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Ecoenzymatic stoichiometry can reflect microbial resource limitation, substrate quality, or both in forest soils

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**ABSTRACT**

Many studies have used the relative activities of extracellular enzymes associated with microbial carbon (C), nitrogen (N) and phosphorus (P) acquisition to infer the relative C vs. nutrient limitation of the microbial community. However, recent experimental and theoretical evidence has shown that the use of ecoenzymatic ratio to infer limiting microbial resources may be invalid. We added the two contrasting leaf litters ash (Fraxinus excelsior L., relatively more labile and nutrient rich) and oak (Quercus robur L.), into samples of mineral soils to validate the use of ecoenzymatic stoichiometry to reflect microbial resource limitation. The litter treatments were also combined with N and P addition treatments to push microbial communities toward stronger C limitation. The microbial resource limitations were examined in all treatments by the responses in microbial respiration, bacterial and fungal growth, microbial community composition, and by detecting the responses of microbial growth to factorial C and nutrient additions in short-term limiting factor assays (LFAs). High ratios of β-1,4-glucosidase (BG) to β-1,4-N-acetylglucosaminidase (NAG) + leucine aminopeptidase (LAP) (>2:1) contrasted with the expected reduction in C limitation after initial litter inputs. The high ratio was mainly driven by an increase in BG activity associated with high energy supply and high microbial rates of metabolism induced by added labile C substrates. During the later incubation period (7–56 days), decreasing respiration and bacterial and fungal growth rates reflected increased microbial C limitation in all treatments. An increasing BG/(NAG + LAP) ratio was in line with increasing microbial C limitation in the ash treatment where cellulose dominated as microbial C source, but a decreasing BG/(NAG + LAP) ratio was inconsistent with the temporal dynamics in microbial C limitation in the oak treatment where substrates other than cellulose (i.e., microbial necromass and lignin) dominated as C source late in the incubation. The increased BG/(NAG + LAP) ratio was in line with intensified microbial C limitation by NP addition, which was mainly due to the stimulated BG activity. In the oak and litter plus NP treatments, fungal growth was stimulated and NAG - an enzyme targeting amino sugars - was increased, presumably to meet the fungal C demand by utilizing microbial necromass - partly consisting of amino sugar polymers. Taken together, these results suggest that variation in substrate quality, availability and how these matched the present microbial energy allocation strategy and resource allocation to enzyme production caused a substantial variation in the BG/(NAG + LAP) ratio, thereby disconnecting the ratio from unambiguously reflecting the microbial resource limitation.

1. Introduction

Microbial resource limitation is an important regulator of ecosystem-level carbon (C) and nutrient cycling (Soong et al., 2019; Tanner et al., 1998). Most studies suggest that soil organic C is the primary growth-limiting resources for soil microbial communities (Demoling et al., 2007; Fanin et al., 2015; Soong et al., 2019), but nitrogen (N) and phosphorus (P) have also occasionally been reported to be limiting resources for microbial growth (Aldén et al., 2001; Cleveland et al., 2002; Schmidt et al., 2000). Several approaches have been developed to investigate nutrient limitation of soil microbes, such as measuring microbial biomass C, N or P contents, microbial respiration, and bacterial and fungal growth responses induced by factorial C and nutrient additions in brief limiting factor assays (LFAs) (Aldén et al., 2001; Rousk and Bååth, 2011). More recently, ecoenzymatic stoichiometry has been proposed to determine nutrient status and limitation of microbes

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The ecoenzymatic stoichiometry approaches are based on the logical expectation that high enzyme activities targeting a particular resource indicate a shortage of that resource (Sinsabaugh et al., 2014; Sinsabaugh and Follstad Shah, 2012). The ecoenzymatic stoichiometry approach, high BG/(LAP + NAG) and BG/AP ratios indicate relatively higher microbial C limitation vs. N limitation and microbial C limitation vs. P limitation, respectively (Sinsabaugh et al., 2008; Sinsabaugh, 1994). Therefore, in the ecoenzymatic stoichiometry approach, high BG/(LAP + NAG) and BG/AP ratios indicate relatively higher microbial C limitation vs. N limitation and microbial C limitation vs. P limitation, respectively (Sinsabaugh et al., 2009). The intuitively appealing rationale of this approach along with high throughput methods to establish activity estimates for the 4 enzymes have led to a broad acceptance and wide application in sediments and soil across many ecosystems (Bai et al., 2021; Chen and Sinsabaugh, 2020; Forstner et al., 2019; Hill et al., 2014; Moorhead et al., 2016; Sinsabaugh et al., 2008). Observations from these studies have revealed differences between ecosystems that largely align with expectations for the variation of limiting resources for plant productivity (Feng et al., 2019; Sinsabaugh et al., 2008). However, only a handful of experimental validations of the ability of ecoenzymatic ratios to indicate limiting resources for microbial growth have been reported to date (Rosinger et al., 2019).

The validity of the ecoenzymatic ratio approaches to inform about microbial resource limitation have recently been questioned (Mori, 2020; Mori et al., 2021; Rosinger et al., 2019). Rosinger et al. (2019) conducted a validation experiment to test the microbial resource limitation by using ecoenzymatic approaches and validating them against conventional C and nutrient addition approaches in two subtropical soils. They found that ecoenzymatic stoichiometry vs. LFAs gave contrasting results both for the untreated soils, and when the soils were experimentally pushed toward C, N, or P limitation. Mori (2020) suggested a conceptual model to distinguish the conditions when the BG/NAG ratio (or BG/(BG + NAG)) reflects microbial C vs. N limitation and when it does not. If cellulose is the predominant microbial C source relative to chitin, peptidoglycan, and protein, then BG/NAG (or BG/(BG + NAG)) would reflect the C vs. N limitation as the ecoenzymatic stoichiometry theory suggests, while if chitin, peptidoglycan, and protein are dominant C sources, C vs N limitation would not be well characterized by BG/NAG (or BG/(BG + NAG)). That is, when C is in low supply, then microbes target organic N compounds mostly to acquire C.

