Making Sense of Chaos: Nutrient Cycling as an Emergent Property of Microbial Biomass and Physiology

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Preface

Soil is frequently referred to as a 'dirt' in colloquial language. According to Thesaurus.com, the synonym for 'dirt' is 'muck'. The same webpage states that the word 'muck' expresses 'a state of chaos or confusion' and, in my opinion, this accurately characterises the true nature of soil. Soil is a mixture of solid particles of various sizes, chemical and structural compositions, gases, soluble or insoluble inorganic and organic compounds, smaller or larger pieces of dead organic material, and many different active or dormant organisms of various shapes, sizes, and taxonomic identity. All that mess somehow interacts sometimes, but not always, and it changes over time and along short distances in all possible directions creating a state of chaos and confusion for anyone who has ever attempted to study any aspect of the soil. Any such person soon gets depressed because navigation through this mess is nearly impossible. Seemingly trivial task such as 'representative' sampling does not feel that trivial once we decide to sample soil. Yet, the soil is undoubtedly important. The human population depends on vast agricultural fields and production forests, while accepting that some soils are more productive than others. Soil represents one of the most important habitats for organisms because roughly 60% of all species that live on Earth (yes, including the aquatic ones) spent all of their life or part of it in soil (Anthony et al., 2023). Soils store a lot of organic carbon (C), which may (Wieder et al., 2015b) or may not (Brovkin and Goll, 2015) get released to atmosphere upon warming and thus, exacerbate (or not, we still do not know) the ongoing climate change. For that reason, it would be unwise to ignore the soil only because it is a muck. Someone must accept the challenge, study the soil and then face the consequences of such a decision.

The consequences are harsh and unavoidable. Even a large multidisciplinary research team, which I was part of during my doctoral studies, cannot cover all aspects of the soil, so simplifications and bold assumptions are integral parts of all published soil studies. Once a single researcher selects a subset of aspects to study in detail and defines the simplifying assumptions, that researcher receives constant critique and denial from other soil researchers studying other subsets of soil aspects because these are, of course, more important. And that is not all. Methods, which are easy to perform and highly reproducible in other scientific disciplines (e.g. microbiology), become inaccurate and/or irreproducible when performed with soil samples. No one can be certain to what extent the data-based conclusions are accurate or simply an artefact of methodological bias. For that reason, it is difficult to convince undergraduate students to work on soil projects and then try to delay the onset of their anxiety over confusing results as long as possible. It is even more difficult to articulate your findings to a family, friends, or the public. It is chaos. Nevertheless, studying soil could be less of a struggle with the right group of people who are fun to be with and who can provide constructive critique and are ready to accept the critique back. I accepted to face the consequences of my decision to be a soil scientist mainly due to colleagues from department of Ecosystem Biology, Institute of Hydrobiology CAS, University of Vienna and Stockholm University represented by Stefano Manzoni. It is appropriate to express my sincere gratitude to all these people.

Now, I am accepting another challenge to write this habilitation thesis, a document which is expected to list scientific achievements of a struggle I went through together with my colleagues over the past years. It is meaningful to expect logical coherency from such a document. This implicit requirement translates into another struggle to find order within the chaos of soil science. Since the challenge has been accepted, I attempted to do so. While reading the studies that I decided to include in this thesis, some order crystalized. However, it is unlikely that the structure of this document would be the same a year ago or a year later, because constant scrutiny requires soil scientists to revise their understanding over again. The order of this thesis with the timestamp 2025-07-18 is as follows. The introduction section defines the aspects of the soil that I decided to study in detail. It provides a rationale for that decision and states the simplifying assumptions, which will be applied first. The following sections will explain the theoretical fundamentals that I frequently use in my research. The theoretical concepts will gradually develop, and some simplifying assumptions will be relaxed making everything more complex and confusing. The following sections will describe the conditions at which the defined concepts do not apply and explain why. This explanation will not be limited to biological phenomena, but it will also consider possible methodological bias. The last section will be, as rational of the thesis dictates, dedicated to future perspectives.

