



Coupling the resource stoichiometry and microbial biomass turnover to predict nutrient mineralization and immobilization in soil

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ABSTRACT

The mineralization of organic nitrogen (N) and phosphorus (P) by the soil microbial biomass, as well as microbial immobilization of their mineral forms, can be predicted from differences between the stoichiometry of organic substrate and the nutrient demand of the microbial biomass. The accuracy of such predictions, however, decreases when the nutrient demand of microbial biomass changes in response to nutrient limitation or excess. We quantified net N and P mineralization/immobilization along gradients of organic substrate stoichiometry in a short-term (2-day) incubation experiment. Gradient of organic substrate stoichiometry (water extractable organic N and P concentrations) was created by mixing soils from two spruce forest soils (from both litter and organic topsoil horizons) at five different ratios. Biological predictors (i.e., microbial carbon (C) use efficiency and microbial biomass C, N, and P) of net nutrient mineralization/immobilization were quantified, and theoretical N and P mineralization/immobilization rates were predicted using known stoichiometric relationship. Measured net N and P immobilization was lower than that predicted. Extended mathematical modelling in combination with stable isotope analysis showed that the capability of microbial community to reduce its demand for external nutrients was responsible for the difference between the predictions and observations. Active part of microbial community instantly recycled N from decaying part of microbial community and very likely utilized internal P sources (i.e. polyphosphates) when the abundance of N and P in available organic compounds was insufficient. Our results suggest that the N recycling from dead microbial biomass and the internal microbial P sources warrant further investigation. Including these mechanisms in soil biogeochemical models based on ecological stoichiometry principles could improve their predictive accuracy.

1. Introduction

Soil microorganisms control the availability of key nutrients such as nitrogen (N) and phosphorus (P) in soils, releasing mineral forms of nutrients into the soil solution (mineralization) and immobilizing mineral nutrients from the soil solution (immobilization; e.g. Manzoni et al., 2010). When nutrient mineralization prevails in soils, it can significantly promote nutrient-limited primary production. On the other hand, when immobilization predominates, primary production can become depressed (Čapek et al., 2018).

Ecological stoichiometry concept has been used to predict the nutrient mineralization and immobilization rates assuming that both processes mainly depend on the difference between the relative abundance of a nutrient in organic compounds that soil microorganisms

consume (referred to as organic substrate), and the actual demand of the microorganisms for nutrients (Manzoni et al., 2017, 2008). Organic substrate is a direct source of organic carbon and nutrients. This source has variable stoichiometry, i.e., it contains variable amounts of nutrients per unit of organic carbon. When this amount exceeds the stoichiometric demand of microorganisms, a particular nutrient is present in an unlimited amount with respect to organic carbon, and the mineral form of the nutrient in excess is released to the soil solution (Manzoni et al., 2017). Vice versa, when a nutrient is in a limited amount with respect to organic carbon, the mineral form of the nutrient is taken up from the soil solution by microorganisms to balance the deficit (Manzoni et al., 2017). However, there are several confounding processes occurring at the same time scales, so the stoichiometry alone might not predict the microbial nutrient mineralization and immobilization with sufficient accuracy.

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First, a significant amount of organic P (McGill and Cole, 1981) as well as N (Geisseler et al., 2010, 2009) can be mineralized extracellularly (e.g., by extracellular or cell surface-bound phosphatases and amino acid oxidases), thus disobeying direct microbial control. Second, P in particular can be adsorbed onto soil minerals and become inaccessible to soil microorganisms (e.g. Olander and Vitousek, 2005). Third, and most importantly, microorganisms can adapt to nutrient limitation. In the short term, they can either manipulate their nutrient demand by changing their organic carbon use efficiency (CUE) and nutrients use efficiency (Manzoni et al., 2017; Mooshammer et al., 2014a, 2014b; Wild et al., 2013), or selectively consume organic substrates with higher nutrient content (Gunina et al., 2017; Reuter et al., 2019; Zhang et al., 2015). In the long term, the species composition of microbial community can shift towards a dominance of species adapted to the organic substrate stoichiometry present (Kaiser et al., 2014). Besides that, there are another two possible ways to avoid nutrient limitation: (i) the recycling of nutrients derived from the dying microbial biomass (Boyle et al., 2012; Chen et al., 2019; Spohn and Widdig, 2017), and (ii) the use of internal storage compounds, namely polyphosphates (Čapek et al., 2016; Kulaev et al., 1999). These mechanisms, however, received less attention so far.

(i) In soils, microbial growth and death occur simultaneously (Chapman and Gray, 1986; Van Veen et al., 1987). Even though no net microbial biomass change is observed over time, one part of microbial biomass is constantly dying, whereas the other part is constantly growing. When gross growth is realized, some of the cellular components of dying microbial biomass, such as protoplasm, can be instantly consumed by the growing part of the microbial community, so some of the nutrients contained in microbial biomass are repeatedly recycled and the microbial biomass demand for external nutrients decreases. For example, soil microorganisms are able to increase the mean residence time of P in their biomass along the gradient of P availability (Chen et al., 2019; Spohn and Widdig, 2017).

(ii) Polyphosphates serve microbes as an internal source of P and energy when their external sources are scarce (Dawes, 1986; Kulaev et al., 1999). They are formed when the energy and P are in excess in the environment and used to supply P for nucleic acid and phospholipid biosynthesis under the conditions of P starvation (Dawes, 1986). It has been shown that a wide variety of soil microbial communities exhibit growth in organic media without P due to the utilization of polyphosphates (Čapek et al., 2016). Thus, analogically to recycling of microbial biomass nutrients, stores of internal polyphosphates decrease the immediate demand of microbial biomass for external P sources. This, however, applies solely to P as the N has no known storage compounds.

In the present study, we tested the ability of the stoichiometric concept to predict N and P mineralization and immobilization in the litter and organic topsoil horizons of the soils from two adjacent mountain catchments, Plešné and Čertovo (Šumava Mountains, Czech Republic), which have similar vegetation (forest dominated by Norway spruce), climatic conditions (Turek et al., 2014) and atmospheric inputs of N and P (Kopáček et al., 2015). However, the stoichiometry of the litterfall (with respect to C:N – Fig. A1A; Kopáček et al., 2015) and the chemical composition of the bedrock (P rich granite at the Plešné catchment vs. P poor mica schist at the Čertovo catchment) differ. Previous studies have demonstrated that soil microbial communities of both catchments have a similar demand for N (Tahovská et al., 2013), but a different demand for P (Čapek et al., 2016). We used these soils to setup two gradients of initial organic substrate stoichiometry, along which the mineralization/immobilization of N and P was expected to change during short-term incubation. The first gradient was represented by two different soil horizons, i.e., litter and organic topsoil, characterizing two different stages of plant litter decomposition, and thus organic substrate stoichiometry. During litter decomposition, organic carbon is lost as CO₂ and nutrients concentrate in the organic substrates. The resulting C-to-nutrient ratio slowly decreases, and this decrease is typically associated with the transition from nutrient immobilization towards nutrient

mineralization (Manzoni et al., 2010). The second gradient was created by mixing of the respective soil horizons from both catchments at five different ratios. Using previously defined stoichiometric relationships (Manzoni et al., 2017), we calculated the expected amount of mineralized or immobilized nutrients by microbial biomass across these gradients, and confronted the predicted values with direct measurements. We hypothesized that recycling of microbial biomass N and P should affect the accuracy of the stoichiometry-based predictions.

2. Materials and methods

2.1. Soils

Soils were sampled in July 2017 in the catchments of the glacial lakes Plešné (PL; 48°77'N, 13°86'E) and Čertovo (CT; 49°16', 13°20'E) located in the Šumava Mountains in the south-western part of the Czech Republic. Between 2004 and 2008, the spruce forest in the PL catchment was damaged by a bark beetle infestation, resulting in an alteration of the soil chemistry and nutrient fluxes (Kaňa et al., 2013, 2015). To avoid any potential effect of this disturbance, soil samples were taken from the remaining mature forest unaffected by the bark beetle attack. The vegetation cover, elevation, slope and aspect of the sampling localities were similar at both catchments. Within these localities, litter and organic topsoil samples were collected at four randomly chosen locations. All soil samples were immediately sieved through 5 mm mesh and kept two days at 15 °C in the dark (pre-incubation).

