



Exploring polyphosphates in soil: presence, extractability, and contribution to microbial biomass phosphorus

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Abstract

Polyphosphates (Poly-P) are known to fulfil several important physiological functions. Many microorganisms can accumulate large amounts of Poly-P in their biomass. Regardless of these facts, systematic research on Poly-P in soil is missing, probably due to the absence of any method of direct Poly-P quantification. In this study, we attempted to unequivocally prove the presence of Poly-P in the biomass of soil microorganisms and quantify their extractability and contribution to microbial biomass phosphorus. To do so, we combined several approaches that can indicate Poly-P presence in soil microbial biomass indirectly, i.e. growth of soil inoculum on media without phosphorus, associated with measurement of changes in the microbial biomass stoichiometry, and the colour of the microbial suspension stained by the Neisser method. All soil microbial communities exhibited growth on media without phosphorus. As the growth on this media depleted Poly-P content, the biomass carbon to phosphorus and nitrogen to phosphorus ratio increased and the colour of the microbial suspension stained by the Neisser method changed predictively. The associated Poly-P addition experiment indicated that the recovery of added Poly-P from soil in form of soluble reactive phosphorus in sodium bicarbonate extract may reach up to 93% mainly due to abiotic depolymerization. Using a simple stoichiometric model applied to measured data, we calculated that the Poly-P content of microbial biomass in our soils may be up to 45 or 70% of total microbial biomass phosphorus depending on the assumptions applied regarding parameter values. We discuss the magnitude of error associated with the measurement of soil microbial phosphorus due to the high extractability of Poly-P.

Keywords Polyphosphates · Soil microbial biomass phosphorus · Fumigation-extraction method · Ecological stoichiometry · Conversion factors

Introduction

The term polyphosphates (Poly-P) refers to a group of compounds, which contain several or many phosphate groups linked together by high energy anhydride bonds (Kulaev et al. 1999). In respect to their chemical properties, these compounds can be linear or branched, may contain various amounts of phosphate groups, and bind various cations due

to their negative charge (Dawes and Senior 1973). They are found in various organisms (Dawes and Senior 1973; Kulaev et al. 1999; Mason-Jones et al. 2021) and are considered “multifunctional”. Their chemical properties make them ideal candidates for fulfilling several important functions in the organisms such as energy and phosphorus (P) storage, homeostatic and osmotic regulation, membrane transport (Kulaev et al. 1999) or promoting the survival under various stress conditions (Mullan et al. 2002; Perdril et al. 2008; Seufferheld et al. 2008; Nikel et al. 2013). Some organisms can accumulate significant amounts of Poly-P in their biomass, a feature, which is commonly used in biotechnology of wastewater treatment (e.g. Egli and Zehnder 1994).

Given the apparent significance of Poly-P in living organisms, it is surprising that systematic research on their presence and function in community of soil microorganisms is scarce (Mason-Jones et al. 2021). So far, Poly-P has been directly identified and studied in few mycorrhizal fungal

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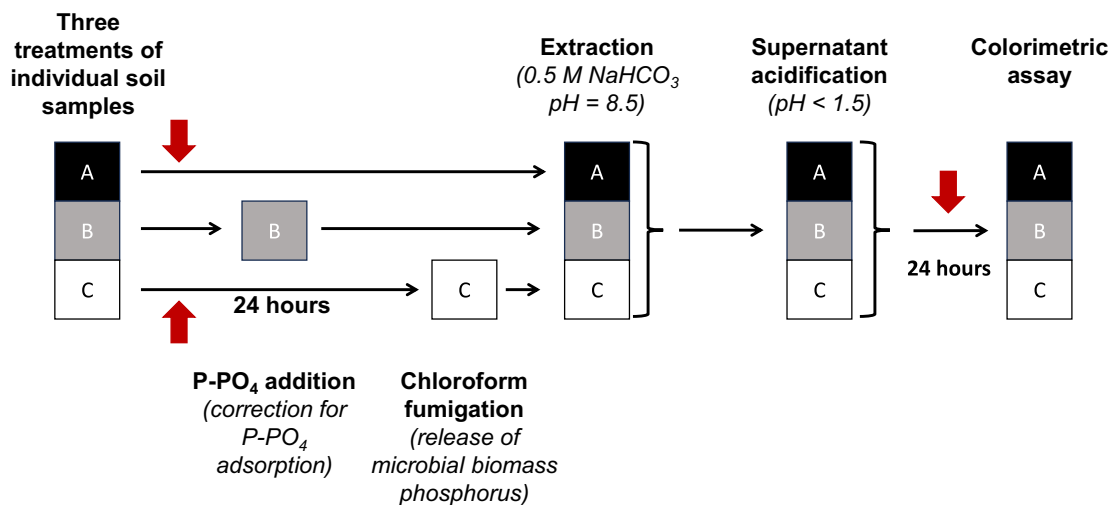
isolates (Frey et al. 1997; Bünemann et al. 2008) and likely they are probably involved in exchange of P between mycorrhizal fungi and plants (Nguyen and Saito 2021). However, indirect evidence suggest that presence of Poly-P can be widespread among all soil microorganisms, not only mycorrhizal fungi (Cheesman et al. 2014; Čapek et al. 2016; Mason-Jones et al. 2021). Cheesman et al. (2014) found a tight relationship between soil microbial biomass P and Poly-P content of alkaline extract of air-dried soils analysed by ^{31}P NMR spectroscopy. When inoculum of soil microorganisms from geographically distant soil samples is supplemented with growth medium without P, a positive growth rate of that community is often observed (Čapek et al. 2016). Moreover, the magnitude of the growth rate scales inversely with carbon (C) to P ratio of microbial biomass, suggesting that the source of P for growth is intracellular (Čapek et al. 2016). Recently, the short-term laboratory incubation data indirectly suggested that net P mineralization and immobilization in soil cannot be predicted with reasonable accuracy without explicit consideration of Poly-P presence and utilization (Čapek et al. 2021). Theoretical implications of Poly-P presence on physiology of soil microorganisms has been explored by Manzoni et al. (2021). This theoretical analysis suggests significant impact of Poly-P presence on microbially-mediated fluxes of P, but also C and nitrogen (N) under P-limiting conditions. Poly-P presence in soil may thus, have ecologically relevant implications for nutrient cycling that await experimental verification.

Systematic research on the presence and function of Poly-P in soil is missing at the moment. One of the possible reasons is that Poly-P cannot be directly visualized and measured in soil (unlike aquatic ecosystems or pure cultures) due to possible interference with soil matrix and compounds located there. The readily available (even though rough) indicator of Poly-P presence in soil is stoichiometry of soil microbial biomass (i.e. its C to N to P ratio), which is frequently measured in various ecosystems. It is because the basic cellular macromolecules containing P, such as DNA, ATP, RNA or phospholipids, as well as their stoichiometry are known (Elser et al. 1996; Vrede et al. 2004). Stoichiometry of microbial biomass is defined by relative abundance of these macromolecules within the biomass. C:P and N:P ratios of microbial biomass can vary within certain boundaries, which are given by maximum possible abundances of the macromolecules within cells (e.g. RNA cannot make 80% of cell biomass). Surpassing these boundaries is possible only by accumulation of storage compounds like Poly-P. One of the P-richest macromolecules that significantly contributes to entire mass of microbial cells are nucleic acids with molar C to N to P ratio 10:3.7:1 and 33:3.3:1 for RNA and DNA, respectively (Vrede et al. 2004). RNA and DNA content changes with growth rate reaching up to 30% and

1.5% of total cell dry mass, respectively (Herbert 1961; Hanegraaf and Muller 2001; Makino et al. 2003; Čapek et al. 2023). Assuming that phospholipids, carbohydrates and proteins contribute to the rest of the cellular mass in relative amounts 1%, 7% and 60.5%, respectively (Herbert 1961; Hanegraaf and Muller 2001; Čapek et al. 2023), C:N:P of microbial cells should be around 40:11.8:1. Slow growing cells with lower RNA content around 15% of cell mass (Hanegraaf and Muller 2001; Makino et al. 2003) should then have a C:N:P around 72:20.6:1. Applying these constraints to the global mean C:N:P of soil microbial biomass 42:6:1 (Xu et al. 2015) either means that most soil microbial communities grow at the rate close to their physiological maximum, which is rather unlikely (Blagodatskaya and Kuzyakov 2013), or contain Poly-P.