The objective of this study was to validate the ability of enzyme ratios to indicate limiting resources for microbial growth in a range of experimental treatments representing a wide variation in limiting resources. The microbial limitations were monitored by determining (i) responses in ecoenzymatic stoichiometry, (ii) responses in microbial growth rates, respiration, biomass and composition, and (iii) responses in limiting factor assays (LFAs) for microbial growth during the course of litter decomposition. We conducted a microcosm experiment where ash (Fraxinus excelsior L.) and oak (Quercus robur L.) litter were added to soil to represent a relatively labile and nutrient rich substrate (ash) and a relatively recalcitrant and nutrient poor (oak) substrate that naturally dominate environments after leaf litterfall in broadleaved forests. The litter additions were also combined with N and P addition treatments to push microbial communities toward stronger C limitation. We hypothesized (H1) that shortly after litter addition, the BG/(NAG + LAP) ratio would be lower for litter treatments than control soil and this would be more pronounced for ash (high in labile contents) than oak (high in recalcitrant contents). These responses were expected because high availability of labile C with litter inputs would reduce C limitation, thus strengthening nutrient limitations, inducing lower BG and higher NAG and LAP activities. We also hypothesized (H2) that the BG/(NAG + LAP) ratio in litter treatments would later increase sharply as C becomes more limiting due to fast depletion of labile C during the early incubation period (<7 days). Then, during the later incubation period (7–56 days), microbes would start to use cellulose as dominant C source by producing BG in the ash treatment since labile C would be exhausted. In the oak treatment, microbes would start to use substrates other than cellulose as C sources, and the dominance of fungi would increase, hereby inducing NAG activity. Thus according to Mori’s concept (Mori, 2020), we hypothesized (H3) that the ratio of BG/(NAG + LAP) in the ash treatment would increase, while the ratio of BG/(NAG + LAP) in the oak treatment would decrease during the later incubation period, leading to an end point where the ratio of BG/(NAG + LAP) would be higher in ash than oak during the later incubation period. Finally, we hypothesized (H4) that mineral nutrient supplements (NP) would increase the C limitation for microbial growth and thus increase the BG/(NAG + LAP) ratio especially for ash.

2. Materials and methods

2.1. Sample collection

Ash and oak leaf litters were collected with litter traps during October–November 2020 in a Danish common garden experiment, in Stokkebjerg Skov, Odsherred, Denmark (55° 50′ N, 11° 42′ E). The two tree species were selected due to their contrasting litter chemistries (Table 1). Cellulose, hemicellulose and water extractable carbohydrates are the dominant C source in ash litter, with a relatively low C/N ratio, while there is a higher fraction of lignin and protein in oak litter, with a relatively high C/N ratio. Leaf litters were ground with a PM-400 ball mill (Retsch Technology Cooperation, Germany) to facilitate easier and faster access of microbes to resources of the substrates (Rinke et al., 2013). Mineral soil for the microcosms was collected by auger to a 0–5 cm layer from the lime (Tilia cordata) stand in same experimental site where the leaf litter were collected. The soil is classified as Oxyaquic Hapludalf with a pH-CaCl2 of 4.3 and a C:N ratio of 15 (Vesterdal et al., 2008). Soils were sieved (2 mm mesh) to remove coarse fractions and roots, thoroughly homogenized, and pre-incubated for two weeks in the dark at 20 °C and at 50% water-holding capacity (WHC).

2.2. Soil and leaf litter chemical properties

Soil pH and electrical conductivity (EC) were analysed in water extracts (soil: water ratio of 1:5) using a pH or electrical conductivity electrode after 1 h extraction. Soil organic carbon and total N concentrations were measured on ground samples with an elemental analyzer (Thermo Fisher Scientific, Waltham, MA, USA) after air-drying to constant mass. Total P concentration in leaf litter was measured on a quadrapole-based ICP-MS (iCAP Qc, Thermo Fisher Scientific, Bremen, Germany) after concentrated HNO3 (sub boiling quality) digestion. The soil P content was determined after 0.1 M H2SO4 extraction as ortho-P (PO4–P) concentration by the salicylate method on an auto-analyzer (AA500, SEAL, Analytical, Germany). Litter chemical composition was determined according to National Renewable Energy Laboratory (NREL) procedures (Sluder et al., 2010). Water and ethanol extraction were performed on a Soxlet apparatus. Sugar determination was performed in an Ultimate HPLC (Thermo Fisher Scientific Inc., Waltham, MA USA). Acid-unhydrolyzable residue (AUR) lignin was measured as the dry weight of the samples (after acid hydrolysis) taking the ash content into account.

2.3. Laboratory microcosms

We conducted five treatments: soil only (control), soil amended with ash or oak leaf litter (denoted as “ash” and “oak”), and soil amended with litters plus nutrients addition (denoted as “ash + NP” and “oak +...
3

NP"). Fifteen soil jars (5 treatments × 3 replicates) were incubated together. Soil and ground litter were mixed in 1000 mL wide mouth plastic jars. Jars for litter input treatments contained 500 g pre-incubated soil and 5 g ground litter, thoroughly mixed. This amount of litter was selected to simulate approximately an input of 3x the annual litter fall (ca. 3.0–3.5 ton/ha) (Vesetardal et al., 2012) which was similar to the amount used in previous studies (Fanin et al., 2016; Rinkes et al., 2013). Treatments with nutrient additions were added 3.75 mL NP solutions containing 0.9 M NH\textsubscript{4} to the amount used in previous studies (Fanin et al., 2016; Rinkes et al., 2013). Treatments with nutrient additions were added 3.75 mL NP solutions containing 0.9 M NH\textsubscript{4}NO\textsubscript{3} and 1.8 M K\textsubscript{2}HPO\textsubscript{4}/KH\textsubscript{2}PO\textsubscript{4}, corresponding to 0.38 mg N · g\textsuperscript{−1} soil and 0.93 mg P · g\textsuperscript{−1} soil (ca 40 kg N ha\textsuperscript{−1} and 115 kg P ha\textsuperscript{−1}), respectively. Soil-only control and litter addition jars were treated with an equivalent volume of demineralized water. Microcosms were incubated at 20 °C in a dark incubator. Soils from microcosms for each treatment were harvested after 1, 3, 7, 14, 28, 42, and 56 days. The harvested soils were divided into four subsamples and were stored at 4 °C until further analyses, and aliquots of 5 g soil were stored at −20 °C for phospholipid fatty acid (PLFA) measurement. Each destructive harvest included analyses of respiration, bacterial growth, fungal growth, extracellular enzyme activity. At three harvest time points (on days 7, 28, 56), soil microbial PLFA and microbial biomass C, soil organic carbon (SOC), total nitrogen (TN), total phosphorus (TP), pH and electrical conductivities were measured. The responses of bacterial and fungal growth to short-term nutrient addition to determine growth limiting resources were measured on days 42.