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Paper 2: Gentsch, N., Mikutta, R., Alves, R.J.E., Barta, J., **Čapek, P.**, Gittel, A., Hugelius, G., Kuhry, P., Lashchinskiy, N., Palmtag, J., Richter, A., Šantrůčková, H., Schnecker, J., Shibistova, O., Urich, T., Wild, B., Guggenberger, G., 2015. Storage and transformation of organic matter fractions in cryoturbated permafrost soils across the Siberian Arctic. Biogeosciences 12, 4525–4542.

Paper 3: **Čapek, P.,** Starke, R., Hofmockel, K.S., Bond-Lamberty, B., Hess, N., 2019. Apparent temperature sensitivity of soil respiration can result from temperature driven changes in microbial biomass. Soil Biol. Biochem. 135, 286–293.

Paper 4: Diáková, K., **Čapek, P.,** Kohoutová, I., Mpamah, P.A., Bárta, J., Biasi, C., Martikainen, P.J., Šantrůčková, H., Šantrůčkova, H., 2016. Heterogeneity of carbon loss and its temperature sensitivity in East-European subarctic tundra soils. FEMS Microbiol. Ecol. 92, 1–17.

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Paper 6: **Čapek, P.,** Manzoni, S., Kaštovská, E., Wild, B., Diáková, K., Bárta, J., Schnecker, J., Biasi, C., Martikainen, P.J., Alves, R.J.E., Guggenberger, G., Gentsch, N., Hugelius, G., Palmtag, J., Mikutta, R., Shibistova, O., Urich, T., Schleper, C., Richter, A., Šantrůčková, H., 2018. A plant–microbe interaction framework explaining nutrient effects on primary production. Nat. Ecol. Evol. 2, 1588–1596.

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Paper 8: Manzoni, S., **Čapek, P.,** Mooshammer, M., Lindahl, B.D., Richter, A., Šantrůčková, H., 2017. Optimal metabolic regulation along resource stoichiometry gradients. Ecol. Lett. 20, 1182–1191.

Paper 9: **Čapek, P.,** Choma, M., Tahovská, K., Kaňa, J., Kopáček, J., Šantrůčková, H., 2021a. Coupling the resource stoichiometry and microbial biomass turnover to predict nutrient mineralization and immobilization in soil. Geoderma 385, 114884.

Paper 10: **Čapek, P.,** Choma, M., Kaštovská, E., Tahovská, K., Glanville, H.C., Šantrůčková, H., 2023. Revisiting soil microbial biomass: Considering changes in composition with growth rate. Soil Biol. Biochem. 184, 109103.

Paper 11: **Čapek, P.,** Tupá, A., Choma, M., 2024. Exploring polyphosphates in soil: presence, extractability, and contribution to microbial biomass phosphorus. Biol. Fertil. Soils. 60, 667 – 680.

Paper 12: **Čapek, P.,** Kotas, P., Manzoni, S., Šantrůčková, H., 2016. Drivers of phosphorus limitation across soil microbial communities. Funct. Ecol. 30, 1705–1713.

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Introduction

The main topic of this habilitation thesis is soil microorganisms and soil processes that the microorganisms either drive directly or affect indirectly by their metabolic activity. Although there are many different organisms in the soil, quantitatively the most important are microorganisms, i.e. bacteria, archea, and fungi. When the biomass (expressed in grams of carbon per square meter) of different soil organisms (excluding plant roots) is compared across biomes, microorganisms typically make 90 - 98% of the biomass of all soil organisms (Bar-On et al., 2018; Fierer et al., 2009; Johnston and Sibly, 2018). It is reasonable to expect that this dominant pool of biomass is functionally important too. That was an assumption, which I started my doctoral studies with. It is, however, surprisingly difficult to provide a rigorous estimate of this functional importance. Before I will attempt to do so, a brief introduction is required.

The function of microorganisms in the soil is inherently determined by their size and physiology. Since they are very small, they are "osmotrophic" – i.e. meaning that they do not ingest the food but absorb the food dissolved in a solution through their membranes. With respect to their physiology, I will apply the first simplifying assumption and hereafter consider all soil microorganisms as heterotrophs. Thus, they require certain organic compounds such as glucose or amino acids to get the energy and material to construct their biomass. Any organic material that ends up on top of the soil or inside the soil may eventually serve soil microorganisms as a source of organic compounds. It could be plant litter, roots, rhizodeposits, dead animals, or microorganisms themselves. The primary function that soil microorganisms fulfil is the transformation of organic material, the process, which is referred to as "decomposition". Soil microorganisms are, therefore, often referred to as "decomposers" (Berg and McClaugherty, 2020a). They decompose organic materials to survive and grow.