2.2. Experimental set-up

After pre-incubation, samples from the PL and CT catchments were mixed at different PL to CT ratios of 0:1, 0.25:0.75, 0.5:0.5, 0.75:0.25 and 1:0 for both litter and organic topsoil horizons separately. All mixtures of each horizon had uniform initial moisture. All samples were immediately analyzed for: concentrations of K₂SO₄ extractable organic carbon (K₂SO₄-EC); microbial biomass carbon (MBC); microbial biomass N (MBN) and P (MBP); and water extractable (hereafter denoted as dissolved) forms of organic carbon (DOC), dissolved organic N (DON), nitrate (NO₃⁻), ammonium (NH₄⁺), dissolved organic P (DOP), and soluble reactive P (SRP). The ^{12/13}C isotope compositions of total soil organic carbon (SOC), K₂SO₄-EC, and MBC were determined alongside. Description of all methods is reported in detail in Appendix (section 1. Analytical methods). The rest of the samples were weighed into glass jars (40 g wet weight) and supplemented either with distilled water or a glucose solution enriched by uniformly labeled ¹³C glucose to a 6.3 atom % ¹³C, and thoroughly mixed. The addition of labelled glucose was performed in order to estimate CUE of microbial community (see section 2.4.). The concentration of added organic carbon in the form of glucose was equal to one half of the microbial biomass carbon [~300 and 100 μmol(C) g(DW)⁻¹ for litter and organic topsoil respectively] to prevent a net increase (net growth) of microbial biomass (Anderson and Domsch, 1986; Sawada et al., 2008). Distilled water and the glucose solution were added in the volumes necessary to reach 60% of the water holding capacity of all soil samples. All glass jars were secured with rubber stoppers and incubated for 48 h at 15 °C in the dark. After 48 h, all analyses were repeated, and the ^{12/13}C isotopic composition and concentration of headspace CO₂ were measured. Abbreviations of all measured variables and respective analytical methods are reported in Table 1.

2.3. CO₂ concentration and its ^{12/13}C isotopic composition

The headspace of glass jars was thoroughly mixed and sampled using a 1 ml plastic syringe. 0.2 ml of the sample was directly injected into a gas chromatograph (Agilent 7820A GC, Agilent Technologies, Santa Clara, USA) equipped with a thermal conductivity detector. The flow rate of the carrier gas (N₂) and the temperature of the separation column were 10 ml/min and 40 °C. A subsample of the headspace atmosphere

Table 1

List of abbreviations used in the text, along with explanations and associated methods, quantifications and calculations. Abbreviations are divided into two groups: the first is associated with specific methods, while the second is associated with the reported equations.

Measurements		
Abbreviation	Explanation	Method
K ₂ SO ₄ -EC	Extractable organic carbon	Organic carbon in 0.5 M K ₂ SO ₄ solution, measured directly (LiquicTOC II)
MBC, MBN, MBP	Micobial biomass carbon, nitrogen and phosphorus	Measured according to Brookes et al. (1985, 1982), Vance et al. (1987)
DOC	Dissolved organic carbon	Organic carbon in water extract, measured directly (LiquicTOC II)
DON	Dissolved organic nitrogen	Organic nitrogen in water extract - total N (LiquicTOC II) with NH ₄ ⁺ and NO ₃ ⁻ subtracted
SRP	Soluble reactive phosphorus	Soluble reactive phosphorus in water extract, measured directly (QuickChem 8500)
Equations		
Abbreviation	Explanation	Quantification/Calculation
ΔM_P , ΔM_N	Net change of mineral phosphorus/nitrogen during incubation	Quantified as a change of SRP/sum of NH ₄ ⁺ and NO ₃ ⁻ concentrations respectively
U	Amount of organic substrate consumed by microbial biomass during the incubation	Calculated as a sum of respired CO ₂ and organic carbon incorporated into microbial biomass (Eq. (3))
G	Amount of organic substrate incorporated into microbial biomass during the incubation	Calculated using respired CO ₂ and CUE (Eq. (4))
R	Amount of organic substrate respired as CO ₂ during the incubation	Quantified as a change in headspace CO ₂ concentration and corrected for CO ₂ dissolution
f_{MBC}	Relative contribution of dying microbial biomass to consumed organic substrate	Calculated using $\delta^{13}C_R$, $\delta^{13}C_{SOC}$ and $\delta^{13}C_{MBC}$ (Eq. (2))
$C:E_S$	Carbon to nutrient (N or P) ratio of organic substrate	Quantified as DOC:DON or DOC:DOP
$C:E_B$	Carbon to nutrient (N or P) ratio of microbial biomass	Quantified as MBC:MBN or MBC:MBP
CUE	Carbon use efficiency	Calculated either using $MBC_{(G)}$ and $R_{(G)}$ ($CUE_{glucose}$; Eq. (5)), or using microbially-explicit model ($CUE_{modelled}$; Eqs. A(1)–A(4))
$R_{(G)}$	Amount of ¹³ C labeled glucose respired as CO ₂ during incubation	Calculated by two-pool mixing model
$\delta^{13}C_R$	^{12/13} C isotopic composition of respired CO ₂	Quantified directly (IR-MS DELTA plus XL)
$\delta^{13}C_{SOC}$	^{12/13} C isotopic composition of organic substrate derived from soil – total soil organic carbon	Quantified directly (IR-MS DELTA plus XL)
$\delta^{13}C_{MBC}$	^{12/13} C isotopic composition of MBC	Calculated by two-pool mixing model
P_X	Amount of chemically adsorbed SRP	Calculated using SRP_0 and $Recovery$ (Eq. (7))
SRP_0	Initial concentration of SRP	Quantified directly (QuickChem 8500)
$Recovery$	Relative proportion of recovered K ₂ HPO ₄ spike	Quantified according to Brookes et al., 1982
k_1	Organic substrate consumption rate normalized to unit of MBC and DOC	Estimated (Eqs. A(1)–A(3))
k_2	Microbial biomass death rate	Estimated (Eqs. A(1), A(2) and A(4))
C_{RES}	Residual soil organic carbon	Not quantified

was further injected into N₂ filled gas-tight 10 ml vials for subsequent isotope analysis on an isotope ratio mass spectrometer (IR-MS DELTA plus XL, Finnigan, Germany).

2.4. Calculations

The net change of mineral N (ΔM_N – represented by the sum of ΔNO_3^- and ΔNH_4^+) and P (ΔM_P – represented by ΔSRP) concentrations in water extracts of the control samples (i.e., not receiving glucose) over the period of 48 h was calculated using the equation presented by Manzoni et al. (2017) and adapted for the purpose of our study by including the parameter f_{MBC} . This parameter defines the contribution of the decaying microbial biomass to the total amount of consumed organic substrate by active part of the microbial community, thus the degree of microbial biomass recycling:

$$\Delta M_E = U \cdot \left\{ 1 / \left[(1 - f_{MBC}) \cdot (C:E)_S + f_{MBC} \cdot (C:E)_B \right] - CUE / (C:E)_B \right\} \quad (1)$$

In Eq. (1), ΔM_E is the net change of a mineral nutrient (i.e., the subscript E denotes either N or P), U is the amount of consumed organic substrate (in moles of organic carbon), $(C:E)_S$ is the molar carbon to nutrient ratio of external (i.e., soil-derived) organic substrate (DOC:DON and DOC:DOP), CUE is the carbon use efficiency, and $(C:E)_B$ is the molar carbon to nutrient ratio of the microbial biomass – both active and decaying (i.e., MBC:MBN or MBC:MBP). See also Table 1 for an explanation of all abbreviations.