2.4. Enzyme extraction and assays

Enzymes that catalyse the degradation of organic carbon (β-1,4-glucosidase [BG]), nitrogen (β-1,4-N-acetyl-glucosaminidase [NAG] and L-leucine aminopeptidase [LAP]), and phosphorus (acid phosphatase [AP]) were quantified with fluorometric assays following the protocol developed by Bell et al. (2013). Briefly, 2.75 g of harvested soil sample from the microcosms was homogenized in 91 mL of 50 mM sodium acetate buffer in a blender for 1 min. 800 μL soil slurries with three technical replicates were then added to a 96-deepwell microplate using an eight-channel electronic pipette (Eppendorf Xplorer Plus, Hamburg, Germany). Additional quench control replicates of the soil slurry, 4-methylumbelliferone or 7-amino-4-methylcoumarin standard curves (200 μL of respectively 0–100 μM and 0–10 μM concentrations) were included with each sample. The microplates were incubated with fluorometric substrates for 4 h at 25 °C. After the incubation, plates were centrifuged for 3 min at 3000 rpm, after which 250 μL of supernatant was transferred from each well into a black flat-bottomed 96-well plate. The fluorescence was measured by a microplate reader (Synergy H1 microplate reader, Biotek, Winooski, USA) using an excitation wavelength at 365 nm and emission wavelength at 450 nm. From the fluorescence values and standard calibration curves, each enzyme activity was calculated and converted to nmol g\textsuperscript{−1} dry soil h\textsuperscript{−1}. Based on the ecoenzymatic stoichiometry theory, high enzyme activities targeting a particular resource in relation to that targeting another indicate the relative limitation by those two resources (Sinsabaugh et al., 2008). The enzymatic ratios of BG/(NAG + LAP), BG/AP and (NAG + LAP)/AP were subsequently calculated to infer C vs. N, C vs. P, and N vs. P limitation respectively. An enzymatic ratio greater than 1 indicate that microorganisms are more limited by the nutrient of the numerator compared to that in the denominator, and vice versa, while an enzymatic ratio of 1 indicates a balanced need (Sinsabaugh and Follstad Shah, 2012; Sinsabaugh et al., 2008, 2009).

2.5. Respiration rate

Subsamples of 1 g harvested soil were weighed into 20 mL glass vials. Vials were purged with pressurized air, then closed with a butyl rubber seal and fixed tight with a crimp cap. The soils were incubated in the dark at 20 °C for approximately 18–20 h. Headspace air was subsampled using an autosampler with a Hamilton gas-tight syringe (2500 μL syringe barrel), and measured using a gas chromatograph with a methanizer and an flame ionization detector (YLG500 GC; YL Instruments, Gyeonggi-do, Korea).

2.6. Bacterial growth rate

To determine bacterial growth rate, we used the \textsuperscript{3}H-leucine incorporation method (Bååth et al., 2001). Subsamples of 1 g harvested soil were mixed with 20 mL of MilliQ-H\textsubscript{2}O, then vortexed for 3 min and centrifuged for 10 min at 1000 × g. 1.5 mL of the supernatant were taken from a predefined depth of the tube and transferred to an Eppendorf tube. 10 μL of \textsuperscript{3}H-labelled (1-[\textsuperscript{4}S,5-\textsuperscript{3}H]-leucine, 5.7 TBq mmol\textsuperscript{−1}, PerkinElmer, USA) and non-labelled Leucine, respectively, were added yielding a final concentration of 280 nM, then mixed using a vortex and incubated for 60 min in the dark at 20 °C. 75 μL 100% TCA solution was then added to terminate incorporation of leucine. Centrifugation and washing was performed as described by Bååth et al. (2001). A scintillation cocktail (Ultima Gold; PerkinElmer, USA) was added to the bacterial pellet and the radioactivity was measured using a liquid scintillation counter. The amount of leucine incorporated into extracted bacteria (pmol Leu incorporated. g soil\textsuperscript{−1} h\textsuperscript{−1}) was used as a measure of bacterial growth.

2.7. Fungal growth rate

Fungal growth rate was estimated using \textsuperscript{14}C labelled acetate incorporation in ergosterol (Bååth, 2001). Subsamples of 0.5 g harvested soil were weighed into 10 mL glass tubes. 20 μL of a \textsuperscript{14}C-acetate solution (1-[\textsuperscript{14}C]acetic acid, sodium salt, 2.04 GBq mmol\textsuperscript{−1}, 7.4 MBq ml\textsuperscript{−1}, PerkinElmer, USA) together with 30 μL of a 16 mM acetate solution were added to the tube, yielding a final acetate concentration of 0.2 mM, was mixed thoroughly and incubated in the dark at 20 °C for 4 h. To terminate fungal growth, 500 μL of a 10% formalin solution were added. Ergosterol was extracted, separated and quantified to determine radioactive content as previously described by Rouxk and Bååth (2007). The ergosterol fraction of each sample was then separated on the HPLC, mixed with 3 mL of scintillation cocktail and \textsuperscript{14}C derived radioactivity was measured using liquid scintillation. The amount of acetate incorporated into ergosterol (pmol Ac incorporated. g soil\textsuperscript{−1} h\textsuperscript{−1}) was used as a measure of fungal growth. Ergosterol concentration was estimated from the UV absorbance at 282 nm compared with an external standard.