It is obvious that there is an incompatibility between microorganisms and their food in terms of size and solubility in many cases. The organic debris such as needle litter is much larger than microorganisms and certainly cannot magically dissolve, so the microorganisms can absorb all the organic compounds it contains right away. The organic debris must fragment, and the soluble organic compounds must be released to become utilisable. This requirement does not apply to soluble rhizodeposites and a certain fraction of dead microbial cells. There are several mechanical, physical, and biochemical processes that either cause fragmentation, directly release some solubles from organic debris, or do both at the same time. Notable is, for example, the fragmentation and transformation of organic debris by soil meso- and macrofauna (Frouz, 2018) or the degradation of plant litter by UV light (King et al., 2012). Although these processes may speed up the decomposition a lot, soil microorganisms are not ultimately dependent on them because they developed their own mechanisms of releasing solubles from organic debris. They can (and often do) produce a wide variety of extracellular enzymes, which are specifically designed to provide soluble organic compounds that can be utilised (as well as inorganic nutrients as discussed later) (Burns and Dick, 2002). In theory, soil microorganisms are capable of decomposing any organic material without any help, but it rarely happens in most ecosystems. That is why quantifying the functional importance of soil microorganisms is so difficult. According to Cebrian (2004), decomposers process 8 to 12 times more plant biomass than herbivores every year. However, this estimate is not "microorganism-specific" because the contribution of soil fauna is included in this estimate. To specifically distinguish the relative contribution of microorganisms from other heterotrophic soil organisms, which also transform organic material, I will perform a simple exercise in which I will apply the metabolic theory of ecology and use data reported in the study of (Johnston and Sibly, 2018).

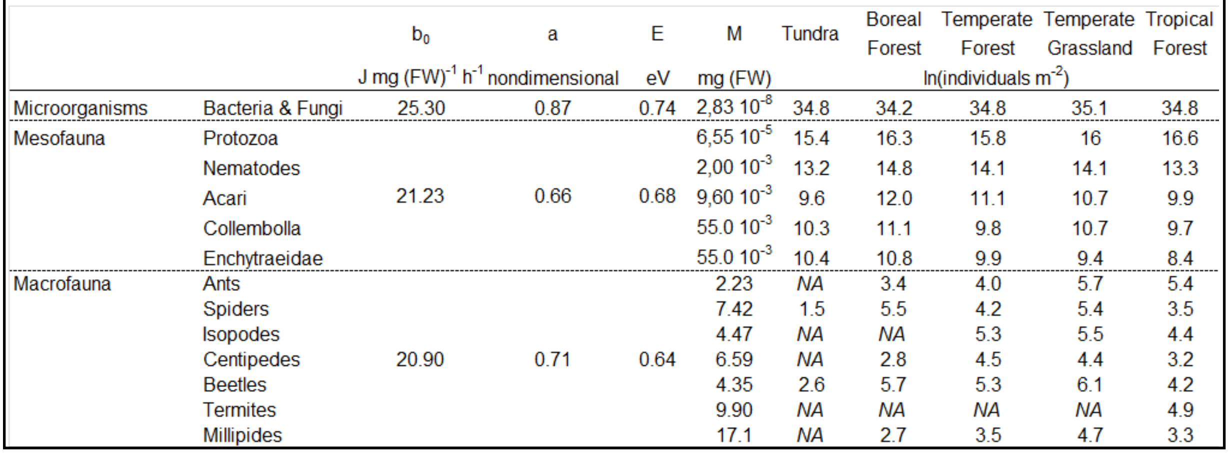
As defined above, the functional importance of soil microorganisms derives from their activity, which I will call the "metabolic rate" for the moment to conform to the terminology of metabolic theory. According to this theory, the metabolic rate (Q in Joules per hour for example – J h^{-1}) of any individual organism i (bacterial cell or an earthworm) can be described by the following equation (Allen et al., 2005; Allen and Gillooly, 2009; Johnston and Sibly, 2018):

