon substrates were used for the determination of potential metabolic microbial diversity [26]. Moist soil (2 g dry weight equivalent) was placed in an Erlenmeyer flask, and 20 ml of a sterile 0.15 M NaCl solution was added. After the suspension was shaken for 30 m, the supernatant was diluted 1:500 with the 0.15 M NaCl solution. This was done separately for each replicate soil sample. Each well of the Eco Microplate was inoculated with 145 µL of the soil suspension. The Eco Microplates were incubated at 22°C for 168 h. The absorbance of the sample in each well was measured with a microplate reader (Versamax, Molecular Devices, USA) at 0 h (when the suspension was added to the well), 12 h, and every 6 h thereafter. A wavelength of 540 nm was used because absorbance at this wavelength was slightly higher than absorbance with the commonly used 590 nm. The values of blanks (inoculated but without carbon source) were subtracted from the values obtained with samples. The mean absorbance per well at the end of the incubation of each
sample was calculated and used for analysis. Average well-color development (AWCD) was calculated as follows equation on the last day of the measurement: \[ AWCD = \frac{\text{sum}(C - R)}{31} \], where C is the optical density within each well and R is the absorbance value of the control well.

2.5. Enumeration of Bacteria

At the same time as when the soil was processed for Biolog and SIR determination, about 0.5 g of soil was weighed and serially diluted. The dilutions were added to a sterile agar medium (R2A) in Petri dishes. The Petri dishes were kept at 20 °C in the dark, and the bacterial colonies were counted after 8 days. The number of colony-forming units (CFU) was calculated based on the dilution. The same serial dilutions were used to obtain direct counts of bacteria, but the dilutions were stained with DAPI (4′, 6-diamidino-2-phenylindole) and examined with a microscope [28].

Soil respiration was measured using the same method as described above for SIR, using the open flow system using IRGA, S151. In soil respiration, 5 g of dry soil was used and water content of each soil was adjusted to 60% of water holding capacity.

2.6. Statistical Analysis

Catabolic diversity, richness, and evenness, were calculated according to Degens et al. [12]. Richness was expressed as the number of substrates used by the microorganisms in the soil sample, and evenness was the variability of microbial response across the range of substrates tested. To find an equation describing the main trend of the data over time, we tested linear and exponential functions. Only functions with significant \((p < 0.05)\) values of \(R^2\) were considered, and the best-fitting functions were plotted. The correlation coefficient between SIR and BIOLOG data was plotted on the percentage of culturable bacteria and the plot was subjected to linear fitting. Curve fitting was performed with IBM SPSS Statistics version 22.

To determine how the SIR and Biolog rank all the 15 plots using their whole catabolic profile, we used method unconstrained by our selection of environmental parameters, as some parameters we have not considered may be crucial drivers of catabolic profile [29]. Since of gradient length, we chose the linear unconstrained method principal component analysis (PCA) computed by CANOCO 4.5 software (Microcomputer Power, Ithaca, USA). Within CANOCO, the options “standardization of sites” and “centering by substrate” were used for both SIR and Biolog data. Environmental factors, site age, soil pH, electrical conductivity (EC), oxidizable carbon, and litter quantity were included in this analysis. These data, which are summarized in Table 1, were obtained from a study by Frouz et al. [19], which was conducted at the same sites and at the same time as the current study.

<table>
<thead>
<tr>
<th>Environmental Parameters</th>
<th>Min</th>
<th>Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site age (year)</td>
<td>3</td>
<td>45</td>
</tr>
<tr>
<td>pH (H₂O)</td>
<td>5.95</td>
<td>8.75</td>
</tr>
<tr>
<td>C ox (%)</td>
<td>3.38</td>
<td>15.23</td>
</tr>
<tr>
<td>EC (µS cm⁻¹)</td>
<td>257</td>
<td>1017</td>
</tr>
<tr>
<td>litter mass (g m⁻²)</td>
<td>0</td>
<td>1080</td>
</tr>
<tr>
<td>root mass (g m⁻²)</td>
<td>0</td>
<td>1187</td>
</tr>
</tbody>
</table>

To determine how the microbial community responded to eight substrates, which were commonly used in both methods, differ between SIR and Biolog, we used constrained analysis, based on length of the gradient we choose linear method [29] redundancy analysis (RDA), using the standardization by sites and centering of substrate options. Microbial responses to the eight substrates that were determined by both Biolog and SIR were compared by RDA, using the centering by substrate, and centering and standardization of site options.
3. Results

The total number of bacteria (as determined by direct counting) and the number of culturable bacteria increased with site age (Figure 1a,b). The percentage of culturable bacteria relative to the total number of bacteria decreased with site age (Figure 1c). Soil respiration (measured at 60% of water holding capacity) did not show any relationship against site age (Figure 1d).