Using microbial biomass stoichiometry as an indicator of Poly-P presence requires the chloroform-fumigation extraction method to be sensitive to changes in Poly-P content. This method determines the soluble reactive P in the CHCl_3 -labile pool as a proxy-parameter for microbial biomass P (MBP, Fig. 1). Because soluble reactive P represents only a fraction of MBP that has been produced by hydrolysis of organic P of microbial origin, conversion factor (denoted as k_{ep}), typically 0.4 is applied (Brookes et al. 1982), although deviations from this value are known (Qiu et al. 2007; Bilyera et al. 2018). Thus, to be detectable by the method, at least some fraction of Poly-P must depolymerize into soluble reactive P. To unambiguously link Poly-P presence to shifts in microbial biomass stoichiometry, this fraction should further be invariant, and, ideally, close to 40% (i.e. equivalent to $k_{ep} = 0.4$). However, there is no a priori reason for why the recovery of Poly-P in the form of soluble reactive P should be 40%. Due to the chemical stability of Poly-P (McCullough et al. 1956), a lower recovery rate can be expected. If the recovery is different from the recovery of other P-containing macromolecules, k_{ep} would be a function of Poly-P content, and using microbial biomass stoichiometry as an indicator of Poly-P presence becomes increasingly difficult.

The aim of this study is to provide evidence for Poly-P presence in microbial biomass of soils from catchments of Plešné and Čertovo lakes (Bohemian forest, Czech Republic), for which the indirect evidence exists (Čapek et al. 2016, 2021). We further aim to quantify the potential extractability of Poly-P from these soils during the standard chloroform-fumigation extraction method (Brookes et al. 1982), determine its mechanism, and estimate the associated error rate. We hypothesize that tested soils host microbial communities accumulating Poly-P and that the presence of Poly-P decreases the overall extractability of microbial biomass P due to low depolymerization of Poly-P to soluble reactive P.



$$MBP(\text{microbial biomass phosphorus}) = \frac{C - A}{\ddagger k_{ep} \times r} \quad r(\text{recovery of added } P - PO_4) = \frac{B - A}{P - PO_4}$$

[‡]correction factor (correction for unextractable fraction of MBP ~ 0.4)

Fig. 1 Schematic representation of standard chloroform-fumigation extraction method for determining soil microbial biomass P (MBP). Each individual soil sample is divided into three subsamples denoted as A, B and C in the scheme. Subsample A determines background soluble reactive P concentration. Subsamples B and C are used to estimate P-PO₄ adsorption to soil particles and CHCl₃-labile soluble reactive P originating from biomass of soil microorganisms, respec-

tively. Two equations reported in the scheme define calculations of P-PO₄ recovery and MBP. Correction factor defining the relative amount of MBP, which can be extracted following chloroform-fumigation, is used in MBP calculation (typically 0.4, i.e. 40% is extractable). Red arrows denote the points at which Poly-P was added in order to estimate Poly-P recovery (see the section “[Recovery of polyphosphates from soil](#)” details)

Material and Methods

Soil samples

Soils from catchments of two glacial lakes (Plešné and Čertovo) located in the Bohemian Forest (Czech Republic) were used in this study. These soils were selected because the presence of Poly-P was indirectly suggested there in two previous studies (Čapek et al. 2016, 2021).

Soil sampling was performed in June 2022. Samples of diagnostic soil horizons denoted hereafter as a litter layer (Ol + Of, i.e., top ~ 5 cm containing fragmented but visible plant litter remnants) and organic horizon (H, i.e., between ~ 5 and 15 cm depth containing amorphous organic material) were collected at three different locations within each catchment. The locations encompass the variability in vegetation cover (of unmanaged spruce forest ecosystem) and nutrient availability recently affected by bark beetle outbreak (Choma et al. 2023, see Fig. S1 and Table 1). According to results of Luo et al. (2023), the variability in tree cover affects the variability in species composition of respective soil microbial communities. The same effect is visible in selected catchments (Choma et al. 2021)(Figs. S2 and S3). In all locations, composite sample of each diagnostic horizon

was created by mixing three subsamples collected from three different soil pits. Composite samples were immediately homogenized by sieving through 5 mm mesh and stored at 4 °C until the start of experiments. Together, 12 different soil samples (2 catchments × 3 locations × 2 horizons) were collected. In all experiments, three laboratory replicates of each sample were used (i.e. 36 sub-samples were analysed). Basic soil characteristics are listed in Table 1.

Presence of polyphosphates in biomass of soil microorganisms

Presence of Poly-P was determined in an experiment that intended to manipulate, i.e. deplete, Poly-P content. It is assumed that Poly-P are depolymerized and used for new microbial biomass construction at the conditions of excessive supply of organic C and organic/inorganic N. Such an assumption is unequivocally met in most pure culture studies (Dawes and Senior 1973) and has been suggested as an explanation for positive growth rate of soil microbial communities under conditions of zero external P supply (Čapek et al. 2016 2021).

Table 1 Basic characteristics of litter and organic topsoil horizons from six different localities (under different vegetation cover) within Plešné and Čertovo catchments: $\text{pH}_{(\text{H}_2\text{O})}$, total organic C (C_T), total N (N_T), total P (P_T), water extractable organic C (DOC) and waterextractable total N (DN), soluble reactive P in 0.5 M NaHCO_3 extract (SRP), microbial biomass C (MBC), N (MBN), and P (MBP). The center-aligned values are means of three replicates and right-aligned values in italics are standard deviations of the mean