$$Q_i = b_0 \times M^a \times e^{\frac{-E}{k \times (T+273.15)}} \qquad \text{(eq. 1)},$$

in which b_0 is a normalization constant, M is mass of an organism (in milligrams of fresh weight for example – mg (FW)), a is nondimensional scaling exponent, E is activation energy (in electron volts -

eV), T is temperature (in °C) and k is Boltzmann's constant (8.62×10^{-5} eV K^{-1}). If the desired units of Q_i are J h^{-1} and M is in mg FW, b_0 must be in J mg $(FW)^{-1}$ h^{-1}. If eq. 1 applies to all soil organisms, it is theoretically possible to calculate contribution of any individual or group of individuals to metabolic activity of entire soil community. The metabolic activity can be conveniently normalized to a single temperature so the differences across biomes with different temperature regimes can be determined as well. The group of individuals we are interested in are microorganisms (mainly bacteria and fungi), and the entire soil community is composed of microorganisms, mesofauna and macrofauna. Since the last two categories are diverse, we may wish to further distinguish different groups of organisms within these categories such as collembolans, isopodes, earthworms etc. To perform the exercise, variables b_0, M, a and E that define metabolic rate of individuals must be known together with number of individuals within each group of organisms per e.g. square meter of an area. All required data are available in study of Johnston and Sibly (2018) and reported here in Table 1.

Table 1: *Equation parameters (b_0 - normalization constant, M - mass of an organism, a - scaling exponent, E - activation energy) of metabolic theory of ecology (eq. 1) reported in Johnston and Sibly (2018) for different groups of soil organisms. The mean number of individuals of a respective group of soil organisms in five different biomes is further reported.*



		b_0	a	E	M	Tundra	Boreal Forest	Temperate Forest	Temperate Grassland	Tropical Forest
		J mg $(FW)^{-1}$ h^{-1}	nondimensional	eV	mg (FW)	ln(individuals m^{-2})				
Microorganisms	Bacteria & Fungi	25.30	0.87	0.74	2,83 10^{-8}	34.8	34.2	34.8	35.1	34.8
Mesofauna	Protozoa	21.23	0.66	0.68	6,55 10^{-5}	15.4	16.3	15.8	16	16.6
	Nematodes				2,00 10^{-3}	13.2	14.8	14.1	14.1	13.3
	Acari				9,60 10^{-3}	9.6	12.0	11.1	10.7	9.9
	Collembolla				55.0 10^{-3}	10.3	11.1	9.8	10.7	9.7
	Enchytraeidae				55.0 10^{-3}	10.4	10.8	9.9	9.4	8.4
Macrofauna	Ants	20.90	0.71	0.64	2.23	*NA*	3.4	4.0	5.7	5.4
	Spiders				7.42	1.5	5.5	4.2	5.4	3.5
	Isopodes				4.47	*NA*	*NA*	5.3	5.5	4.4
	Centipedes				6.59	*NA*	2.8	4.5	4.4	3.2
	Beetles				4.35	2.6	5.7	5.3	6.1	4.2
	Termites				9.90	*NA*	*NA*	*NA*	*NA*	4.9
	Millipides				17.1	*NA*	2.7	3.5	4.7	3.3

In all biomes for which the data are available, the contribution of soil microorganisms to the metabolic rate of the entire soil community is dominant (fig. 1A). These results apparently allow a straightforward conclusion that the dominant pool of biomass in soil, i.e. soil microorganisms, is functionally very important.