According to SIR data, average activity was weakly related to site age ($r = 0.49$, $P = 0.06$) and evenness was unrelated to site age (Figure 2). Since the soil microorganisms at all sites responded to all substrates in the SIR assay, richness based on the SIR data was the same for sites of all ages. According to the Biolog data, AWCD, evenness, and richness increased with site age (Figure 2). Since the soil microorganisms at all sites responded to all substrates in the Biolog assay, richness based on the Biolog data was the same for sites of all ages.

Figure 1. Relationship between site age and the total number of bacteria as determined by direct counts (a), number of culturable bacteria (b), percentage of culturable relative to the total number of bacteria (c), and soil respiration (d).

According to the Biolog data, AWCD, evenness, and richness increased with site age (Figure 2). According to SIR data, average activity was weakly related to site age ($p = 0.06$) and evenness was unrelated to site age (Figure 2). Since the soil microorganisms at all sites responded to all substrates in the SIR assay, richness based on the SIR data was the same for sites of all ages.
was detected for N-acetyl-D-glucosamine but not for the other seven substrates (Figure 3, Table 2).

In the RDA, the microbial response of eight substrates chosen from Biolog substrates explained 74.4 % variation of all substrates used in Biolog data. When we compared the SIR and Biolog data for the eight substrates that were common to both methods, a positive correlation between SIR and Biolog methods was negatively correlated with total number of bacteria (r = -0.60).

Figure 2. Relationship between site age and average microbial catabolic activity (a,b) and catabolic evenness (c,d) as determined by Biolog and substrate-induced respiration (SIR) methods, and between site age and catabolic richness as determined by Biolog (e). Richness is not presented for the SIR data because the soil microbial communities responded to every substrate at every site. Average microbial catabolic activity in Biolog indicates average well-color development (AWCD).

Figure 3. Redundancy analysis (RDA) diagram comparing SIR (black bold arrow) and Biolog (grey, thin arrow) data with the options "centering and standardization of sites" and "centering by a substrate" along a successional gradient of unclaimed post-mining soils (circles and numbers indicate site age).
The first two ordination axis of PCA for both SIR and BIOLOG data explain more than 50% of data variability (Figure 4). In the PCA of the Biolog data, a substantial proportion of the variation was explained by EC, on the 1st axis and by soil carbon content, pH, and litter quantity on the 2nd axis (Figure 4, Table 3). In the PCA of the SIR data, a substantial proportion of the variation on the 1st axis was explained by pH, soil carbon content, and litter quantity (Figure 4, Table 3), the eigenvalue of the 1st axis was 0.628.

### Table 2. Interrelation between SIR and BIOLOG data on 8 substrates. ** Correlation significant for \( p < 0.01 \).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>SIR</th>
<th>BIOLOG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tween 80</td>
<td>r</td>
<td></td>
</tr>
<tr>
<td>4-Hydroxy Benzoic Acid</td>
<td>0.48</td>
<td></td>
</tr>
<tr>
<td>D-Mannitol</td>
<td>-0.15</td>
<td></td>
</tr>
<tr>
<td>D-Galacturonic Acid</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>L-Asparagine</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>α-Cyclodextrin</td>
<td>0.47</td>
<td></td>
</tr>
<tr>
<td>N-Acetyl-D-Glucosamine</td>
<td>0.72**</td>
<td></td>
</tr>
<tr>
<td>Phenylethylamine</td>
<td>-0.13</td>
<td></td>
</tr>
</tbody>
</table>