Catchment	Vegetation cover	Horizon	$\text{pH}_{(\text{H}_2\text{O})}$	C_T	N_T	P_T	DOC	DN	SRP	MBC	MBN	MBP
				mmol g^{-1}	mmol g^{-1}	$\mu\text{mol g}^{-1}$						
Plešné	Young closed canopy	Litter Layer	4.00	39.8	1.33	32.8	87.5	30.8	1.6	477	58.1	25.5
			<i>0.16</i>	<i>0.65</i>	<i>0.05</i>	<i>4.49</i>	<i>1.43</i>	<i>0.58</i>	<i>0.16</i>	<i>26.31</i>	<i>3.18</i>	<i>0.80</i>
	Organic Horizon		3.47	37.6	1.33	26.8	49.2	28.1	0.5	313	35.9	20.7
			<i>0.07</i>	<i>2.39</i>	<i>0.21</i>	<i>7.05</i>	<i>0.92</i>	<i>0.66</i>	<i>0.06</i>	<i>13.76</i>	<i>2.29</i>	<i>0.28</i>
	Dense tree regeneration	Litter Layer	4.65	39.4	1.40	32.7	46.4	16.3	0.2	261	29.5	13.8
			<i>0.16</i>	<i>0.64</i>	<i>0.08</i>	<i>1.49</i>	<i>1.12</i>	<i>0.37</i>	<i>0.04</i>	<i>21.23</i>	<i>2.63</i>	<i>0.49</i>
Organic Horizon		3.76	36.3	1.50	27.7	45.6	5.9	0.0	140	15.5	8.0	
		<i>0.19</i>	<i>0.25</i>	<i>0.08</i>	<i>1.28</i>	<i>0.99</i>	<i>0.02</i>	<i>0.02</i>	<i>8.21</i>	<i>1.05</i>	<i>0.72</i>	
Open canopy	Litter Layer		4.22	38.5	1.60	28.1	63.4	26.9	0.4	435	49.9	19.6
			<i>0.16</i>	<i>0.92</i>	<i>0.08</i>	<i>5.53</i>	<i>1.15</i>	<i>0.53</i>	<i>0.03</i>	<i>22.53</i>	<i>2.14</i>	<i>0.24</i>
	Organic Horizon		3.62	31.0	1.37	47.6	26.4	6.3	0.1	176	22.0	11.2
			<i>0.02</i>	<i>2.38</i>	<i>0.05</i>	<i>4.44</i>	<i>0.42</i>	<i>0.08</i>	<i>0.02</i>	<i>7.49</i>	<i>1.32</i>	<i>0.53</i>
Čertovo	After tree dieback	Litter Layer	4.32	38.8	1.50	35.3	60.1	13.8	0.6	378	46.1	13.4
			<i>0.17</i>	<i>0.21</i>	<i>0.00</i>	<i>2.62</i>	<i>0.84</i>	<i>0.14</i>	<i>0.04</i>	<i>5.88</i>	<i>0.91</i>	<i>1.09</i>
	Organic Horizon		3.71	28.7	1.13	28.3	41.1	8.8	0.3	260	29.7	14.4
			<i>0.14</i>	<i>5.44</i>	<i>0.17</i>	<i>3.46</i>	<i>0.52</i>	<i>0.19</i>	<i>0.07</i>	<i>13.17</i>	<i>0.97</i>	<i>0.44</i>
	Mature closed canopy	Litter Layer	4.17	40.3	1.40	30.3	90.9	23.7	1.2	492	58.5	20.2
			<i>0.13</i>	<i>0.45</i>	<i>0.00</i>	<i>3.02</i>	<i>1.73</i>	<i>0.25</i>	<i>0.02</i>	<i>27.91</i>	<i>3.87</i>	<i>1.34</i>
	Organic Horizon		3.34	35.3	1.33	25.9	29.5	5.1	0.4	211	26.0	12.1
			<i>0.08</i>	<i>1.15</i>	<i>0.09</i>	<i>0.62</i>	<i>0.97</i>	<i>0.33</i>	<i>0.03</i>	<i>13.83</i>	<i>2.07</i>	<i>1.75</i>
	Dense tree regeneration	Litter Layer	4.01	35.7	1.47	34.3	66.9	35.0	2.4	337	46.9	23.3
			<i>0.15</i>	<i>1.51</i>	<i>0.12</i>	<i>6.09</i>	<i>1.22</i>	<i>1.24</i>	<i>0.21</i>	<i>20.99</i>	<i>1.77</i>	<i>1.47</i>
	Organic Horizon		3.73	24.1	1.07	29.4	35.6	6.6	0.6	175	21.5	11.0
			<i>0.09</i>	<i>2.44</i>	<i>0.17</i>	<i>5.85</i>	<i>1.39</i>	<i>0.30</i>	<i>0.03</i>	<i>4.72</i>	<i>0.37</i>	<i>0.55</i>

The growth rate experiment was performed as described in Čapek et al. (2016). Briefly, soil samples, hereafter referred to as “soil inoculum”, containing 70 μmol of microbial biomass C (Tab. S1) were mixed with sterile C-free sand in 1:25 (w/w) ratio. The mixture was moistened to 75% of water holding capacity by supplying basal medium designed by Veldkamp (1970) but without P. The chemical composition of medium was normalized to a unit of microbial biomass C (MBC) so that the 20 mol of organic C per one mol of MBC was added (Čapek et al. 2016). Samples were incubated for one week in the dark at 20 °C. Oxygen consumption was measured continuously during the incubation using Oxitop system (WTW Wissenschaftlich-Technische Werkstätten, Weilheim, Germany). Growth rate of the microbial community was calculated as a slope of increase of oxygen consumption over time (see the section “Statistical analyses”). At the beginning and end of incubation, CHCl_3 -labile C, N and P, microbial biomass N (MBN) and P (MBP) were analysed, and

slurries of samples were stained by Neisser method (see the section “Analytical methods”).

Recovery of polyphosphates from soil

In previous studies (Čapek et al. 2016, 2021), the Poly-P were assumed to be detectable by the chloroform fumigation-extraction method, which is commonly employed to determine MBP as a soluble reactive P in CHCl_3 -labile pool following 24 h fumigation of soil by chloroform vapours (Brookes et al. 1982). Since Poly-P is polymeric substance, it requires depolymerization into orthophosphates to become detectable by colorimetric assay of soluble reactive P as described in Murphy and Riley (1962). Here we aimed to test if the Poly-P depolymerization is large enough to significantly increase soluble reactive P concentrations. We also seek to identify the conditions at which the depolymerization occurs.

To accomplish both aims, chloroform fumigation-extraction method was performed as described in Brookes et al.

(1982). The method consists of several steps, which are graphically presented in Fig. 1. Every individual soil sample is exposed to three treatments – (i) immediate extraction of P by 0.5M NaHCO₃ with pH adjusted to 8.5 (see the section “Analytical methods” for details) to determine baseline extractable soluble reactive P concentration (Fig. 1, treatment A), (ii) extraction with P-PO₄ spiked 0.5M NaHCO₃ that accounts for any possible adsorption of P released from soil microbial biomass (Fig. 1, treatment B) during the extraction step (immediate adsorption of released P during 24 h chloroform fumigation is considered negligible; Brookes et al. 1982), and (iii) extraction after 24 h fumigation of soil by amylene-stabilized chloroform, which releases content of microbial cells (Fig. 1, treatment C). Each soil extract is subsequently centrifuged and acidified by 4.5M H₂SO₄. Acidified soil extracts are left to react for 24 h. Then, the extract is filtered, and soluble reactive P concentration measured. The term soluble “reactive” P is used to indicate that certain fraction of organic P can be hydrolysed to P-PO₄ in acidic conditions that are required for development of molybdate blue colour.

Poly-P can be theoretically depolymerized to some extent in each step of chloroform fumigation-extraction method. Two mechanisms of depolymerization are possible – biotic (i.e. enzymatic) and abiotic (i.e. without presence of enzymes). Abiotic depolymerization can occur in soil during the extraction, and in acidified soil extract. The low pH of acidified soil extracts prevents any biotic depolymerization (i.e. because all enzyme activities are close to zero under such conditions) and magnifies rate of abiotic depolymerization (McCullough et al. 1956). Biotic depolymerization may occur directly in the soil and/or during chloroform fumigation step, which exposes Poly-P as well as variety of enzymes that conduct Poly-P depolymerization within the cells. To distinguish between biotic and abiotic Poly-P depolymerization, spike of Poly-P solution was added either directly to soil, which is immediately extracted, to soil before chloroform-fumigation or to acidified soil extracts obtained before and after chloroform-fumigation (Fig. 1). Poly-P was added to the soil as Graham’s salt (sodium polyphosphate, Merck Millipore EMPLURA®, product no. 1.06529). The solution was prepared so that the amount of added P in form of Poly-P was close to 15 µmol per gram of dry soil (approximate amount of CHCl₃-labile P, Tab. 1). The solution was added in volume 0.5 ml to three grams of fresh soil (~ 1.15 g of dry weight). Applying the chemical formula of Graham’s salt (NaPO₃)_n, the stock solution of Poly-P with a concentration of 3.5 g L⁻¹ was prepared. To fully dissolve Graham’s salt, ultrasonication was applied and the potential depolymerization caused by ultrasonication controlled for (approx. 0.02% of total P was converted to P-PO₄). The equivalent amount of Poly-P was added into acidified soil extract, because the Poly-P depolymerization