The first term in the braces (i.e., $1 / [(1 - f_{MBC}) \cdot (C:E)_S + f_{MBC} \cdot (C:E)_B]$) denotes the relative abundance of a nutrient in the consumed soil-derived organic substrate or consumed decaying microbial biomass. The second term (i.e., $CUE / (C:E)_B$) denotes the nutrient demand of the

active microbial biomass, which depends on the concentration of a nutrient in the microbial biomass and CUE . When the nutrient demand is higher than its abundance in the consumed organic substrate, ΔM_E is negative and the mineral nutrient is consumed in an amount proportional to U (i.e., net nutrient immobilization occurs to balance the nutrient deficit). Vice versa, when the nutrient demand is lower than the nutrient abundance in the consumed organic substrate, ΔM_E is positive and organic nutrient is mineralized in an amount proportional to U (i.e., net nutrient mineralization occurs to release nutrients in excess). The $(C:E)_S$ and $(C:E)_B$ values were assumed to be time independent constants in Eq. (1), because they exhibited no statistically significant temporal changes during our experiment (see Appendix; Fig. A2A, B, C, D).

The parameter f_{MBC} can range from zero to one. When f_{MBC} is zero, the active part of microbial community consumes only the organic substrate derived from the soil and the first term of Eq. (1) in the braces simplifies to $1 / (C:E)_S$. When f_{MBC} is one, only the decaying part of microbial biomass is consumed and the term simplifies to $1 / (C:E)_B$. The f_{MBC} value was calculated using the equation:

$$f_{MBC} = (\delta^{13}C_R - \delta^{13}C_{SOC}) / (\delta^{13}C_{MBC} - \delta^{13}C_{SOC}), \quad (2)$$

in which $\delta^{13}C_R$ is the isotopic signal of respired CO₂, $\delta^{13}C_{SOC}$ is the isotopic signal of soil-derived organic substrate (isotopic signal of SOC), and $\delta^{13}C_{MBC}$ is the isotopic signal of MBC (Table 1). The closer the isotopic signal of respired CO₂ resembles the isotopic signal of MBC, the greater f_{MBC} and thus, greater microbial biomass recycling. When $\delta^{13}C_R$ equals $\delta^{13}C_{MBC}$, f_{MBC} is one and vice versa.

Symbol U represents the total amount of organic substrate consumed by the active microbial biomass over a period of 48 h (Table 1). It comprises the measured total amount of carbon respired as CO₂ (R) and the estimated total amount of carbon incorporated into MBC (G) over

Table 2

Chemical analyses (pH_(KCl), DOC – water extractable organic carbon, DON – water extractable organic nitrogen, NH₄⁺ – water extractable ammonium, NO₃⁻ – water extractable nitrate, and SRP – water extractable phosphate, DOP – water extractable organic phosphorus, $\delta^{13}\text{C}_{\text{SOC}} -^{12/13}\text{C}$ isotopic composition of total soil organic carbon) and biological characteristics (MBC – microbial biomass carbon, MBN – microbial biomass nitrogen, and MBP – microbial biomass phosphorus) of litter and organic topsoil horizons of two spruce forest soils that was mixed at five different ratios. The center-aligned values are means of four replicates and right-aligned values in italics are standard deviations of the mean.

Horizon	Soil		pH _(KCl)	MBC	MBN	MBP	DOC	DON	NH ₄ ⁺	NO ₃ ⁻	SRP	DOP	$\delta^{13}\text{C}_{\text{SOC}}$
	Plešné	Čertovo		$\mu\text{mol g (DW)}^{-1}$			nmol g (DW)^{-1}						
Litter	0	1	3.0	482	43.2	12.2	51.6	2.6	5.6	0.3	39.7	36.6	-27.4
			<i>0.2</i>	<i>86</i>	<i>10.6</i>	<i>2.6</i>	<i>9.9</i>	<i>1.0</i>	<i>2.3</i>	<i>0.3</i>	<i>9.1</i>	<i>2.7</i>	
			3.0	551	48.9	14.0	45.9	2.2	4.7	0.2	32.6	39.3	-27.3
	0.25	0.75	<i>0.2</i>	<i>98</i>	<i>11.4</i>	<i>2.5</i>	<i>8.0</i>	<i>0.6</i>	<i>2.4</i>	<i>0.1</i>	<i>8.2</i>	<i>5.9</i>	
			3.1	592	54.3	14.0	55.6	2.6	3.6	0.2	30.3	59.8	-27.3
			<i>0.1</i>	<i>114</i>	<i>12.8</i>	<i>2.7</i>	<i>5.1</i>	<i>0.2</i>	<i>1.6</i>	<i>0.1</i>	<i>4.7</i>	<i>34.2</i>	
	0.5	0.5	3.1	656	56.3	15.2	52.0	2.3	2.4	0.2	27.3	40.2	-27.2
			<i>0.1</i>	<i>83</i>	<i>9.7</i>	<i>2.1</i>	<i>7.9</i>	<i>0.4</i>	<i>0.7</i>	<i>0.1</i>	<i>6.1</i>	<i>8.5</i>	
			3.2	734	63.1	17.1	58.2	2.2	1.9	0.2	36.1	55.3	-27.1
	0.75	0.25	<i>0.1</i>	<i>163</i>	<i>16.1</i>	<i>4.89</i>	<i>11.6</i>	<i>0.5</i>	<i>0.6</i>	<i>0.1</i>	<i>2.5</i>	<i>13.9</i>	
			3.1	734	63.1	17.1	58.2	2.2	1.9	0.2	36.1	55.3	-27.1
			<i>0.1</i>	<i>163</i>	<i>16.1</i>	<i>4.89</i>	<i>11.6</i>	<i>0.5</i>	<i>0.6</i>	<i>0.1</i>	<i>2.5</i>	<i>13.9</i>	
	1	0	2.6	205	17.4	9.3	26.4	1.9	0.6	0.30	28.5	24.6	-26.3
			<i>0.1</i>	<i>51</i>	<i>5.0</i>	<i>2.4</i>	<i>4.0</i>	<i>0.2</i>	<i>0.1</i>	<i>0.14</i>	<i>8.2</i>	<i>14.5</i>	
			2.6	254	19.9	13.5	28.6	1.9	0.5	0.23	28.9	27.1	-26.3
0.25	0.75	<i>0.1</i>	<i>41</i>	<i>4.5</i>	<i>5.9</i>	<i>8.1</i>	<i>0.3</i>	<i>0.2</i>	<i>0.08</i>	<i>8.2</i>	<i>8.1</i>		
		2.7	260	22.6	12.9	27.1	1.8	0.4	0.2	26.7	26.0	-26.2	
		<i>0.1</i>	<i>66</i>	<i>6.1</i>	<i>4.3</i>	<i>11.9</i>	<i>0.5</i>	<i>0.2</i>	<i>0.1</i>	<i>10.2</i>	<i>9.1</i>		
0.5	0.5	2.7	315	27.0	17.1	26.6	1.8	0.4	0.2	32.5	31.9	-26.1	
		<i>0.1</i>	<i>106</i>	<i>8.8</i>	<i>5.2</i>	<i>8.4</i>	<i>0.6</i>	<i>0.2</i>	<i>0.1</i>	<i>11.1</i>	<i>1.4</i>		
		2.7	323	28.4	20.0	28.8	2.0	0.3	0.1	35.8	36.0	-26.1	
0.75	0.25	<i>0.1</i>	<i>83</i>	<i>6.6</i>	<i>8.4</i>	<i>14.1</i>	<i>0.7</i>	<i>0.2</i>	<i>0.1</i>	<i>14.6</i>	<i>7.8</i>		
		2.7	323	28.4	20.0	28.8	2.0	0.3	0.1	35.8	36.0	-26.1	
		<i>0.1</i>	<i>83</i>	<i>6.6</i>	<i>8.4</i>	<i>14.1</i>	<i>0.7</i>	<i>0.2</i>	<i>0.1</i>	<i>14.6</i>	<i>7.8</i>		
1	0	2.6	205	17.4	9.3	26.4	1.9	0.6	0.30	28.5	24.6	-26.3	
		<i>0.1</i>	<i>51</i>	<i>5.0</i>	<i>2.4</i>	<i>4.0</i>	<i>0.2</i>	<i>0.1</i>	<i>0.14</i>	<i>8.2</i>	<i>14.5</i>		
		2.6	254	19.9	13.5	28.6	1.9	0.5	0.23	28.9	27.1	-26.3	
0.25	0.75	<i>0.1</i>	<i>41</i>	<i>4.5</i>	<i>5.9</i>	<i>8.1</i>	<i>0.3</i>	<i>0.2</i>	<i>0.08</i>	<i>8.2</i>	<i>8.1</i>		
		2.7	260	22.6	12.9	27.1	1.8	0.4	0.2	26.7	26.0	-26.2	
		<i>0.1</i>	<i>66</i>	<i>6.1</i>	<i>4.3</i>	<i>11.9</i>	<i>0.5</i>	<i>0.2</i>	<i>0.1</i>	<i>10.2</i>	<i>9.1</i>		
0.5	0.5	2.7	315	27.0	17.1	26.6	1.8	0.4	0.2	32.5	31.9	-26.1	
		<i>0.1</i>	<i>106</i>	<i>8.8</i>	<i>5.2</i>	<i>8.4</i>	<i>0.6</i>	<i>0.2</i>	<i>0.1</i>	<i>11.1</i>	<i>1.4</i>		
		2.7	323	28.4	20.0	28.8	2.0	0.3	0.1	35.8	36.0	-26.1	
0.75	0.25	<i>0.1</i>	<i>83</i>	<i>6.6</i>	<i>8.4</i>	<i>14.1</i>	<i>0.7</i>	<i>0.2</i>	<i>0.1</i>	<i>14.6</i>	<i>7.8</i>		
		2.7	323	28.4	20.0	28.8	2.0	0.3	0.1	35.8	36.0	-26.1	
		<i>0.1</i>	<i>83</i>	<i>6.6</i>	<i>8.4</i>	<i>14.1</i>	<i>0.7</i>	<i>0.2</i>	<i>0.1</i>	<i>14.6</i>	<i>7.8</i>		