** \( p < 0.01 \).

**Figure 4.** Principal component analysis (PCA) ordination diagram of individual substrates in Biolog and SIR with the options “standardization of sites” and “centering by substrate”. Circles represent individual sites. Narrow arrows represent substrates, bold arrows represent environmental variables. age: site age, C ox: oxidizable carbon, litter: litter mass, root: root mass, methyl glucoside: \( \beta \)-methyl-D-glucoside, galactonic ac lactone: D-galactonic acid g-lactone, arginine: L-arginine, pyruvic ac methyl ester: pyruvic acid methyl ester, xylose: D-xylose, galacturonic ac: D-galacturonic acid, asparagine: L-asparagine, tw40: tween 40, erythritol: i-erythritol, 2-hydroxy benzoic ac: 2-hydroxy benzoic acid, phenylalanine: L-phenylalanine, tw80: tween 80, mannitol: D-mannitol, hydroxy benzoic ac: 4-hydroxy benzoic acid, serine: L-serine, cyclodextrin: \( \alpha \)-cyclodextrin, acetyl glucosamine: N-acetyl-D-glucosamine, hydroxybutyric ac: \( \gamma \)-hydroxybutyric acid, threonine: L-threonine, glucosaminic ac: D-glucosaminic acid, itaconic ac: itaconic acid, glycy glutamic ac: glycy-L-glutamic acid, cellobiose: D-cellobiose, glucose phosphate: glucose-1-phosphate, keto butyric ac: \( \alpha \)-keto butyric acid, lactose: \( \alpha \)-D-lactose, glycerol phosphate: D,L-\( \alpha \)-glycerol phosphate, malic ac: D-malic acid.
Table 3. Correlation between each parameter and 1st and 2nd axis of PCA analysis results in each method. Bold and black characters are statistically significant. age: site age, C ox: oxidizable carbon, litter: litter mass, root: root mass.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>BIOLOG Environmental Properties</th>
<th>SIR 1st Axis</th>
<th>2nd Axis</th>
<th>1st Axis</th>
<th>2nd Axis</th>
</tr>
</thead>
<tbody>
<tr>
<td>age</td>
<td>age</td>
<td>-0.69</td>
<td>-0.04</td>
<td>-0.20</td>
<td>-0.39</td>
</tr>
<tr>
<td>pH (H₂O)</td>
<td>pH (H₂O)</td>
<td>0.73</td>
<td>0.01</td>
<td>0.49</td>
<td>0.62</td>
</tr>
<tr>
<td>C ox</td>
<td>C ox</td>
<td>-0.74</td>
<td>-0.08</td>
<td>-0.33</td>
<td>-0.63</td>
</tr>
<tr>
<td>EC</td>
<td>EC</td>
<td>0.14</td>
<td>-0.06</td>
<td>0.52</td>
<td>0.27</td>
</tr>
<tr>
<td>litter</td>
<td>litter</td>
<td>-0.65</td>
<td>0.30</td>
<td>-0.19</td>
<td>-0.56</td>
</tr>
<tr>
<td>root</td>
<td>root</td>
<td>-0.60</td>
<td>0.16</td>
<td>-0.25</td>
<td>-0.64</td>
</tr>
</tbody>
</table>

4. Discussion

4.1. Soil Biological Properties

The results of the total number of bacteria, the number of culturable bacteria and the percentage of culturable bacteria relative to the total number of bacteria along the forest chronosequence are consistent with those of Frouz et al. [1]. Frouz et al. [1] reported that the total number of bacteria increased with time in the same sites as those sampled in the current study. Generally, soil carbon content has increased with site age in the area of the current study [30]. A large number of total bacteria in the older sites of the current study resulted in a decrease in percentage of culturable bacteria. In other words, the increase with site age was greater for the total number of bacteria than for the number of culturable bacteria. Krištůfek et al. [31] found same trend in the same post-mining site. Similar results have been found in glacial forefield soils [32]. The culturable bacterial strategy would be an opportunistic growth strategy. Actually Krištůfek et al. [31] found bacterial community structure shifted from r strategy to K strategy species along the chronosequence. Soil respiration in the current study was unrelated to site age. Working in the same post-mining area, Helingerova et al. [30] also reported the absence of a relationship between soil respiration and succession. Respiration did not show any relationship against site age (Figure 1d). This may be supported by fact that based on earlier data of Helingerova from the same forest did not change much along chronosequence only the oldest site differ from the younger ones, despite continual increase in total C. Helingerova et al. [30] inferred that carbon availability and consequent microbial activity in pioneer, reclaimed sites may be supported by certain sources of energy which are plentiful in initial sites but decreased in older sites namely herb and grass litter and root exudates, and fossil C. Several studies have documented the microbial use of fossil organic matter (e.g., Kříbek et al. [33]). In addition, the use of stable C from fossil remains can be enhanced by the priming effect of root exudates [34]. Understory vegetation in early and intermediate may be also denser than in older sites with closed canopy. In later successional stage, this C source gets replaced by C from decomposing tree litter. Replacement of various C sources over succession cause that amount of available C is quite stable during succession.