can be concentration dependent (McCullough et al. 1956). To do so, the stock solution of Poly-P was diluted by factor 4/9. During the extraction step, three grams of fresh soil is extracted with 45 mL of 0.5 M NaHCO₃ (see the section “Analytical methods” for details). After centrifugation (3000 g), 20 mL aliquot (4/9 of the original volume) is then transferred to a clean beaker and acidified. Therefore, the addition of 0.5 ml of Poly-P stock solution diluted by factor 4/9 prior to acidification of soil extract makes the amount of added P in form of Poly-P to the soil and the soil extract equal. This approach allows us to directly compare Poly-P recoveries from soil and acidified extracts. The recovery of Poly-P as soluble reactive P (r_{PP} , in %) was calculated for soil and acidified soil extract according to equation:

$$r_{PP} = \frac{SRP_{PP} - SRP}{PP_{add} \times r} \times 100, \quad (1)$$

in which PP_{add} is the amount of added P in form of Poly-P (in µmol (P) g (DW)⁻¹), SRP_{PP} and SRP are amounts of soluble reactive P (in µmol (P) g (DW)⁻¹) extracted from soil with and without Poly-P addition, respectively. r is recovery of P-PO₄ spike (Fig. 1, treatment B). Poly-P recovery from acidified soil extract was calculated setting r equal to 1 because of the absence of soil particles that P can adsorb to.

Analytical methods

The basic characteristics of soil samples reported in Table 1 were measured for the first time before the start of experiments described in previous sections. Sub-samples of collected soils were dried (at 60 °C to a constant weight) and finely ground (using a Mixer Mill MM 200, Retsch, Germany) in order to determine total organic C (C_T), total N (N_T) and P (P_T). C_T and N_T were analysed using NC 2100 soil analyzer (Thermo Quest Italia S.p.A., Rodano, MI). P_T was determined by HNO₃ and HClO₄ digestion according to Kopáček et al. (2001). All other analyses were performed with field-moist (i.e. fresh) soil samples. Soil pH was measured in 1:2.5 (w/v) soil–water slurry using portable pH meter 330i (Xylem Analytics, Weilheim, Germany). To analyse dissolved organic C (DOC) and dissolved N (DN), 4 g of fresh soil samples were shaken with 40 mL of distilled water for 1 h at laboratory temperature in the dark. Extracts were centrifuged (3000 g) and filtered through 0.45µm glass fiber filters (Watrex, Prague, Czech Republic). DN and DOC in water extracts were measured with a TOC/TN analyzer (LiquicTOC II, Elementar, Germany). To analyse soluble reactive P, 3 g of fresh soil samples were shaken with 45 mL of 0.5 M NaHCO₃ for 1 h at laboratory temperature in the dark. As described above, extracts were centrifuged (3000 g), and aliquot was acidified by H₂SO₄ to reach pH below 1.5. Acidified extract was filtered through paper filter after

24 h and soluble reactive P was determined according to Murphy and Riley (1962).

In addition to MBP (Fig. 1), MBC and MBN were determined by chloroform-fumigation extraction method at the same time (Brookes et al. 1985; Vance et al. 1987). The MBC and MBN were calculated as the difference in concentrations of organic C and total N, respectively, measured in 0.5 M K_2SO_4 soil extract of fumigated and unfumigated soil samples. MBC, MBN and MBP data are reported without correction for incomplete recovery of microbial biomass, i.e. correction factors k_{ec} , k_{en} and k_{ep} , in the result section only, not in Table 1. To avoid confusion, uncorrected MBC, MBN and MBP estimates are hereafter denoted as $CHCl_3$ -labile C, N and P. Their relationship to true MBC, MBN and MBP values and associated errors are discussed later in the discussion section (see section “Implications of polyphosphates presence”).

Poly-P staining by the Neisser method, a direct method for visualizing the presence of Poly-P in the biomass of soil microorganisms, was performed according to Nielsen et al. (2016). This technique was used as a method independent of two indirect approaches of Poly-P identification in a growth rate experiment (see section “Presence of polyphosphates in biomass of soil microorganisms”) – progress curves of oxygen consumption and microbial biomass stoichiometry. To verify the degree of convergence of all three approaches, Neisser staining was first performed with all soil samples at the beginning of incubation with growth media without P, and then with soil inoculum added to sterile sand after one week of incubation. Because the incubation was expected to deplete any Poly-P reserves in microbial biomass, the convergence of the methods is reached if Neisser staining indicates presence of Poly-P before incubation and their absence at the end of incubation. Diluting soil inoculum with sand, however, makes the comparison challenging. To allow reasonable comparison, the number of visible cells in the slurries of soil samples and the soil inoculum in the sand must be similar. Therefore, soil slurry of samples before the incubation was prepared by 30 s ultrasonication (10 μ m amplitude; Ramsay 1984) of 50 ml 0.8% NaCl with 2 g of fresh soil. After the incubation, 10 g of soil inoculum in sand was mixed with 50 mL 0.8% NaCl. The appropriate volume of NaCl and the amount of soil/soil inoculum-sand mixture was determined in the trial experiments. The intensity of staining was visualised using Olympus BX61 (Olympus Corporation, Shinjuku, Japan) applying 100 \times magnification. The microscope pictures were obtained using Canon DS126571 (Canon, Ota, Japan).

Statistical analyses

Growth rate of soil microbial community following addition of growth media without P was estimated from progress

curves of O_2 consumption normalized to unit of initial MBC over time (Fig. S4) using R package growthrates (version 0.8.4; Petzoldt 2022). The estimation was performed using linear regression on log-transformed data, which searched for the exponential part of the progress curve and then, estimated the slope of increase.

In general, the effect of different treatments (i.e. effect of incubation in growth experiment, effect of different steps of fumigation-incubation method on Poly-P recovery), catchments, soil horizons, or their combinations on the variables measured in this study was determined using generalized linear models with gamma distribution. The analysis was performed by stepwise forward selection thus, only the significant categorical predictors were included in a regression model and their effect quantified. To determine differences among levels of a categorical predictor(s), pairwise comparison using Tukey’s honestly significant difference test was conducted in R package emmeans (version 1.8.9; Lenth 2023). In the incubation experiment, we further specifically tested the significance of the change in $CHCl_3$ -labile C, N and P due to growth on media without P. To do so, we tested whether the observed difference of $CHCl_3$ -labile C, N and P at the beginning and at the end of incubation is statistically different from zero using Student’s t-test. In addition to the effects of different treatments, catchments, soil horizons or their combinations, we further tested the significance of the negative linear relationship between the initial C to P or N to P ratio in the $CHCl_3$ -labile pool (i.e. calculated for the samples before mixing with sterile sand and growth media, see section “Presence of polyphosphates in biomass of soil microorganisms”) and the growth rate of soil microbial community without external P (Čapek et al. 2016).

The change in the C to P and N to P ratio of the $CHCl_3$ -labile pool due to growth on media without P was confronted with the change in the colour of bacterial cells stained by the Neisser method as acquired by the microscope. Pictures of stained microbial cells representative to each soil sample before and after incubation were selected and the respective data extracted using R package magick (version 2.7.4; Ooms 2023). R, G and B channels data extracted from the pictures were collapsed into vectors using unconstrained principal components analysis (PCA) applied onto dissimilarity data matrix (across R, G and B channels data of all pictures) calculated using Bray–Curtis dissimilarity index. The PCA constructs axis of the greatest variability in the multidimensional data space. The position of soil samples along the first PCA axis was correlated with C to P or N to P ratio of $CHCl_3$ -labile pool before and after the incubation on media without P. All analyses were performed in the statistical program R version 4.3.0 (R Core Team 2023). All data as well as code to all analyses performed within this study are publicly available at <https://github.com/petacapek/Polyphosphates>.