However, an interesting characteristic of the soil microbial community needs to be considered in view of these results. The number of individuals of soil microorganisms reported in Tab. 1 is derived from total soil microbial biomass carbon and the mean carbon (C) content of a single microbial cell. Therefore, it is assumed (among others) that all individuals are metabolically active. Nevertheless, only a fraction of the microbial community, roughly 40% (often less), is active, the rest being dormant (Blagodatskaya and Kuzyakov, 2013). When the dormancy is considered, microorganisms still remain the most metabolically active group of soil organisms but their contribution to overall activity is no longer as dominant, especially in boreal forests and temperate grasslands (fig. 1B). The situation becomes confusing once considering additional argument. While ignoring dormancy, Johnston and Sibly (2018) converted metabolic activities of soil communities to respiration rates and confronted the predicted respiration rates with independent dataset of measured respiration rates (per square meter of an area and year). They found surprisingly accurate correspondence suggesting that their calculations, which ignored the fact that 60% of microbial community or more is dormant, match the independent data well. By considering the dormancy, the contribution of soil microorganisms to entire metabolic activity becomes surprisingly underestimated. At this point, one may ask why to bother a reader with the dormancy issue just to argue that it is very likely not an issue at the end. This exercise is meant to serve several purposes. The main purpose is to show that soil microorganisms are very likely very important. The second purpose is to prove that the rigorous estimate of microbial functional importance, something that intuitively feels self-evident, is not easy to provide. Finally, the third purpose is to document the degree of uncertainty that every soil scientist must face when trying to make responsible

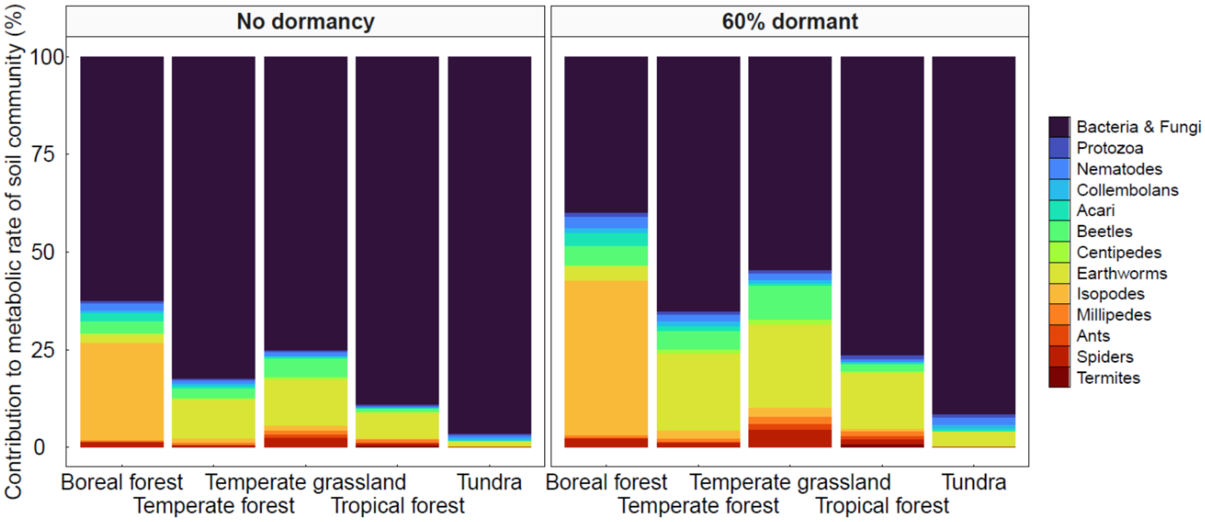


Figure 1: *Relative contribution of different categories of soil organisms (highlighted by different colours) to metabolic rate of entire soil community assuming that all (A) or only 40% (B) of soil microorganisms are active.*

conclusions. Johnston and Sibly (2018) made several simplifying assumptions. Not all of them could be considered reasonable and some may even be considered counterintuitive. Nevertheless, the erroneous assumptions very likely cancelled each other out (as documented in last section of this thesis) so the confrontation with independent dataset successfully proved authors' point. This exercise is symptomatic of soil science. Its various aspects will resonate throughout the rest of this document. I will apply simplifying assumptions, which may feel too simplifying or counterintuitive, but they may eventually cancel each other out and provide a reasonable conclusion or representation of empirical observations. I will attempt to highlight all uncertainties I am aware of at this moment. At the same time, I will need to focus on methodological as well as terminological limitations as a significant source of uncertainty.