time:

$$U = R + G \quad (3)$$

G is proportional to R via the parameter CUE (e.g. Hagerty et al., 2014; Manzoni et al., 2018) and was calculated as:

$$G = R * CUE / (1 - CUE) \quad (4)$$

CUE is an essential variable of the Eq. (1). Its estimation, however, is associated with uncertainty and bias (Geyer et al., 2019). To avoid miscalculation due to error associated with CUE estimation, we estimated CUE using two independent approaches:

- (i) CUE was calculated as the relative amount of ¹³C-labelled glucose incorporated into the MBC over the period of 48 h using the equation:

$$CUE = MBC_{(G)} / (R_{(G)} + MBC_{(G)}) \quad (5)$$

in which $MBC_{(G)}$ and $R_{(G)}$ are respectively defined as the amount of glucose-derived carbon found in MBC and the respired CO₂ over the 48-hour period in samples receiving labeled glucose (Table 1). $MBC_{(G)}$ and $CO_{2(G)}$ were calculated as previously described by Geyer et al. (2019). Using this CUE estimate in Eq. (1) assumes the same CUE for the glucose, decaying microbial biomass and DOC. This estimate is denoted as CUE_{glucose} further in the text (see also Table 1).

- (ii) Adapted microbially-explicit model presented by Allison et al. (2010) was used to estimate CUE . The model consisted of four carbon pools that exchange carbon – DOC representing the directly available source of soil organic carbon, MBC, CO₂, and passive organic carbon (C_{RES} – unavailable to MBC within 48 h). This model was calibrated against DOC, MBC and CO₂ measured in samples not supplemented by glucose using the Differential Evolution algorithm (Mullen et al., 2011) and the CUE , as one model parameter, was estimated. For more details, see the Appendix (section 3. Mass balance). Using this CUE estimate assumes the same CUE for decaying microbial biomass and DOC. This estimate is denoted as CUE_{modelled} further in the text (see also Table 1).

2.5. Statistical analysis

To estimate the effect of the soil horizon (i.e., litter or organic topsoil) and the ratio between PL and CT in the soil mixture on chemical and biological soil characteristics, a generalized linear model with the Gamma distribution was used. The statistical model had a nested design, i.e., the ratio between PL and CT was nested within the soil horizon. The same statistical model with the added factor “time” was used when the temporal changes of chemical and biological soil characteristics were evaluated.

To quantify the effect of microbial biomass recycling on N and P mineralization/immobilization, predictions of Eq. (1) with ($f_{\text{MBC}} > 0$) and without ($f_{\text{MBC}} = 0$) the contribution of decaying microbial biomass consumption were confronted with the observations separately. The correspondence between these two predictions and observations was then compared. The correspondence was calculated as a log likelihood (LL):

$$LL = -n/2 \ln(2\pi\sigma^2) - \sum (o_i - p_i)^2 / 2\sigma^2 \quad (6)$$

In Eq. (6), n is the number of observations, σ is the standard deviation, o_i and p_i represent the observation and corresponding prediction i , respectively. Correspondence between the observations and two predictions of Eq. (1) (i.e., with and without the contribution of decaying microbial biomass consumption) were compared using the log likelihood ratio test. The probability that both predictions correspond with the observations in the same way was drawn from the chi-squared distribution.

The statistical analyses as well as all calculations described above were done in the statistical program R (R Core Team, 2020). The code and data necessary to reproduce our results are available at https://github.com/petacapek/mixing_experiment/manuscript_data_and_code.

3. Results

3.1. Initial conditions

The gradients in chemical and biological characteristics manifested across soil mixtures and horizons are all listed in Table 2 and Appendix