Results

Presence of polyphosphates in biomass of soil microorganisms

Progress curves of O₂ consumption over time are shown for all 36 samples in Figure S4. All curves exhibited exponential increase of O₂ consumption after approximately 24 h of incubation following addition of growth media without P. Growth rate was thus estimated to be higher than zero in all samples. Magnitude of the growth rate was significantly affected only by soil horizon ($F_{(1,34)}=31.4$, $p < 0.001$, Fig. 2). Even though the trend of decreasing growth rate with increasing initial C to P (Fig. 2A) and N to P ratio (Fig. 2B) of CHCl₃-labile pool occurred, the relationship was not statistically significant.

The initial C to P and N to P ratios in CHCl₃-labile pool were low in all samples, below 30 (mol/mol) and 4 (mol/mol), respectively. There was no statistically significant difference in these ratios between catchments,

localities within catchments or soil horizons. Both ratios, however, significantly increased ($F_{(1,70)}=208.6$, $p < 0.001$ for CHCl₃-labile C to P ratio; and $F_{(1,70)}=66.5$, $p < 0.001$ N to P ratio) towards the end of incubation with growth on media without P (Fig. 3). The increase in respective ratios was caused by significant increase in CHCl₃-labile C ($t = 14.5$, $df = 35$, $p < 0.001$) and N ($t = 7.87$, $df = 35$, $p < 0.001$). Change in CHCl₃-labile P was not statistically significant from zero ($t = 1.11$, $df = 35$, $p = 0.27$). Similarly to initial conditions (i.e. before incubation), there was no significant difference between catchments, localities within catchments or soil horizons in respective ratios. It means that the net change in CHCl₃-labile C and N, and thus C to P and N to P ratios of CHCl₃-labile pool was independent of soil sample identity.

Observed trend of increasing C to P and N to P ratios of CHCl₃-labile pool due to incubation was partially reflected in change of the colour of bacteria in suspensions stained by Neisser method. Figure 4 shows an example of four different samples – litter layer and organic horizons from localities within Plešné and Čertovo catchment.

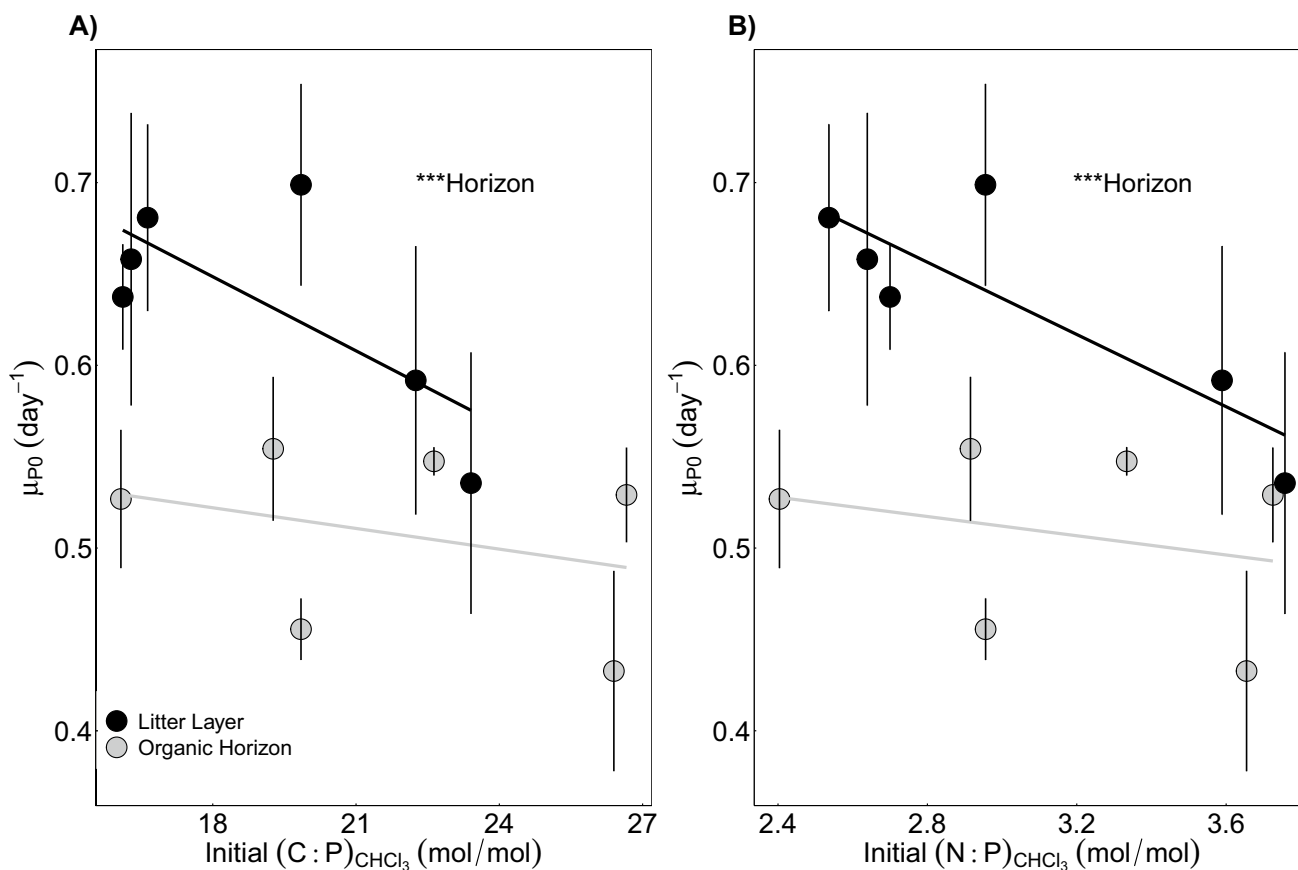


Fig. 2 Growth rate of soil inoculum on media without P (μ_{p0}) plotted against initial C to P ($(C:P)_{\text{CHCl}_3}$; panel A) and N to P ($(N:P)_{\text{CHCl}_3}$; panel B) ratios of CHCl₃-labile pool. Filled and empty circles represent means ($n=3$) calculated for samples of litter layer and organic

horizon, respectively. Error bars represent standard errors of the means. Solid lines represent linear trends fitted to the data. The only significant factor affecting the variability in μ_{p0} was soil horizon

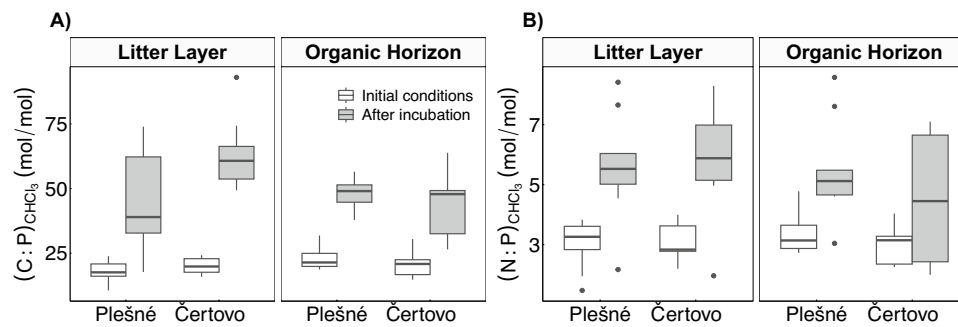


Fig. 3 C to P ($(C : P)_{CHCl_3}$; panel A) and N to P ($(N : P)_{CHCl_3}$; panel B) ratio of $CHCl_3$ -labile pool measured in soil inoculum before incubation on media without P (white bars) and after one week incubation (grey boxes). Each panel (A and B) is divided into two facets representing two soil horizons, litter layer and organic horizon. The lower

and upper hinge of the box represents 25% and 75% quantile, respectively. Horizontal line in the box represents median. Lower and upper whiskers extend lower and upper quartiles by 1.5 times the interquartile range. Black dots represent outliers extending above or below the whiskers