2.8. Determination of limiting resources for bacterial, fungal, and total microbial growth in limiting factor assays (LFAs)

To determine limiting resources for growth, the short-term responses

| Table 1 | Average foliar litter chemistry in the ash (Fraxinus excelsior L.) and oak (Quercus robur L.) leaf litter (n = 6). |
|---------|---------------------------------------------------------------------------------------------------|
| Extractives | Cellulose (%) | Hemicellulose (%) | Lignin (%) | TOC (g kg\textsuperscript{−1}) | TN (g kg\textsuperscript{−1}) | TP (g kg\textsuperscript{−1}) | C/N | DOC (g kg\textsuperscript{−1}) |
| Oak | 19.7 | 12.2 | 9.5 | 30.3 | 472 | 17.6 | 1.60 | 26.9 | 1.43 |
| Ash | 30.8 | 10.5 | 8.5 | 18.7 | 472 | 17.6 | 1.60 | 26.9 | 1.43 |

Extractives include water and ethanol soluble material. TOC, total organic carbon; TN, total nitrogen; TP, total phosphorus; C/N, carbon to nitrogen ratio; DOC, dissolved organic carbon.
to nutrient amendments were performed in a full-factorial design as previously established (Demoling et al., 2007; Göransson et al., 2011) with modifications. Subsampled soils were subdivided as 1.5 g aliquots into 50 ml test tubes. 60 μl of C (as D-Glucose; 0.5 mg C·g soil−1), N (as NH4NO3 0.029 mg N·g soil−1) and P (as KH2PO4/K2HPO4 in a 1:1 mol ratio, 0.065 mg P·g soil−1) solutions were added in a in a full factorial design (denoted C, N, P, CP, NP and CNP, and where H2O indicates the no nutrient addition control with addition of water). Soils were incubated for 24 h at 20 °C before determination of bacterial growth and fungal growth and the sum of these growth rates (total microbial growth) as described above. An increase in growth in soils to which nutrients were added, compared with the no addition control soils, indicates that growth was limited by the nutrient added. Relative responses of bacterial, fungal and total microbial growth rates to short-term nutrient additions were calculated as response of the treatment (nutrient addition) minus the control (H2O addition) and standardized by control ((Growth treatment − Growth control)/Growth control).

2.9. Microbial community composition

Microbial biomass and community composition were analysed by determination of phospholipid fatty acids (PLFAs). PLFAs were extracted from freeze dried subsamples using the method described by Frostegård et al. (1993) with modifications (Cruz-Paredes et al., 2017). The PLFA 18:2ω6:9 was used as an indicator of fungal biomass and PLFAs for estimation of bacterial biomass were i14:0, i15:0, a15:0, i16:0, 16:1ω7c, 16:1ω7c, 17:0, 17:1ω8c, cy17:0, 10Me17:0, 18:1ω7, 10Me18:0 and cy19:0. The fungal to bacterial ratio was achieved from the 18:2ω6:9 to bacterial PLFA ratio (Frostegård et al., 2011). The ratio between Gram-positive (GP) and Gram-negative (GN) bacteria (the GP/GN ratio) was based on the sum of the PLFAs i14:0, i15:0, a15:0, i16:0, i17:0, a17:0, as indicators of Gram-positive bacteria, and the sum of the PLFAs 16:1ω9c, 16:1ω7c, cy17:0, 18:1ω7 and cy19:0 for Gram-negative bacteria. The sum of bacterial PLFAs, fungal PLFAs, and unclassified PLFAs was used as a measure of total microbial biomass.

2.10. Data analyses

All the analyses were performed in R (version 3.6.0; R Development Core Team, 2018). Based on the deviation from a balanced enzyme ratio of 1:1 (Sinsabaugh et al., 2008, 2009), ecoenzymatic stoichiometry of BG/(LAP + NAG) were plotted against (LAP + NAG)/AP to show microbial resources limitation (Hill et al., 2012; Schmidt et al., 2016). Repeated measures ANOVA was performed to determine differences in respiration, bacterial and fungal growth, enzymatic ratios, microbial PLFAs between treatments over different harvesting points in time. Individual differences in responses of bacterial and fungal growth to short-term nutrient addition were tested in each treatment and time point by Tukey post-hoc tests when the ANOVA model was significant. To study differences between microbial communities in different treatments over incubation time, the molar percentage of individual phospholipid fatty acids (PLFAs) was subjected to principal component analyses (PCA). The data were standardized to unit variance before PCA.

3. Results

3.1. Soil chemical properties

Litter treatments (ash and oak) and litter plus NP treatments (ash + NP and oak + NP) increased soil organic carbon (SOC) concentrations compared to control during 56 days incubation (Table 2). Litter plus NP treatments had higher phosphorus (P) concentrations (p < 0.05), but lower C/N ratio compared to corresponding litter treatments (p < 0.05). Litter treatments significantly increased soil pH and decreased soil electrical conductivity (EC) (p < 0.05) compared to control. Compared to litter treatments, litter plus NP treatments significantly decreased soil pH but increased soil EC (p < 0.05). The ash treatment resulted in higher pH, but lower C/N ratio and EC than the oak treatment after 7 days of incubation (p < 0.05). SOC and soil pH showed no significant differences between ash + NP and oak + NP during 56 days of incubation.

3.2. Microbial respiration and growth rate

Rates of respiration and bacterial and fungal growth in litter and litter plus NP treatments were all higher than control during 56 days incubation (Fig. 1). For respiration rates, maximum rates for all four treatments occurred after approximately 1 day of incubation (Fig. 1A). Fungal growth rate peaked on day 3, except for the ash + NP treatment that had highest rates and already peaked on day 1 of incubation (Fig. 1C). Bacterial growth rate peaked on day 7, except for the ash treatment that peaked on day 3 (Fig. 1B).