4.2. Changes in the Catabolic Activity during Spontaneous Succession

AWCD, evenness, and richness increased with site age in Biolog analysis, but in SIR, only average activity was weakly related to site age (p = 0.06). Only N-acetyl-D-glucosamine showed positive co-relationship between SIR and Biolog from eight substrates that explained 74.4 % variation of all substrates used in Biolog data. This result indicates that the two assays may be measuring the catabolic activities of different components of the microbial community. There was negative relationship (r = 0.65 in exponential function) between the percentage of culturable bacteria relative to the total number of bacteria. Also, correlation coefficients between SIR and Biolog methods were negatively correlated with total number of bacteria (r = -0.60). Since the response of culturable bacterial community in substrate
solution is detected in Biolog, and in SIR response of whole community are detected, we expect results of two methods will agree more in samples with high percentage of culturable bacteria. However the correlation coefficient was high in samples with low percentage of cultivable bacteria and was low in high percentage of cultivable bacteria. Krištufek et al. [31] investigate growth strategy of bacterial population along successional sequence on same post-mining site and showed K-strategy bacterial species dominant in late succession. As percentage of cultivable bacterial decreased in later succession site (Figure 1c), different species would respond to substrate in two methods. On the other hand, some culturable bacterial species may become dominant in late succession sites, and these species would decompose each substrate in Biolog and SIR measurement.

4.3. Driving Factors of Microbial Catabolic Profile along Chronosequence

The first two ordination axis explain more than 50% of data variability of PCA for both SIR and BIOLOG data (Figure 4) which can be assumed as good results [29]. In the Biolog result, a substantial proportion of the variation was explained by EC on the 1st axis and by soil carbon content, pH, and litter quantity on the 2nd axis (Figure 4, Table 3), suggesting that these parameters would affect microbial catabolism. In SIR result, a substantial proportion of the variation on the 1st axis was explained by pH, soil carbon content, and litter quantity (Figure 4, Table 3), suggesting that these parameters would affect microbial catabolism. In other studies, higher soil carbon content leads to greater catabolic diversity, evenness, and average activities [11,13,35]. Increasing soil carbon content generally enhances microbial catabolism. In a previous study of post-mining sites, moreover, catabolic diversity increased late in succession [35]. The PCA and other analyses in the current study also indicated that catabolic diversity, evenness, and AWCD increased with succession and that these increases may have involved soil carbon content.

Previous research on succession at the same post-mining sites found that fungal biomass and enzymatic activity increased from early to middle successional stages, that bacterial numbers increased with succession, and that fungal biomass and some enzymatic activities decreased in late stages [9,19]. In these studies, food web structure changed during succession. In the early stages of succession, the bacterial channel dominated the food web. Later, in shrub-dominated stands, the fungal channel dominated. Even later, in the forest stage, the bacterial channel prevailed again [36]. Later succession on these post-mining heaps would be preferable condition to bacterial community due to increasing carbon stock and processing soil formation. Microbial catabolic profiles were different between Biolog and SIR methods. However, each profile was explained by pH, soil carbon content, and litter quantity. Soil biological and physicochemical changes in chronosequence of post-mining site would be driving factors of microbial catabolic profile change.

5. Conclusions

The Biolog data obtained in this study showed that the diversity, evenness and richness, and AWCD of the microbial catabolic profiles in Sokolov post-mining soil increased with the successional stage and forest development. Although Biolog and SIR results differed for seven of the eight substrates that were common to both determinations, ordination analyses of the microbial catabolic profiles were similar. For both methods, PCAs indicated that much of the variation in the microbial catabolic profiles was explained by soil carbon content, pH, and litter quantity.


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Conflicts of Interest: The authors declare no conflict of interest.
References


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