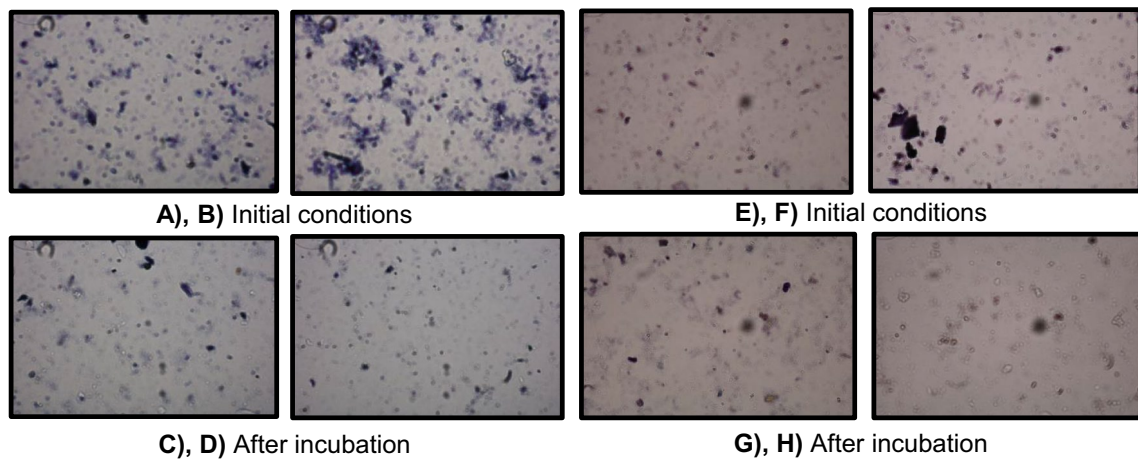


Fig. 4 Slurries stained by Neisser method. Representative samples of litter layer and organic horizons from Plešné and Čertovo catchment. The first row of picture (A, B, E and F) shows slurries of soil inocu-

lum before the incubation on media without P. The second row of picture (C, D, G and H) shows slurries after one week of incubation on media without P

The pictures of all samples are shown in supplementary Fig. S3. In most of the samples, cells in suspension of soil inoculum before incubation appeared purple as expected if the cells contain Poly-P (Fig. 4A, B, E, F). The purple colour was usually reduced (Fig. 4C, D) or disappeared completely (Fig. 4G, H) when suspension of samples after incubation were stained and the colour of most cells appear brown or colourless suggesting absence of Poly-P. When data of pictures were analysed as vectors by principal component analysis (PCA), the position of samples along the first PCA axis correlated with C to P ratio of $CHCl_3$ -labile pool (Fig. 5), especially in organic horizon. There was, however, one plot – mature closed canopy in Čertovo catchment (Fig. S5), which had to be removed for statistical analyses to be significant. The correlation between first PCA axis and N to P ratio of $CHCl_3$ -labile pool was not statistically significant.

Recovery of polyphosphates from soil

The recovery of Poly-P added to soil and acidified soil extract was high in all samples, usually exceeding 60% (Fig. 6) and the highest value was reached when Poly-P was added to soil samples, which were then exposed to chloroform vapours for 24 h. In this treatment, the recovery was found significantly higher in organic horizon (93%) as compared to litter layer (84%). Recoveries of Poly-P added to soil samples, which were immediately extracted, or to acidified soil extracts of fumigated and unfumigated soil samples were similar. In these treatments, there was also no difference among different soil horizons, catchments or localities within catchments.

Fig. 5 Relationship between C to P ((C : P)_{CHCl₃}; panel A) and N to P ((N : P)_{CHCl₃}; panel B) ratio of CHCl₃-labile pool and position of respective samples along first PCA axis constructed from vectors of microscope pictures after Neisser staining (see Figs. 4 and S3). White and grey circles represent microbial biomass stoichiometry before and after incubation on media without P. Solid black line represents regression line. The black dots denote one outlier plot – locality with mature closed canopy in Čertovo catchment. Each panel (A and B) is divided into two facets representing two soil horizons, litter layer and organic horizon

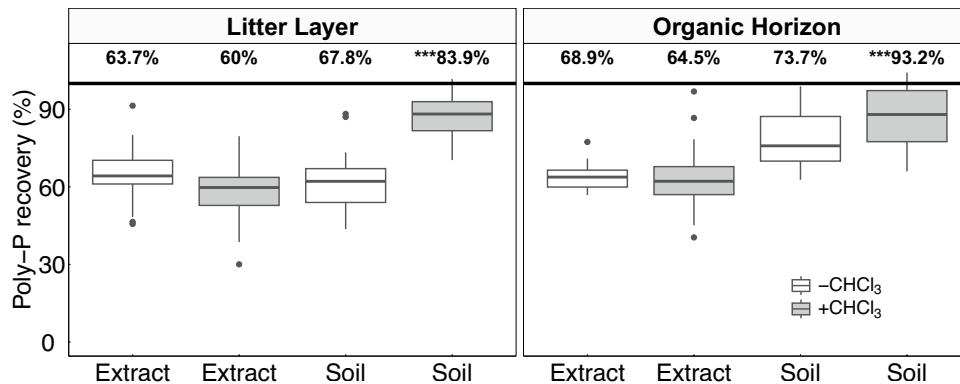
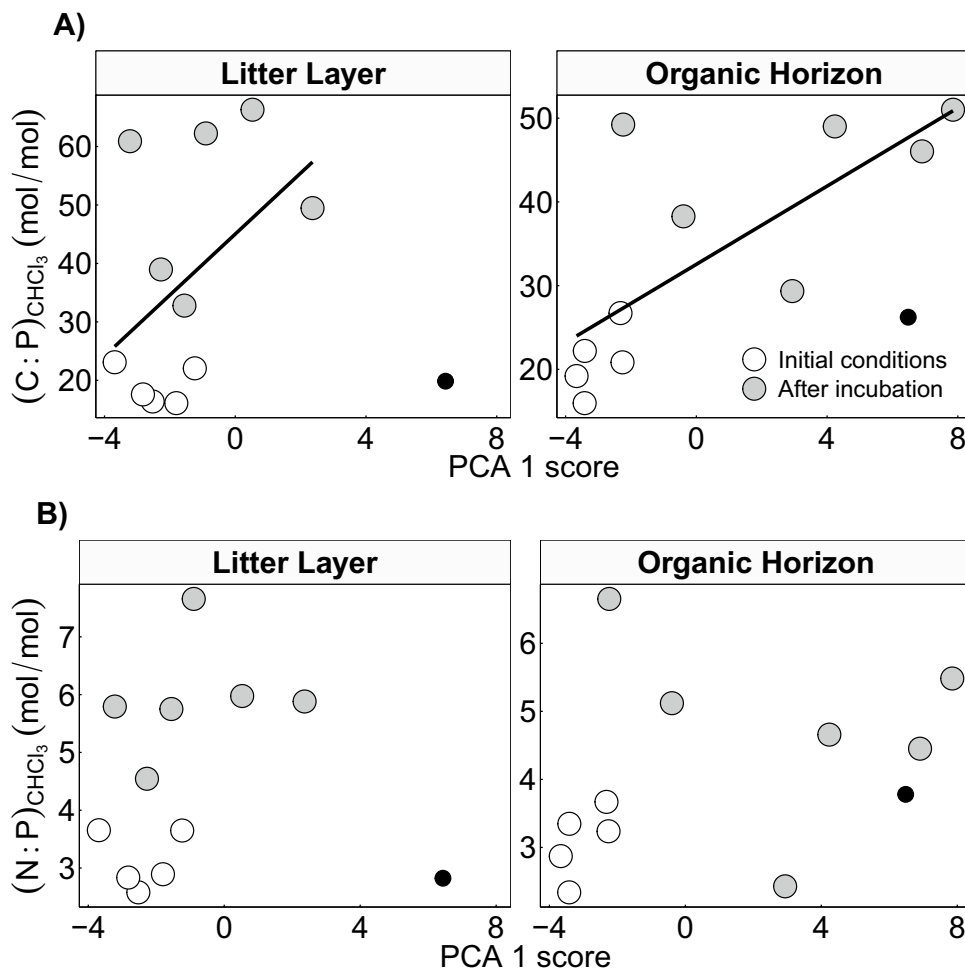