Respiration rates in ash and ash + NP treatments were two to three fold higher than in oak and oak + NP treatments between 1 and 3 days of incubation, and NP addition reduced respiration in both ash and oak treatments from 14 to 56 days of incubation (Fig. 1A). Bacterial growth rates in ash and ash + NP were higher than those in oak and oak + NP from 7 to 56 days of incubation. Both ash and oak litter had reduced bacterial growth rates between 1 and 3 days of incubation when supplemented with NP, while NP addition increased bacterial growth rate for ash between 7 and 28 days (Fig. 1B). Fungal growth rates and the

| Table 2 Soil chemical properties after 7, 28, 56 days incubation for soils in five treatments. | Incubation Time (d) | Treatments | SOC g kg−1 | TN g kg−1 | P mg kg−1 | C/N | pH | EC μS cm−1 |
|---|---|---|---|---|---|---|---|---|
| 7 | Control | 63 ± 2.4 | 4.2 ± 0.1 | 35 ± 0.7b | 15.1 ± 0.1b | 4.4±0.1d | 137±1b |
| | Ash | 66 ± 3.8 | 4.4 ± 0.2 | 36 ± 2.6b | 15.2 ± 0.1b | 5.2±0.1a | 82±2d |
| | Oak | 75 ± 4.2 | 4.8 ± 0.3 | 40 ± 1.2b | 15.9 ± 0.1a | 4.7±0.1b | 101±1c |
| | Ash + NP | 66 ± 3.6 | 4.5 ± 0.2 | 519 ± 6.2a | 14.5 ± 0.2b | 4.6±0.1bc | 335±6a |
| | Oak + NP | 76 ± 1.5 | 5.1 ± 0.1 | 513 ± 7.8a | 14.8±0.01b | 4.6±0.1c | 336±2a |
| 28 | Control | 58 ± 0.7b | 4.0 ± 0.1 | 47 ± 1.5b | 14.8±0.01b | 4.3±0.1c | 178±1c |
| | Ash | 61 ± 1.4 ab | 4.1 ± 0.1 | 41 ± 0.8bc | 15.0±0.01b | 5.0±0.1a | 80±1d |
| | Oak | 64 ± 0.8 ab | 4.1 ± 0.1 | 39 ± 1.5c | 15.7±0.1a | 4.8±0.1a | 81±1d |
| | Ash + NP | 61 ± 0.6 ab | 4.4 ± 0.1 | 531 ± 3.2a | 13.9±0.3c | 4.6±0.1b | 77±5a |
| | Oak + NP | 66 ± 2.5a | 4.4 ± 0.2 | 538 ± 9.8a | 14.8 ± 0.1b | 4.5±0.1b | 354±1b |
| 56 | Control | 56 ± 0.2b | 3.8±0.1b | 47 ± 1.6b | 14.8 ± 0.1b | 4.3±0.1d | 200±2c |
| | Ash | 59 ± 1.2 ab | 4.0 ± 0.1b | 35.9 ± 0.8c | 14.8 ± 0.1b | 5.0±0.1a | 94±4d |
| | Oak | 60 ± 2.0 ab | 3.8 ± 0.1b | 38 ± 1.1c | 15.6 ± 0.1a | 4.8±0.1b | 101±2d |
| | Ash + NP | 62 ± 0.2a | 4.4±0.1a | 538 ± 25a | 14.2 ± 0.1c | 4.6±0.1c | 397±4a |
| | Oak + NP | 60 ± 1.2 ab | 4.0 ± 0.1b | 526 ± 7.6a | 14.9 ± 0.1b | 4.7±0.1c | 362±1b |

Values are means with standard errors (n = 3), and different letters denote significant differences among treatments in each sampling time at the p < 0.05 level. SOC, soil organic carbon; TN, total nitrogen; P, phosphorus; C/N, carbon to nitrogen ratio; EC, electrical conductivity.
ratio of fungal growth to bacterial growth rates in the ash treatment were significantly higher than in the oak treatment between 1 and 3 days of incubation, but slightly lower later on (Fig. 1 C and D). Added NP increased fungal growth rate and the ratio of fungal growth to bacterial growth rates for both ash and oak litter between 1 and 3 days (Fig. 1 C and D).

3.3. Ecoenzymatic ratios

The BG/(NAG + LAP) ratios were greater than 1 and the BG/AP and (NAG + LAP)/AP ratios were both lower than 1 in all treatments during the 56-day incubation (Fig. 2), reflecting that microbes were mainly P limited or co-limited by C and P with high activities of C- and P-acquiring enzymes (Fig. S1). The BG/(NAG + LAP) and BG/AP ratios were strongly elevated after one day in litter treatments, and were higher in the ash than the oak treatment. Moreover, the BG/(NAG + LAP) and BG/AP ratios decreased sharply between 1 and 7 days of incubation (Fig. 2B and C). Thereafter, the BG/(NAG + LAP) ratio in the ash treatment increased slightly (linear regression; \( p < 0.01 \)) during the later incubation period (14–56 days), while in the oak treatment this ratio continued to decrease during the later incubation period (Fig. 2B). This led to higher BG/(NAG + LAP) ratio in the ash than in the oak treatment during the later incubation period. The litter plus NP treatments had higher ratios of BG/(NAG + LAP), BG/AP, and (NAG + LAP)/AP than those in corresponding litter treatments during the incubation, except that the (NAG + LAP)/AP ratio converged in oak and oak + NP treatments during the later incubation period (Fig. 2).

3.4. Limiting resources for microbial growth

In control soil, microbial growth responded mainly to additions of C alone and in combination with nutrients after 42 days incubation (panel A in Fig. 3). Patterns for bacterial and fungal growth were similar (Fig. S2). This indicated that the microbial growth in the control soil was primarily C limited. In the ash treatment, microbial growth responded moderately positively to addition of CN and CNP (panel B in Fig. 3 and Fig. S2) indicating that microbes had shifted their growth limitation to be co-limited by C and N. In the oak treatment, microbial growth responded most positively to addition of C, CN, CP and CNP (panel D in Fig. 3 and Fig. S2) which suggested C limitation. Moreover, the microbial growth rates increased less in litter treatments without mineral nutrients supplements compared to the growth rate in the control soil when C, CN, CP and CNP were added. This indicated that litter inputs had alleviated an initial C limitation for microbial growth. Following litter plus NP treatments, microbial growth (especially fungal growth, Fig. S2) showed stronger responses to additions of C alone and C combined with nutrients (i.e. C, CN, CP and CNP) compared to that in corresponding litter treatments. This showed that NP addition aggravated C limitation of microbial growth.