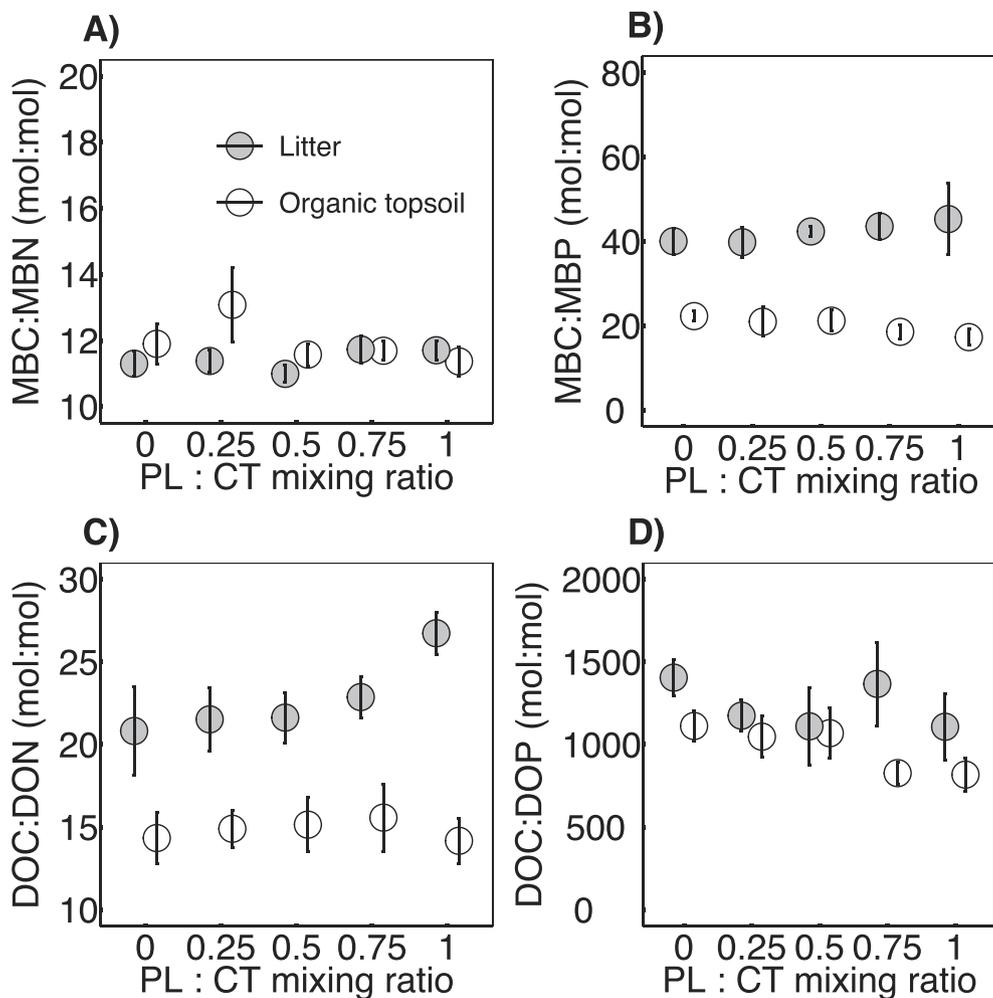


Fig. 1. Initial values of microbial biomass carbon to (A) nitrogen (MBC:MBN) and (B) phosphorus (MBC:MBP) ratios, and water extractable organic carbon to (C) nitrogen (DOC:DON) and (D) phosphorus (DOC:DOP) ratios in two spruce forest soils (PL - Plešné and CT - Čertovo catchments) mixed at five different ratios (i.e., 0:1, 0.25:0.75, 0.5:0.5, 0.75:0.25, and 1:0 in respect to PL). Grey circles denote the litter horizon and empty circles the topsoil organic horizon. Symbols show mean values and error bars standard error of the mean (n = 4). Notice that plots have different y-axes scales.

(Section 4. Initial conditions). Here we highlight the variables relevant to the results calculated by Eq. (1). In general, the litter horizon had a greater amount of MBC ($F_{(1,40)} = 143.6, p < 0.001$), MBN ($F_{(1,40)} = 125.9, p < 0.001$), DOC ($F_{(1,40)} = 61.2, p < 0.001$), DON ($F_{(1,40)} = 10.7, p = 0.002$), DOP ($F_{(1,40)} = 19.1, p < 0.001$), and NH_4^+ ($F_{(1,40)} = 166.7, p$

< 0.001) than the organic topsoil. Soil horizons from the PL catchment had higher concentration of MBC, MBN and MBP, but lower concentrations of NH_4^+ and NO_3^- . Accordingly, MBC ($F_{(1,40)} = 17.4, p < 0.001$), MBN ($F_{(1,40)} = 12.9, p < 0.001$), and MBP ($F_{(1,40)} = 16.0, p < 0.001$) decreased with the decreasing proportion of PL soil in the mixed

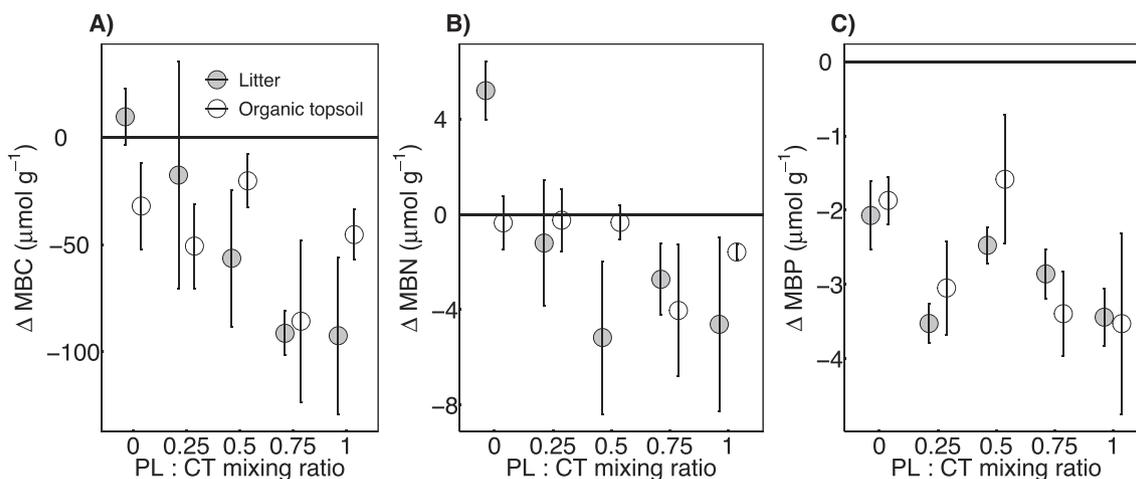


Fig. 2. Net changes of soil microbial biomass (A) carbon (MBC), (B) nitrogen (MBN) and (C) phosphorus (MBP) after 48-hour incubation of two spruce forest soils (PL - Plešné and CT - Čertovo catchments) mixed at five different ratios (i.e., 0:1, 0.25:0.75, 0.5:0.5, 0.75:0.25, and 1:0 in respect to PL). Grey circles denote the litter horizon and empty circles the topsoil organic horizon. Symbols show mean values and error bars standard error of the mean (n = 4). Notice that plots have different y-axes scales.

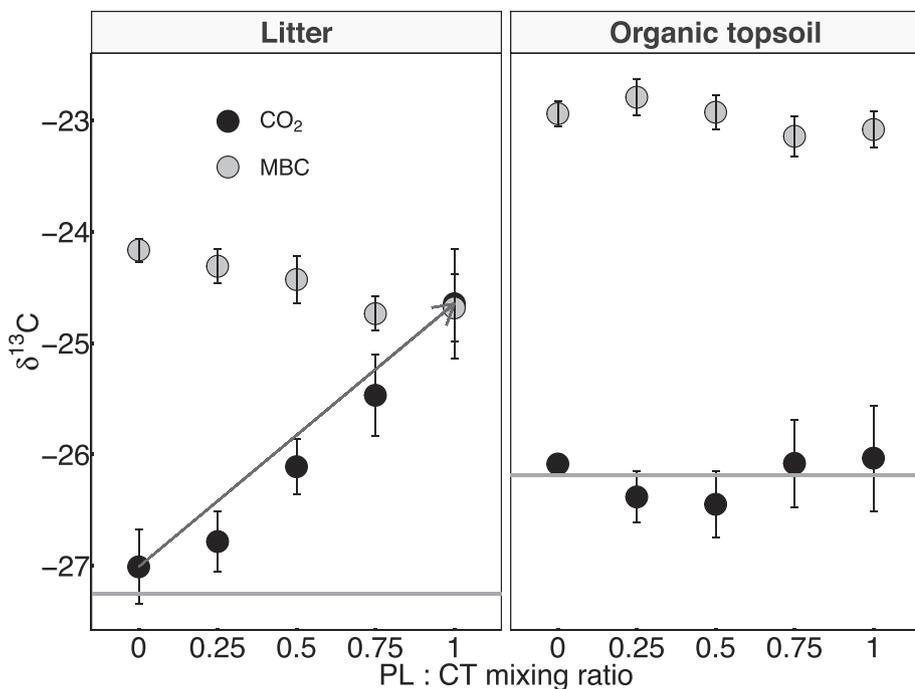


Fig. 3. Isotopic signals of respired CO₂ (black symbols) and microbial biomass carbon (MBC, empty symbols) in the litter and topsoil organic horizons of two spruce forest soils (PL - Plešné and CT - Čertovo catchments) mixed at five different ratios (i.e., 0:1, 0.25:0.75, 0.5:0.5, 0.75:0.25, and 1:0 in respect to PL). Symbols show mean values and error bars standard error of the mean (n = 4). The grey horizontal line represents an approximation of the isotope signal of total soil organic carbon (SOC, see also Tab. 2). The solid arrow shows the change in the isotopic signal of respired CO₂ across litter mixtures that was used to calculate f_{MBC} (see section 2.4. for details).