To move forward from abstract discourse towards concrete terms, four key statements have been made so far: (i) the most important groups of soil organisms in respect to biomass and activity are soil microorganisms, (ii) they are responsible for transformation of most of the organic material in an ecosystem, (iii) they transform organic material to survive and eventually grow, and (iv) the transformation is conducted by intracellular metabolism and extracellular enzymes. The likelihood that these statements match the reality reasonably well is high. However, the uncertainty is not negligible. Selected consequences of the presence and activity of soil microorganisms, namely production of carbon dioxide (CO_2), transformation of organic forms of nutrients to inorganic forms, and vice versa, will be specified and discussed in the following chapters. Since the quantitative estimation of these consequences will be attempted, the short introduction to microbially-explicit soil models follows first.

Representing microorganisms in soil models

All primary research is conducted to expand our understanding of the world (or universe). From an anthropocentric point of view (the point of government agency financing the research), such an understanding is required because we want/need to know what may happen in future so we can get ready for any possible scenario in advance. For that reason, the available knowledge is gradually converted to mathematical models, and the simulations of these models are consulted with. The two most influential examples are weather forecasts or IPCC reports (IPCC, 2014). Since soil research is no exception, it is reasonable to acknowledge the modelling aspect. The additional benefit of doing so is that simplifying assumptions are central to all models, so their presence does not cause so much confusion, and, more importantly, the model simulations can be confronted with available data in order to quantify the magnitude of error caused by the presence/absence of simplifying assumptions. Unlike soil, the models are well-organised units so starting with the modelling aspect will hopefully help to increase clarity of further discussion.

Even though the first models describing organic material transformation in soil date back to early seventies (Manzoni and Porporato, 2009), and the significance of soil microorganisms was recognized more than seventy years before that (Darbishire and Russell, 1907), microorganisms were rarely considered in these models explicitly (Manzoni and Porporato, 2009). Until today, there are still several models such as CENTURY (Zhang et al., 2018) or OCHIDEE (Sun et al., 2021) that do not contain any specific biological component. These so called 'classical' models are based on simple first order decay kinetic of various abstract pools, categorised according to the rate of their decay (decomposition) – e.g. denoting them as active, passive and/or recalcitrant. Counterintuitively again, these models perform just fine in many cases (Lawrence et al., 2009; Lennon et al., 2024) so there was, and still is, no big pressure to abandon them completely. The situation changed considerably after a series of influential studies authored by Steven Allison and William Wieder were published (Allison, 2012; Allison et al., 2010; Wieder et al., 2015a, 2013). These studies showed three important things – *(i)* integration of microbial component to Earth system models is feasible (Allison, 2012; Allison et al., 2010; Wieder et al., 2015a), *(ii)* accuracy of microbially-explicit models simulations may outperform classical models at global scale (Wieder et al., 2013), and *(iii)* microbially-explicit models are flexible enough to explain various responses of heterotrophic soil respiration to long-term warming as observed in discrete experiments performed all over the world (Allison et al., 2010). Research interest in microbial component(s) has increased a lot since then.

Figure 2 shows schematic representation of typical microbially-explicit model (on C-1 molar or mass basis). There are heterotrophic microorganisms that live in the soil (all active). Taxonomic and/or functional differences between different species of microorganisms are usually not considered (although not always, e.g. Kaiser et al., 2015). The microorganisms are treated as a pool, so the size of the pool increases or decreases, i.e. microbial cells do not strictly divide or die. Microorganisms produce extracellular enzymes, which depolymerize complex soil organic material (denoted as SOC – soil organic C) into assimilable dissolved organic C (DOC) for some time, and then become DOC. DOC gets assimilated and is used by microorganisms to grow, produce extracellular enzymes, and get the energy for growth and extracellular enzyme production. The energy gain is associated with the production of carbon dioxide (CO_2). When microorganisms die, one fraction of the dead biomass becomes SOC and the other becomes DOC. It is assumed that all soils are well-aerated, optimally wet, and homogenously mixed. The scheme depicted in fig. 2 represents the greatest extent of simplification I will start with to show that considering the microbial component can explain significant variability in heterotrophic CO_2 production along gradients of soil types and temperatures.