3.5. Microbial community composition and microbial biomass C

The first principal component (PC1) in a PCA of microbial PLFA composition explained 47.3% of the variation, while PC2 explained another 16.2% (Fig. 4). Both treatment and incubation time were key
drivers for the microbial PLFA composition, as revealed by the separation of samples along PC1 (treatment) and PC2 (time) of the PCA (Fig. 4A). Ash litter with NP addition was associated with higher relative abundances of fungi (18:2ω6,9). Both pure litter treatments (ash and oak) were related to higher relative abundances of gram-negative bacteria (16:1ω7c, 16:1ω9 and 18:1ω7) (Fig. 4B). Total and bacterial PLFA concentrations in the ash treatment were significantly higher than those in the oak treatment during the entire incubation period except bacteria...
after 7 days (Table 3). The higher ratio of fungi to bacteria (F/B) in the ash than the oak treatment showed up after 7 days of incubation (Table 3) but was reversed later in the incubation period. The ash + NP and oak + NP treatments tended to have higher ratios of F/B and gram-positive to gram-negative bacteria (GP/GN) compared to ash and oak treatments (Table 3).

4. Discussion

According to ecoenzymatic theory (Sinsabaugh et al., 2008, 2009), the enzymatic ratios indicate that microbes were co-limited by C and P in all treatments (Fig. 2). This is not consistent with the limiting factor assays (LFAs), which suggested that microbes were primarily C limited (Fig. 3). These results add more support to recent claims that ecoenzy

Table 3

Microbial phospholipid fatty acid (PLFA) characteristics after 7, 28, 56 days incubation for soils in five treatments.

| Incubation Time (d) | Treatments | Bacterial PLFA nmol g soil⁻¹ | Fungal PLFA nmol g soil⁻¹ | Total PLFA nmol g soil⁻¹ | F/B ratio | GP/GN ratio |
|---------------------|------------|-----------------------------|---------------------------|-------------------------|----------|------------|
| 7                   | CK         | 155±2b                      | 5.6±0.4d                  | 244±4c                  | 0.04±0.01d | 0.79±0.05  |
|                     | Ash        | 189±6a                      | 19.9±0.9b                 | 350±12a                 | 0.11±0.01b | 0.70±0.01  |
|                     | Oak        | 169±4 ab                    | 14.3±0.5c                 | 287±7b                  | 0.08±0.01c | 0.71±0.02  |
|                     | Ash + NP   | 167±8 ab                    | 29.1±1.9a                 | 348±17a                 | 0.17±0.01a | 0.76±0.01  |
|                     | Oak + NP   | 166±5 ab                    | 18.4±1bc                  | 290±10b                 | 0.11±0.01b | 0.72±0.01  |
| 28                  | CK         | 132±4c                      | 5.9±0.2d                  | 205±6c                  | 0.04±0.01c | 0.73±0.01a |
|                     | Ash        | 175±2a                      | 14.2±0.5b                 | 300±4a                  | 0.08±0.01b | 0.65±0.01c |
|                     | Oak        | 155±6 b                     | 12.3±0.4c                 | 254±10b                 | 0.08±0.01b | 0.67±0.01b |
|                     | Ash + NP   | 155±1b                      | 17.7±0.2a                 | 286±3a                  | 0.11±0.01a | 0.74±0.01a |
|                     | Oak + NP   | 143±1bc                     | 15.4±0.3b                 | 245±2b                  | 0.11±0.01a | 0.71±0.01b |
| 56                  | CK         | 126±5b                      | 6.4±0.7c                  | 198±9c                  | 0.05±0.01d | 0.7±0.02a  |
|                     | Ash        | 188±3a                      | 13.8±0.3ab                | 313±6a                  | 0.07±0.01c | 0.63±0.01b |
|                     | Oak        | 137±9b                      | 11.5±0.7b                 | 223±14bc                | 0.08±0.01bc| 0.57±0.02b |
|                     | Ash + NP   | 134±2b                      | 17.0±0.2a                 | 248±4b                  | 0.13±0.01a | 0.65±0.01a |
|                     | Oak + NP   | 126±2b                      | 12.0±0.3b                 | 212±4bc                 | 0.1±0.01bc | 0.68±0.03a |

Values are means with standard errors and different letters denote significant differences among treatments at each time point at the p < 0.05 level. PLFA, phospholipid fatty acid; F/B ratio, fungi to bacteria ratio; GP/GN ratio, gram-positive to gram-negative bacteria ratio.

Fig. 4. Principal component analysis (PCA) of microbial phospholipid fatty acid (PLFA) composition (% mol) in different treatments after 7, 28 and 56 days of incubation (A). Loading plots of the PCA (B) show the PLFA biomarkers that drive the separation among treatments and incubation time.