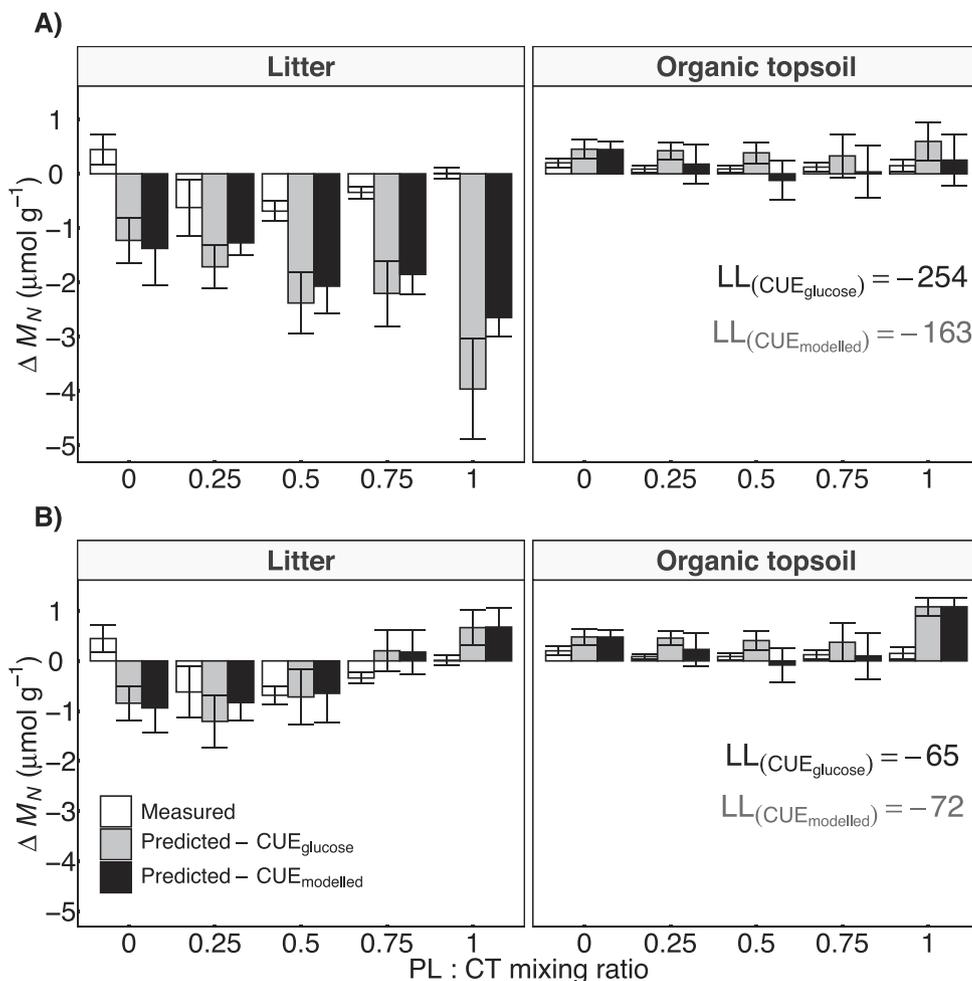


Fig. 4. Net changes of water extractable mineral nitrogen (ΔM_N) in the litter and topsoil organic horizons of two spruce forest soils (PL - Plešné and CT - Čertovo catchments) mixed at five different ratios (i.e., 0:1, 0.25:0.75, 0.5:0.5, 0.75:0.25, and 1:0 in respect to PL). White bars represent measured ΔM_N , black and grey bars represent ΔM_N calculated using Eq. (1) with CUE estimated using isotopic approach (denoted as $CUE_{glucose}$) or mass balance approach (denoted as $CUE_{modelled}$), respectively. ΔM_N was calculated with (A) and without (B) f_{MBC} (see section 2.4. for details). Error bars represent standard error of the mean (n = 4). Correspondence between the predictions and observations are reported as log likelihood (LL, Eq. (6)). The higher the LL (less negative), the better the correspondence.

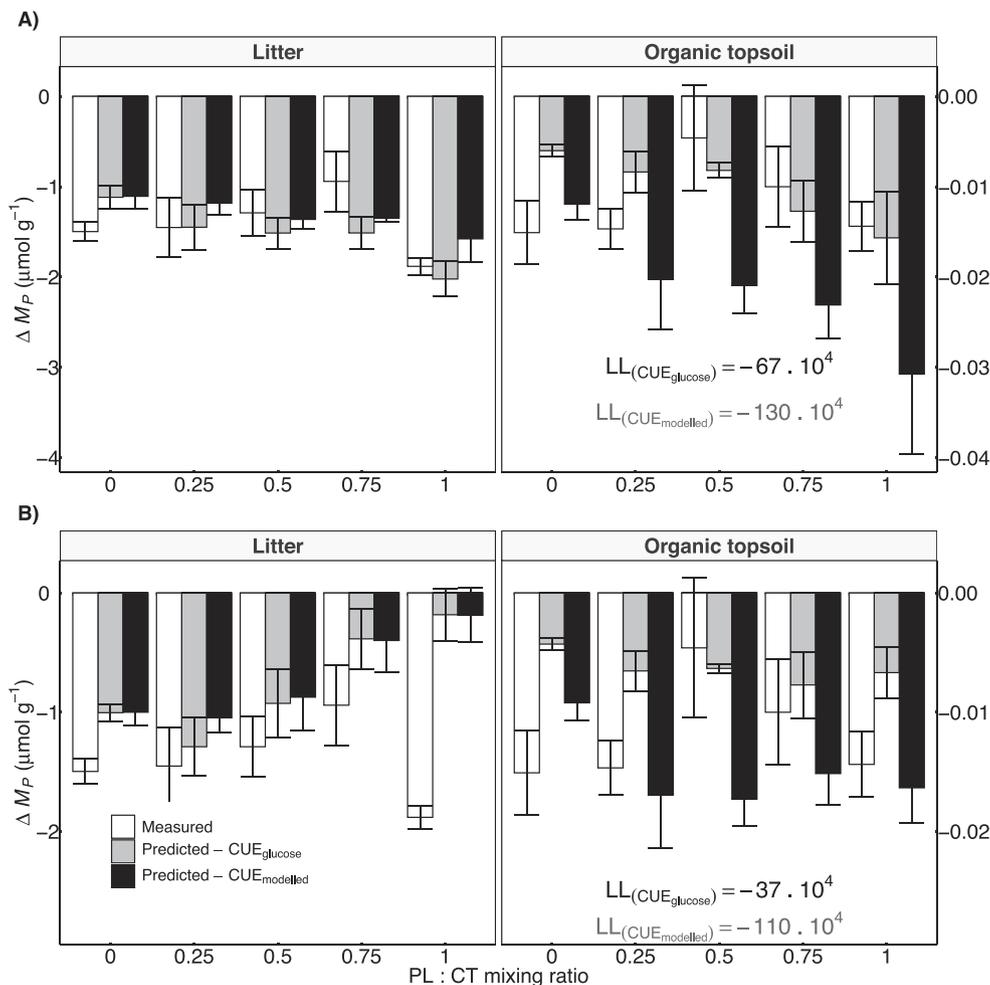


Fig. 5. Net changes of water extractable soluble reactive phosphorus (ΔM_P) in the litter and topsoil organic horizons of two spruce forest soils (PL - Plešné and CT - Čertovo catchments) mixed at five different ratios (i. e., 0:1, 0.25:0.75, 0.5:0.5, 0.75:0.25, and 1:0 in respect to PL). White bars represent measured ΔM_P , black and grey bars represent ΔM_P calculated using Eq. (1) with CUE estimated using isotopic approach (denoted as $CUE_{glucose}$) or mass balance approach (denoted as $CUE_{modelled}$), respectively. ΔM_P was calculated with (A) and without (B) f_{MBC} (see section 2.4. for details). Error bars represent standard error of the mean ($n = 4$). Correspondences between the predictions and observations are reported as log likelihood (LL, Eq. (7)). The higher the LL (less negative), the better the correspondence. Note the different scales of left and right y-axis. The scale of left y-axis is related to predicted ΔM_P , whereas the scale of right y-axis is related to observed ΔM_P .

samples, while NH_4^+ ($F_{(1,40)} = 20.6$, $p < 0.001$) and NO_3^- ($F_{(1,40)} = 14.4$, $p < 0.001$) increased in the same direction (Table 2).