Fig. 6 Recovery of polyphosphates from soil as soluble reactive P. The recoveries from soil and acidified soil extract of fumigated (grey boxes) or unfumigated (white boxes) soil samples are shown. The lower and upper hinge of the box represent 25% and 75% quantile, respectively. Horizontal line in the box represents median. Lower and upper whiskers extend lower and upper quartiles by 1.5 times the interquartile range. Black dots represent outliers extending above

or below the whiskers. The solid horizontal line denotes complete recovery (100%). The mean recovery is reported above the boxes. The value followed by symbol “***” represents the value significantly different from all the others within the designated soil horizon. Figure is divided into two facets representing two soil horizons, litter layer and organic horizon

Discussion

Presence of polyphosphates in biomass of soil microorganisms

Although Poly-P have been identified in soil extracts applying ^{31}P NMR spectroscopy (Cheesman et al. 2014; Lang et al. 2017), to the best of our knowledge, there is no method to quantify the Poly-P content of soil microbial biomass directly. Therefore, we designed an experiment that combines several approaches, which have a potential to indicate Poly-P presence indirectly, i.e. growth on media without P, and associated measurement of net changes in microbial biomass stoichiometry, and colour of microbial suspension stained by Neisser method. As we discuss below, the results of all three methods converge and all together, they suggest the presence of Poly-P in the microbial biomass in all soil samples from different localities, under different vegetation covers within Plešné and Čertovo catchments.

In previous study, the Poly-P presence was inferred from the ability of soil inoculum to exhibit growth on media without P and the fact that the growth rate scaled inversely with microbial biomass C to P ratio (Čapek et al. 2016). In this study, soil inoculum from all samples exhibited significant growth too but there was no relationship to microbial biomass C to P or N to P ratio (Fig. 2) expressed as a stoichiometry of CHCl_3 -labile pool (biomass uncorrected for incomplete recovery). The absence of significant relationship could be caused by low variability in the initial biomass stoichiometry, i.e. with interquartile range 17–22 and 3–4 for C to P and N to P ratio of CHCl_3 -labile pool, respectively. There are, however, other two indices suggesting that the growth on medium without P was achieved due to Poly-P utilization:

- i) C to P and N to P ratio of CHCl_3 -labile pool significantly increased towards the end of incubation on media without P (Fig. 3). The increase was uniform, i.e. there were no differences among soil samples post incubation, and the increase was achieved due to significant increase of both CHCl_3 -labile C and N. CHCl_3 -labile P remained unchanged. Even though microbial storage of elements in excess could theoretically cause the increase in CHCl_3 -labile C, it cannot cause parallel increase in CHCl_3 -labile N because N storage compounds are rare (Mason-Jones et al. 2021). Moreover, storage of C in excess, cannot by itself cause an exponential increase of oxygen consumption (Manzoni et al. 2021). This can be caused only by the increase of the number of actively metabolizing microbial cells, i.e. microbial growth. Thus, the observed patterns leave us with the most rea-

sonable explanation that certain part of CHCl_3 -labile P at the beginning of the incubation was composed of Poly-P, which were utilized during incubation with excess of external C and N to produce new microbial biomass. As we discuss below (see section “[Recovery of polyphosphates from soil](#)”), Poly-P are principally detectable in the CHCl_3 -labile P pool.

- ii) Neisser staining of microbial cells in suspension after incubation of soil inoculum on media without P was weak or even completely negative (Figs. 4 and S5), again suggesting Poly-P utilization. Moreover, the greatest variability among microscope pictures expressed in a first principal component seems to be associated with the change in microbial stoichiometry (Fig. 5). It must be noted, however, that this relationship was not absolutely straightforward (Fig. 5B). The pictures suggest some interference of Neisser staining with soil matrix, which could cause the absence of a statistically significant relationship between PCA1 and microbial biomass stoichiometry across all localities. In several samples, we observed smears of purple colour, which were seemingly not bacterial conglomerates. We also tried alternative staining, i.e. DAPI method (data not shown) (Li and Dittrich 2019), but the interference of fluorescence with fine sand particles was too excessive to allow identification of microbial cells.

Interestingly, we found significant difference in growth rate exhibited by soil inoculum on media without P among soil horizons regardless of similar microbial biomass stoichiometry (Fig. 2). This result suggests that microbial communities of different horizons have either different capacity to grow or to utilize Poly-P, or they have different initial Poly-P content regardless the same initial stoichiometry. The difference in growth rates among horizons most likely results from combination of different specific growth rates of microbial communities, which typically decreases with increasing sampling depth (Spohn et al. 2016), and different initial Poly-P contents (see section “[Implications of polyphosphates presence](#)”).

Recovery of polyphosphates from soil

In contrast to our hypothesis, recovery of Poly-P from soil as soluble reactive P in sodium bicarbonate extract was high (Fig. 6). Graham’s salt solution used in this recovery test contained only approx. 0.02% of total P in form of soluble reactive P. However, once the stock solution was added to soil or acidified sodium bicarbonate extract, the Poly-P recovery in form of soluble reactive P increased dramatically. The recoveries of Poly-P from acidified soil extracts as well as from the soil before fumigation were uniformly around 65% (Fig. 6), which allows us to conclude that

abiotic depolymerization is primarily responsible for Poly-P recovery during the soil unexposed to chloroform vapours. Higher recovery, reaching up to 93%, was observed when Poly-P was added to soil, which was then treated by chloroform vapours (Fig. 6). This approx. 19% and 28% increase of recovery rate in litter and organic horizon, respectively, can be attributed to biotic (enzymatic) depolymerization during 24 h of fumigation. The difference in biotic depolymerization rate between horizons is probably caused by different amounts/activity of enzymes responsible for Poly-P depolymerization, which are released from microbial communities by chloroform treatment. It must be, however, stressed that these results apply only to Graham's salt, which is a linear Poly-P (McCullough et al. 1956). It is possible that recovery of branched Poly-P can be slightly different. It is also possible that the length of Poly-P chain(s) can affect the recovery rate.