4.1. High BG/(NAG + LAP) ratio but low microbial C limitation after litter addition

In contrast with H1, the BG/(NAG + LAP) ratios in litter treatments were very high compared to control soil after the initial litter addition (Fig. 2), suggesting that microbial C limitation in litter treatments had significantly strengthened according to ecoenzymatic stoichiometry (Sinsabaugh et al., 2008, 2009). In sharp contrast to this result, the high respiration rate, bacterial growth rate and fungal growth rate all indicated that C limitation was alleviated or even removed by leaf litter addition in the early phase of decomposition (Fig. 1). The high respiration and growth rates with litter addition were mainly attributed to the alleviation of C limitation rather than reduced nutrient limitation, since LFAs showed the control soil was primarily C limited (Fig. 3). Moreover, the microbial C limitation was indicated to be higher in the ash than the oak treatment due to higher BG/(NAG + LAP) ratio in ash treatment (Fig. 2), which contrasted with the higher microbial respiration and growth rates in the ash treatment indicating lower C limitation (Fig. 1). The difference in microbial respiration between ash and oak treatments was likely due to differences in C availability rather than nutrient availability, as NP addition did not diminish the difference in microbial respiration between the two types of substrate (Fig. 1).
microbial growth and respiration rates matched with previous studies in which microbial C limitation was alleviated with inputs of labile C with litter addition (Kamble and Bååth, 2014; Soong et al., 2019), and where microbes tended to be nutrient limited rather than C limited during the early decomposition stage. The lower microbial C limitation in ash than oak treatment (Figs. 1 and 3) also coincided with much higher water extractive carbohydrates (Table 1) and higher microbial growth rates in ash forest than oak forest in the field experiment (Hedeneček et al., 2020). We hypothesized (H2) that the BG/(NAG + LAP) ratio in litter treatments would later increase as C became more limiting following the fast depletion of labile C during the early incubation period (<7 days). In contrast to H2, the BG/(NAG + LAP) ratios in litter treatments were markedly decreased during the early incubation period (Fig. 2), which ecoenzymatic theory would suggest is a consequence of decreased microbial C limitation. This clearly contradicts increasing microbial C limitation revealed by decreasing microbial growth and respiration rates (Fig. 1), presumably due to depletion of labile C contained in the litter, including monomer sugars, amino acids and soluble protein (Bray et al., 2012; Soong et al., 2019). The decreasing microbial growth and respiration rates in litter treatments occurred both with and without the NP additions, thus indicating lack of nutrients as an unlikely explanation for the reduction in microbial growth rates (Fig. 1). Moreover, the LFAs suggested that microbes were primarily limited by C rather than NP in litter treatments as well as litter plus NP treatments (Fig. 3), even in the later incubation period when the rates of microbial respiration and growth had become low (Fig. 1).

The high BG activity just after litter addition was likely induced by that ample supply of fresh labile C stimulated microbial growth and provided an energy source to produce enzymes needed for the hydrolysis of litter C compounds (Weintraub et al., 2012). Hernández and Hobbs (2010) found that the addition of labile C (i.e., glucose) increased BG activity and microbial respiration, which was potentially caused by labile C stimulating microbial biomass production and shifts in microbial community in favour of particular groups, such as gram-negative bacteria. Indeed, we observed a rapid increase in total microbial biomass and gram-negative bacteria, often associated with labile substrate decomposition (Bray et al., 2012; Fanin et al., 2015; Hedeneček et al., 2020), were more abundant in the litter treatment than in control soil (Table 3 and Fig. 4). These findings indicate that resource-driven changes in soil microbial community composition play important roles to mediate decomposition of specific C substrates and enzyme allocations (Leff et al., 2012; Nemergut et al., 2016; Weintraub et al., 2012). In summary, in contrast to the concept of ecoenzymatic stoichiometry, higher BG/(NAG + LAP) ratio did not reflect higher microbial C limitation when labile C dominates as microbial C source, but instead reflected the greater availability of C (energy) in labile substrates and higher rates of microbial metabolism (Fig. 1).

4.2. Increasing microbial C limitation induces higher BG/(NAG + LAP) ratios in ash litter, but lower ratios in oak litter

As we hypothesized (H3), the BG/(NAG + LAP) ratio increased in the ash treatment but decreased in the oak treatment during the later incubation period (7–56 days, Fig. 2). These responses indicated microbial C limitation increased in the ash treatment but decreased in the oak treatment. However, decreasing respiration rates, and bacterial and fungal growth rates all indicated that C limitation increased in both ash and oak treatments during later decomposition (Fig. 1). Moreover, NP addition exacerbated the decrease in respiration rates during the later incubation period (Fig. 1), which is also consistent with microbes being progressively more C-limited during the later incubation period. Seven days after litter addition, the microbial respiration and growth rates slowed down sharply due to depletion of labile C contained in the litter as discussed above, whereafter microbes probably shifted their resource use toward the remaining dominant C substrates by modulating enzyme production (Allison et al., 2010; Sinsabaugh and Follstad Shah, 2012). Accordingly, microbes had relatively high BG activity and low NAG activity in the ash treatment (Fig. S1) where cellulose is the dominant C source, while microbes had relatively high NAG activity and low BG activity in the oak treatment (Fig. S1) where substrates other than cellulose (i.e., lignin or lignin-embedded substrates) probably are the dominant C source. Consequently, the BG/(NAG + LAP) ratio was consistent with the temporal dynamics of microbial C limitation in the ash treatment, but was inconsistent with the temporal dynamics of microbial C limitation in oak treatment. These results supported the concept proposed by (Mori, 2020), i.e., that the BG/(NAG + LAP) ratio is a reliable indicator of relative microbial C vs. N limitation when cellulose dominates as microbial C source but is not a reliable indicator for this when substrates other than cellulose dominate as C sources.

The continuously increasing NAG activity (an enzyme targeting polymers of amino sugars) likely resulted from microbes producing NAG to acquire C through increased consumption of microbial necromass-derived C sources (i.e. chitin and muramic acids; both polymers of amino sugars) (Mori, 2020; Mori et al., 2021), which contain both C and N (Moorhead et al., 2012; Olander and Vitousek, 2000), and such C sources might be more abundant in oak litter with a more fungal dominated community and higher fungal growth rates (Table 3 and Fig. 1D). Firstly, soil fungi are the primary producers of extracellular enzymes (such as NAG and laccases) that break down substrates including both C and N (Morrison et al., 2019; Talbot and Treseder, 2012). Secondly, two-thirds of microbial necromass (residue-derived C sources) typically originates from fungi (Wang et al., 2021). Thirdly, fungal growth was utilizing much more C than bacterial growth in soils of all treatments (Fig. 1D). Lastly, microbes invested most of their energy to acquire C since microbial growth was primarily C rather than N limited in soils of all treatments as revealed by LFAs (Fig. 3). Indeed we found that NAG activity in the oak treatment was higher than in the ash treatment during the later incubation period, which was consistent with a shift in microbial community toward greater fungal abundance and higher ratio of fungal to bacterial growth rates in oak treatment than in ash treatment (Table 3 and Fig. 1D).