In respect to stoichiometric predictors of N and P mineralization/immobilization (see Eq. (1)), there was no difference in MBC:MBN ratios between soil horizons or across soil mixtures. However, the MBC:MBP ratio was significantly higher in the litter compared to the organic topsoil ($F_{(1,40)} = 132.62$, $p < 0.001$; Fig. 1). The opposite trend was found in stoichiometry of water extractable organic compounds. Whereas the DOC:DON ratio was significantly lower in the organic topsoil as compared to the litter horizon ($F_{(1,40)} = 56.7$, $p < 0.001$), there was no difference in DOC:DOP ratio between horizons or across soil mixtures (Fig. 1). The DOC:DON ratio further tended to increase with the increasing proportion of the PL soil in litter mixtures. This increase was, however, only marginally significant ($F_{(1,40)} = 3.1$, $p = 0.086$; Fig. 1).

3.2. Net changes over time

3.2.1. Microbial biomass and organic substrate utilization

Generally, the MBC ($F_{(1,40)} = 5.3$, $p = 0.025$) and MBP ($F_{(1,40)} = 11.4$, $p = 0.001$) decreased during the incubation (Fig. 2). Estimated CUEs of the soil microbial community ranged between ~ 0.6 and 0.8 and depended on the approach of estimation. Whereas the $CUE_{glucose}$ differed between the litter and organic topsoil horizons (~ 0.8 and ~ 0.6 , respectively), the $CUE_{modelled}$ was almost constant (~ 0.8) in all samples (see Appendix, Fig. A6).

The isotope analysis of the samples not supplemented by ^{13}C labeled glucose displayed a significant trend of increasing $\delta^{13}C_R$, with the

increasing proportion of the PL soil in the litter mixture ($F_{(1,20)} = 27.1$, $p < 0.001$; Fig. 3). Whereas the $\delta^{13}C$ of CO_2 respired from the CT litter was on average 3‰ lower than the $\delta^{13}C$ of MBC and almost similar to $\delta^{13}C$ of SOC, the $\delta^{13}C$ of CO_2 respired from the PL litter matched the $\delta^{13}C$ of MBC (Fig. 3). As a result, the parameter f_{MBC} (Eqs. (1) and (2)) changed almost linearly across the litter mixtures. In contrast, $\delta^{13}C_R$ was similar to $\delta^{13}C$ of SOC and approximately 3‰ lower compared to $\delta^{13}C$ of MBC in all mixtures of organic topsoil (Fig. 3).

3.2.2. Mineral nitrogen

There was no significant change of dissolved mineral N during the incubation (Fig. 4). The measured ΔM_N was $< 1 \mu mol g^{-1}$ on average. Without the contribution of decaying microbial biomass consumption (i. e., $f_{MBC} = 0$, Fig. 4A), Eq. (1) predicted that up to five $\mu mol g^{-1}$ of mineral N should be immobilized to the microbial biomass in the PL litter (Fig. 4A). This result was primarily given by the almost 10-times lower MBC:MBN compared to DOC:DON ratios, and the high cumulative CO_2 (Fig. A7), and $CUE_{glucose}$ and $CUE_{modelled}$ (Fig. A6), resulting in values of organic substrate consumption (U) of approximately $60 \mu mol (C) g^{-1}$ independently of the CUE estimation approach. The difference between MBC:MBN and DOC:DON ratios were lower in the CT litter, and thus, Eq. (1) without the contribution of decaying microbial biomass consumption predicted a more than two times lower amount of immobilized N compared to the PL litter (Fig. 4A).

With the contribution of decaying microbial biomass consumption (i. e., $f_{MBC} > 0$, Fig. 4B), the amount of immobilized mineral N predicted for the PL litter by Eq. (1) was lower than without this contribution. The predicted values corresponded with the observations significantly better

irrespective of the CUE estimation approach (likelihood ratio test for $CUE_{\text{glucose}} = 372$, $p < 0.001$; and for $CUE_{\text{modelled}} = 165$, $p < 0.001$; Fig. 4B). There was, however, still some disagreement between measured and predicted ΔM_N (Fig. 4B). Low net N mineralization was predicted for the PL litter and low N immobilization for the CT litter. These predictions did not entirely correspond with the measured data (Fig. 4B).

Compared to the litter horizon, the difference between the MBC:MBN and DOC:DON ratios was negligible in the organic topsoil horizon, and thus, Eq. (1) predicted small net N mineralization. Net N mineralization was indeed observed, but it was lower than the predicted values (Fig. 4B).

3.2.3. Mineral phosphorus

The SRP concentration significantly and consistently decreased during incubation in both horizons ($F_{(1,20)} = 62.4$, $p < 0.001$; Fig. 5). The difference between MBC:MBP and DOC:DOP ratios was substantial (>1000) in both horizons (Fig. 1). Because of this difference, Eq. (1) without the contribution of decaying microbial biomass consumption (i. e., $f_{MBC} = 0$) predicted that high amounts of SRP should be immobilized by the microbial biomass (Fig. 5A). However, the measured decrease of SRP in the soil solution was two orders of magnitude lower. When the contribution of decaying microbial biomass consumption was acknowledged ($f_{MBC} > 0$; Fig. 5B), the correspondence between predictions of Eq. (1) and observations significantly improved (likelihood ratio test for $CUE_{\text{glucose}} = 58 \cdot 10^4$, $p < 0.001$; and for $CUE_{\text{modelled}} = 40 \cdot 10^4$, $p < 0.001$). However, the predicted P immobilization was still much higher than the measured data (Fig. 5B).

4. Discussion

The difference between the stoichiometry of organic substrate and microbial demand predicts the N and P mineralization and immobilization rates well across large gradients of initial plant litter stoichiometry and over long time scales (Manzoni et al., 2010, 2008). At the finer scale, represented here by two soil horizons of two mountain forest catchments, individually mixed in five different ratios, the predictive capability was low. It implies that the stoichiometric concept needs to be generalized carefully as the effect of other factors on nutrients mineralization/immobilization can become dominant at certain conditions. Our analysis highlighted the importance of microbial biomass recycling. When it was taken into account, it helped to better explain the temporal changes in mineral N concentration in soil solution across the litter mixtures than the stoichiometric relationship itself. The temporal changes in P concentrations were, however, not explained by the same mechanism as in case of N, suggesting that some additional P pools were used by microbial community for growth.

4.1. Microbial biomass recycling

4.1.1. Quantification

Gross growth of microbial biomass is frequently measured in soil using various methods (Chen et al., 2019; Demoling et al., 2007; Spohn et al., 2016; Spohn and Widdig, 2017). The extent, to which the gross growth is realized from an instant recycling of the decaying part of microbial biomass, is considered significant (Chapman and Gray, 1986; Van Veen et al., 1987) and therefore, it is directly acknowledged in some microbially-explicit soil biogeochemical models (Chapman and Gray, 1986; Hagerty et al., 2014). It is, however, difficult to quantify directly. The quantification typically employs indirect mathematical analysis upon substrate addition (Chapman and Gray, 1986). Here we used a direct isotopic analysis approach. According to Šantrůčková et al. (2000), MBC is typically $\sim 2\%$ ^{13}C -enriched as compared to SOC. Thus, $^{12/13}\text{C}$ isotopic composition of respired CO_2 can determine whether the SOC or MBC is being metabolized by the active part of microbial community. The steep increase in $\delta^{13}\text{C}_R$ with the increasing proportion of PL

soil in the litter mixture and the remarkable similarity between $\delta^{13}\text{C}_R$ and $\delta^{13}\text{C}_{MBC}$ in the PL litter suggested that the CO_2 originated from decaying microbial biomass when the litter mixture became dominated by the PL soil (Fig. 3). It is unlikely that the difference in isotope discrimination during respiration between PL and CT litters was responsible for this pattern. Isotope discrimination has been shown to mostly depend on the incubation temperature (Lehmeier et al., 2016), which was identical for all soil samples. Moreover, we found no indication of different isotopic discrimination for the PL and CT organic horizons.