Implications of polyphosphates presence

Our results suggest that the presence of Poly-P in the microbial biomass increases the extractability of MBP. Typical conversion factor (usually denoted as k_{ep}) between CHCl_3 -labile P and MBP 0.4 (Brookes et al. 1982) could thus, not apply when Poly-P are present. The magnitude of change of k_{ep} due to Poly-P presence theoretically depends on the contribution of Poly-P to MBP and can be expressed by the following equation:

$$k_{ep} = r_{basal} \times n_{basal} + r_{Poly-P} \times n_{Poly-P}, \quad (2)$$

in which n_{basal} and n_{Poly-P} represent relative contributions of microbial P, that is not Poly-P (all macromolecules containing P), and Poly-P to MBP, respectively. n_{basal} and n_{Poly-P} sum up to 1 because they represent two different pools of total MBP. r_{basal} and r_{Poly-P} then represent respective recoveries of the two MBP pools. Without explicit knowledge of n_{Poly-P} for soil microbial communities, the magnitude of k_{ep} underestimation cannot be determined. In natural cyanobacterial cultures (Li and Dittrich 2019) and different bacteria in activated sludge (Mino et al. 1985; Hiraishi and Morishima 1990), n_{Poly-P} varies widely between ~6% and 95%. Within such a range and assuming r_{basal} and r_{Poly-P} being 0.4 (Brookes et al. 1982) and 0.93 (representative to organic horizon, Fig. 6), k_{ep} is expected to vary within 0.43 and 0.90, respectively.

Studies of Bilyera et al. (2018) and Qui et al. (2007) suggest that k_{ep} could be either lower or higher than 0.4 in different soils. Bilyera et al. (2018) provide an empirical equation for k_{ep} calculation. In this equation, k_{ep} decreases with increasing total soil organic C and total P. Applying the reported equation to our organic rich and acidic soils, negative k_{ep} is obtained. Data presented by Bilyera et al. (2018),

however, suggest that the k_{ep} also increases with increasing soil acidity, the most acidic soils having k_{ep} around a value of 0.7 (Fig. S6). Such a trend can be theoretically explained by increasing Poly-P content of the soil microbial biomass along the gradient of increasing acidity as a reaction to increasing mobility of toxic Al^{3+} . Poly-P has been shown to increase with increasing Al^{3+} concentration (Gerlitz 1996), which is a function of soil pH. The Poly-P presence in our acidic soils could thus, be caused by the acidity (Table 1).

Since the Poly-P affect the stoichiometry of CHCl_3 -labile pool (Fig. 3), n_{Poly-P} can be theoretically estimated from the stoichiometry of CHCl_3 -labile pool. The C to P ratio of CHCl_3 -labile pool (i.e. $(C:P)_{\text{CHCl}_3}$) is a function of MBC to MBP ratio, and the ratio of respective conversion factors k_{ec} and k_{ep} :

$$(C:P)_{\text{CHCl}_3} = \frac{\text{MBC}}{\text{MBP}} \times \frac{k_{ec}}{k_{ep}}. \quad (3)$$

Under the assumption of constant microbial growth rate and absence or constant concentration of C storage compounds, $(C:P)_{\text{CHCl}_3}$ changes only due to changing Poly-P content. Because Poly-P is not bound to C, it is convenient to define C to P ratio of basal microbial biomass— $(C:P)_{\text{MB}}$, which can be used to calculate microbial biomass P, that does not belong to Poly-P, from the measured CHCl_3 -labile C and respective k_{ec} . A general equation for n_{Poly-P} estimation can then be defined as:

$$n_{Poly-P} = \frac{\frac{(C:P)_{\text{MB}}}{(C:P)_{\text{CHCl}_3}} \times k_{ec} - r_{basal}}{\frac{(C:P)_{\text{MB}}}{(C:P)_{\text{CHCl}_3}} \times k_{ec} - r_{basal} + r_{Poly-P}}. \quad (4)$$

Equation 4 shows that four different variables have to be known to calculate n_{Poly-P} — k_{ec} , $(C:P)_{\text{MB}}$, r_{basal} and r_{Poly-P} . All of these parameters are associated with some uncertainties. The most commonly used k_{ec} is 0.45 (Wu et al. 1990; Joergensen 1996). Other studies, however, suggest that k_{ec} can differ from that value due to changing soil conditions (Dictor et al. 1998), microbial community composition (Eberhardt et al. 1996; Meyer et al. 2019) or microbial growth rate (Bremer and van Kessel 1990; Nguyen and Guckert 2001; Glanville et al. 2016; Čapek et al. 2023). Conservative range for k_{ec} between 0.2 and 0.6 could be expected. $(C:P)_{\text{MB}}$ changes as a function of growth rate (Makino et al. 2003) as described in introduction section, and also as a function of microbial community composition (Mouginot et al. 2014). In soils from Plešné and Čertovo catchments, microbial growth has been shown to switch between C and P limitation at C to P ratio of external resources of 95 and 119, respectively (Čapek et al. 2016). Assuming that 20% of organic C is used for energy production (Čapek et al. 2021), $(C:P)_{\text{MB}}$ is expected to be 95

and 76 for Plešné and Čertovo soils, respectively. These estimates are close to the C to P ratio of the non-growing culture of *Escherichia coli* at low inorganic P concentrations (Makino et al. 2003) and the mean C to P ratio of bacterial (88) and fungal isolates (106) from grassland soil (Mouginot et al. 2014). $(C : P)_{CHCl_3}$ of microbial biomass after one week incubation on growth media without P, which should reflect $(C : P)_{MB}$ according to Eq. 3, was on average 50 (Fig. 3) across all samples. Substituting expected $(C : P)_{MB}$ for Plešné and Čertovo soils into Eq. 3, measured $(C : P)_{CHCl_3}$ around 50 can be obtained using a ratio between k_{ec} and r_{basal} (i.e. k_{ep} at n_{Poly-P} equal to zero) of 0.53 and 0.66, respectively. These estimates provide limits to possible values of k_{ec} and r_{basal} . If we arbitrarily select r_{basal} to be 0.4, k_{ec} must be 0.21 and 0.26 for Plešné and Čertovo soil, respectively, i.e. values within the expected range. Using expected values of k_{ec} , $(C : P)_{MB}$ and r_{basal} , together with estimates of r_{Poly-P} measured in this study, contribution of Poly-P to total soil microbial biomass P before incubation on growth media without P should be between 33 – 45%. It must be stressed, however, that these are first order estimates whose accuracy depends on applied assumptions. If we, for example, apply commonly used k_{ec} and r_{basal} of 0.45 and 0.4 while keeping rest of the parameters unchanged, the maximum contribution of Poly-P to total microbial biomass P increases to almost 70%. In either way, the calculations suggest that the amount of P stored in the biomass of soil microorganisms in the form of Poly-P should not be considered negligible.

The Poly-P content could be of particular interest because MBP has been shown to represent hydrolysable pool of organic P (De Sena et al. 2022), which is easily available to plants and, as such, affects plant nutrition (Peng et al. 2022; Shi et al. 2023). On the other hand, storing P in excess increases the retention capacity of microbial biomass for P, which could affect plant nutrition negatively (Shi et al. 2023). To distinguish between the two hypothetical scenarios, the content of Poly-P and its turnover rate needs to be known. Without direct method of microbial Poly-P determination, however, such task is impossible at the moment.

Conclusions

The results of our study allow us to conclude that accumulation of Poly-P in the microbial biomass can be common. Poly-P may significantly contribute to microbial biomass P. The depolymerization of Poly-P in soil and/or acidified sodium bicarbonate soil extracts is high, and thus, their recovery from soil in the form of soluble reactive P is almost quantitative. Such a high recovery rate associated with a high Poly-P content of microbial biomass can affect the value of the conversion factor microbial biomass P and $CHCl_3$ -labile P.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00374-024-01829-6>.

Author contributions Petr Čapek designed the experiment, evaluated data and wrote the manuscript. Adéla Tupá conducted all experiments and measured data. Michal Choma selected sampling localities and commented on first versions of the manuscript. All authors read and approved the final manuscript.

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Data availability All data as well as code to all analyses performed within this study are publicly available at <https://github.com/petacapek/Polyphosphates>.

Declarations

Conflict of interests The authors declare that they have no conflict of interest.

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