As we hypothesized in H3, the BG/(NAG + LAP) ratio in the ash treatment was higher than oak treatment during later incubation period, which suggest greater relative C limitation in the ash treatment than the oak treatment (Fig. 2). However, this contradicted with the weak microbial C limitation in the ash treatment as revealed by LFAs for microbial growth compared to the oak treatment (Fig. 3B vs. 3D). The higher microbial C limitation in oak treatments was mainly attributed to microbial composition shifting to higher fungal dominance in community structure and growth rates due to microbial utilization of relatively recalcitrant substrates which are energetically expensive, and microbes decreasing the energy investment in bacterial growth. Indeed, we found bacterial growth in the oak treatment was more C limited than in the ash treatment (Fig. S2). Therefore, our results suggest that variation in substrate quality, availability and how these matched the present microbial energy allocation strategies and resources allocation to enzyme production caused a substantial variation in the BG/(NAG + LAP) ratio, thus disconnecting the ratio from the microbial C limitation.

The results were synthesized in combination with previous studies in a conceptual figure of the dynamic pattern of the BG/(NAG + LAP) ratio and microbial C limitation with litter inputs (Fig. 5). Increasing microbial C limitation (solid lines) is supported by the temporal dynamics of microbial respiration, bacterial and fungal growth rates with time after input (dashed line in Fig. 1), and corroborated with numerous studies (Soong et al., 2019). However, the BG/(NAG + LAP) ratio (dashed line; fitted in this study) has different trends in indicated microbial C limitation after the initial period of litter inputs (<7 days), when high availability of C in simple substrates (i.e., monomer sugars, amino acids and soluble protein) exist. During the later incubation period, the microbial respiration and growth rates slowed down sharply due to depletion of labile C contained in the litter, whereafter microbes probably shifted their resource use toward the remaining dominant C
substrates, cellulose in ash and other than cellulose (i.e., lignin or lignin-embedded substrates and necromass) in oak. Consequently, the BG/(NAG + LAP) ratio was consistent with the temporal dynamics of microbial C limitation in the ash treatment, but was inconsistent with the temporal dynamics of microbial C limitation in oak treatment. These results supported the concept proposed by (Mori, 2020), i.e., that BG/(NAG + LAP) ratio is reliable to indicate microbial relative C vs. N limitation when cellulose dominated as microbial C source but not consistently to indicate when substrates other than cellulose dominated as C sources.

4.3. Ecoenzymatic ratios can capture microbial C limitation induced by NP addition

In line with our last hypothesis (H4), addition of NP in both the ash and the oak treatment intensified C limitation for microbial growth as indicated by the inhibited respiration rates (Fig. 1) and increased responses in total microbial growth to short-term C addition (Fig. 3C vs. 3B and 3E vs. 3D). The ecoenzymatic ratio revealed a similar picture, i.e. that NP addition exacerbated microbial C limitation, as indicated by the higher BG/(NAG + LAP) ratio in litter plus NP treatments than in litter treatments after 54 days of incubation (Fig. 2). The aggravated C limitation of microbial growth with NP addition is in concert with previous findings that NP addition accelerate C loss during the course of litter decomposition, which in turn increased microbial C demand to fulfill stoichiometric needs of microbial communities (Fanin et al., 2016; Hobbie, 2000; Knorr et al., 2005). The increased BG/(NAG + LAP) ratio was mainly explained by increasing BG activity with NP addition (Fig. S1), since imbalanced nutrient additions increases investment in enzymes to acquire the limiting resources under the new conditions (Allison et al., 2010; Geisseler and Horwath, 2009; Hernández and Hobbie, 2010). However, NP addition depressed activities of LAP and AP, but stimulated or showed no difference in activity of NAG (Fig. S1). This indicates that NAG was an ambiguous indicator to reflect microbial N acquisition, and provides some evidence that microbes also produce NAG to alleviate the strengthened C limitation by NP addition. For instance, NP addition stimulated fungal growth rate (ca.150 times higher than bacteria growth; Fig. 1), fungal biomass (Table 3) as well as fungal C demand (Fig. S2), which in turn increased NAG production (Fig. S1) to fulfill C demand by degrading substrates including both C and N such as necromass. These observations are also consistent with fungi having a competitive growth advantage over bacteria in the use of added substrates (i.e., straw or cellulose), especially combined with mineral N (i.e., NH$_4$NO$_3$) (Fontaine et al., 2011; Kamble and Bååth, 2016; Rousk and Bååth, 2007; Soares et al., 2017). The increased microbial necromass C with N addition has been found in a recent meta-analysis, and was more pronounced in fungal than bacterial necromass C (Liao et al., 2020). These results support that NAG activity was tightly correlated with fungal C acquisition from necromass and substrates other than cellulose as discussed above (4.2).

5. Conclusion

The high BG/(NAG + LAP) ratio contrasted with alleviated C limitation during initial periods of litter inputs, which was mainly attributed to high BG activity along with high energy supply and high microbial metabolism in abundant labile C substrates. During later incubation period (7–56 days), microbial C limitation increased as reflected by decreasing respiration, bacterial and fungal growth rates. The increasing BG/(NAG + LAP) ratio was in line with increasing microbial C limitation in the ash treatment where cellulose dominated as microbial C source, but the decreasing BG/(NAG + LAP) ratio was inconsistent with increasing microbial C limitation in the oak treatment where substrates other than cellulose, such as necromass components like chitin, probably dominated as a C source. In the oak and litter plus NP treatments, fungal growth was stimulated, making it likely that NAG was induced to meet the fungal C demand by utilizing microbial necromass (e.g., chitin). These findings suggest that the ecoenzymatic ratio of BG/(NAG + LAP) can indicate limiting resources for microbial growth when cellulose dominates as C source, but not when labile forms of carbon or substrates other than cellulose (i.e., microbial necromass) dominate as microbial carbon sources.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled.

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Appendix A. Supplementary data

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