We are, however, aware that the calculated f_{MBC} was not absolutely accurate. This can be seen in the temporal changes of DOC concentrations. Even though the calculated f_{MBC} was close to one in the PL litter, DOC decrease was observed during incubation. This decrease suggests that the decaying microbial biomass was not an entire source of organic substrate for active part of microbial community (Fig. A8), but also some DOC was consumed by microbial biomass. This inaccuracy could have caused some deviations in the predicted ΔM_N from observations in the CT and PL litter horizons (Fig. 4A), but cannot undermine the effect of microbial biomass recycling.

4.1.2. Reaction to nitrogen limitation

In agreement with the observed differences in the litterfall C to N ratios between the PL and CT catchments (Fig. A1A), the DOC:DON ratio increased with the increasing proportion of PL soil in the litter mixture. Given the same MBC:MBN ratios (Fig. 1A) across all litter mixtures and high CUE, organic N was in greater shortage as the mixture became dominated by the PL litter. Consequently, the microbial biomass would have needed to consume a significant amount of mineral N to cope with the stoichiometric imbalance (Fig. 4A). Concentrations of mineral N forms were, however, low in the PL litter already at the beginning of incubation (Table 2, Fig. A5D, C), so the concentrations of organic as well as mineral N forms were limiting. Facing this limitation, the active part of microbial community used N derived from the decaying microbial biomass (Figs. 2 and 4B). In contrast, NO_3^- and NH_4^+ concentrations were significantly higher in the CT litter (Table 2, Fig. A5D, C). Therefore, the microbial community needed to recycle less microbial biomass N in the mixed samples dominated by the CT litter. In all mixtures of organic soil horizons, DOC:DON ratios were very close to MBC:MBN suggesting that the microbial biomass received sufficient amount of organic N and therefore, similarly as in the CT litter, did not need to rely on microbial biomass N recycling (Fig. 3).

It could be argued that our conclusions are partly biased by inaccurate CUE estimates as DOC is typically utilized with lower efficiency than glucose. Even though the value of 0.8 estimated for glucose in the litter horizon (Fig. S5A) is very close to the theoretical maximum (Geyer et al., 2019; Heijnen, 1991; Manzoni et al., 2017), similarly high CUE values were estimated by mass balance approach, which was independent of substrate addition. Assuming lower CUE values would not improve predictions of temporal changes in MBC, DOC and CO_2 . Moreover, the lower CUE values would worsen the correspondence between the measured and predicted ΔM_N , regardless the parameter f_{MBC} (data not shown).

4.2. Phosphorus

The measured molar DOC:DOP ratios of > 1000 reflected the high C:P ratios of litterfall in both catchments (Fig. A1B). Such a great stoichiometric imbalance between the organic substrate and microbial biomass (Fig. 1B, D) should have theoretically led to high SRP immobilization (Fig. 5A, B). However, the observed ΔM_P values were two orders of magnitude lower. In contrast to results of N, as well as to some other studies (Spohn and Widdig, 2017; Chen et al., 2019), we did not find an indication that the microbial biomass recycling was able to offset the observed stoichiometric imbalance (Fig. 5B). Based on our previous

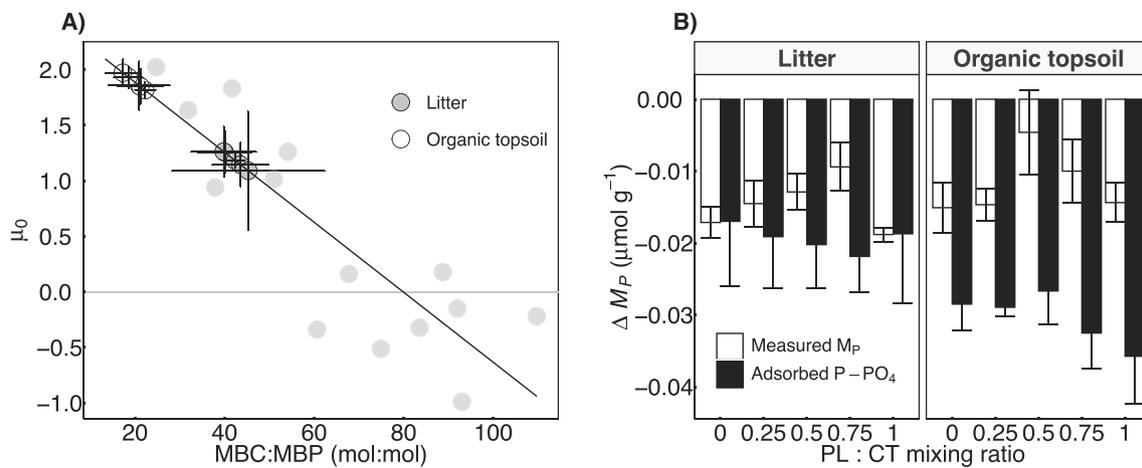


Fig. 6. A) Expected growth rates of the soil microbial community in the litter (grey circles) and organic topsoil (empty circles) horizons without an external source of phosphorus (i.e., phosphorus demand is covered exclusively from internal resources). Symbols show mean values and error bars standard error of the mean ($n = 4$). The expected growth rate (solid black line) was calculated from measured MBC:MBP ratios using a previously derived relationship between MBC:MBP ratios and growth rate in the absence of external sources of phosphorus (light grey points; Čapek et al., 2016). The horizontal solid line denotes a zero growth rate. B) Net changes of water extractable soluble reactive phosphorus (ΔM_P) in the litter and topsoil organic horizons of two spruce forest soils (PL - Plešné and CT - Čertovo catchments) mixed at five different ratios (i.e., 0:1, 0.25:0.75, 0.5:0.5, 0.75:0.25, and 1:0 in respect to PL). White bars represent measured ΔM_P , black bars represent theoretical amount of chemically adsorbed SRP. Error bars represent standard error of the mean ($n = 4$).

results (Fig. 6; Čapek et al., 2016), we argue that the most plausible explanation for the difference between measured and calculated ΔM_P values is that a significant part of the P used by active part of microbial community did not originate from the non-microbial part of the soil (i.e., SRP and DOP) but from internal microbial P storage compounds. The low MBC:MBP ratio measured in all samples suggests that the active part of microbial community was able to grow the whole 48-hour incubation period without external P (Fig. 6A). In such case, the chemical adsorption, and not the microbial consumption, had to be responsible for the observed decrease of SRP concentration over time (Fig. 6B). The relative recovery of the K_2HPO_4 spike measured during MBP quantification can be used to estimate theoretical amount of adsorbed SRP using the equation:

$$P_X = \text{SRP}_0 \{1 - \exp[-(1 - \text{Recovery}) 48/0.75]\} \quad (7)$$

in which P_X is the amount of adsorbed SRP, SRP_0 is the SRP concentration at the beginning of the incubation, and *Recovery* is the relative proportion of the recovered K_2HPO_4 spike (Table 1). The calculated theoretical amount of chemically adsorbed SRP was closely similar to the measured decrease of SRP during the incubation (Fig. 6B), which supports our conclusion. It remains unclear, however, under what conditions the P storage compounds can accumulate in the soil microbial biomass. This question warrants further investigation.

5. Conclusions

Stoichiometry of organic substrate controls the way, the soil microorganisms use organic and mineral forms of nutrients. However, the predictive accuracy of stoichiometric approach is affected by the capabilities of soil microorganisms to deal with the nutrient limitation. For this reason, nutrient recycling and use of storage compounds should be acknowledged to improve the predictive accuracy of stoichiometry-based models at the local scales.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.geoderma.2020.114884>.

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