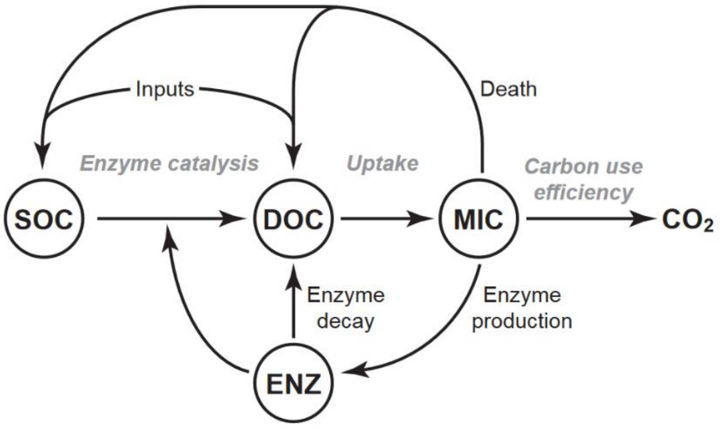


Figure 2: *Schematic representation of microbially-explicit soil model. Different circles represent different carbon (SOC – soil organic carbon, DOC – dissolved organic carbon, MIC – microbial biomass carbon, ENZ – extracellular enzymes in units of carbon) pools. Arrows represent carbon fluxes. Adapted according to Allison et al. (2010).*

Loss of soil organic carbon via respiration

The most prominent consequence of microbial activity is the loss of SOC by respiration. As defined above, heterotrophic soil respiration (hereafter denoted as respiration for simplicity) is expected to be a function of microbial biomass because all soil microorganisms are assumed to be heterotrophs whose metabolic activity dominates the activity of entire soil community and depends on temperature approximately according to eq.1 (applying all assumptions stated above). This definition contrasts with many previous studies, which showed that respiration can be conveniently defined as a function of temperature, quantity and quality of SOC instead of microbial biomass (Ågren and Bosatta, 2002; Bosatta and Ågren, 1999; Conant et al., 2011; Craine et al., 2010; Davidson and Janssens, 2006). The two concepts are not strictly in contradiction if an equilibrium exists between microbial biomass and SOC (the ratio between them is constant). Soils with greater SOC content should host more microorganisms (because SOC is their food) so the respiration rate should increase with SOC and microbial biomass, which is in equilibrium with SOC (being approx. 1% of SOC; Fierer et al., 2009). From this point of view, knowing the microbial biomass provides no additional benefit for understanding and predicting SOC loss. However, that applies only as long as we do not focus on the temperature sensitivity of respiration, i.e. the magnitude of change of respiration with the temperature. If we do, the two concepts provide contradictory predictions. If SOC determines respiration, its temperature sensitivity within a reasonable temperature range (say 0 – 25°C, see below) only depends on SOC quality. The SOC pools that transform to CO_2 at slower rate (i.e. passive pools) have greater temperature sensitivity because their so called "activation energy" is higher (see e.g. Bosatta and Ågren, 1999). If microbial biomass determines respiration, the temperature sensitivity is independent of SOC quality. It is determined by activation energy E in eq. 1 instead of SOC quality.

In addition to various studies contradicting the SOC quality effect on temperature sensitivity of heterotrophic soil respiration (e.g. Fang et al., 2005; Gershenson et al., 2009), two of our studies provided direct evidence that the respiration is more accurately predicted by microbial biomass **(Čapek et al., 2019, 2015)**. The first study was conducted in the Arctic where soils are often cryoturbated (Bockheim and Tarnocai, 1998; Van Vliet-Lanoe, 1998). The cryoturbation forms pockets of soil with high SOC content inside the mineral soil. The assumption of homogeneously mixed soils must be relaxed in this case to allow the soil to be stratified. There is a high SOC content on top of the soil and this content decreases with increasing soil depth unless the pocket of so called "subducted" or "cryoturbated" organic soil horizon appears in greater depths where it seemingly doesn't belong (according to SOC content). The subducted organic horizon is derived from top soil, but it is much older (Bockheim, 2007; Kaiser et al., 2007). Because of that, the SOC content and quality are lower in the subducted organic compared to the top soil horizon **(Gentsch et al., 2015)**. Top soil, subducted organic soil and mineral soil form gradient of SOC content and quality, which is ideal for quantifying accuracy of predictions of SOC and microbial biomass centered concepts described above.

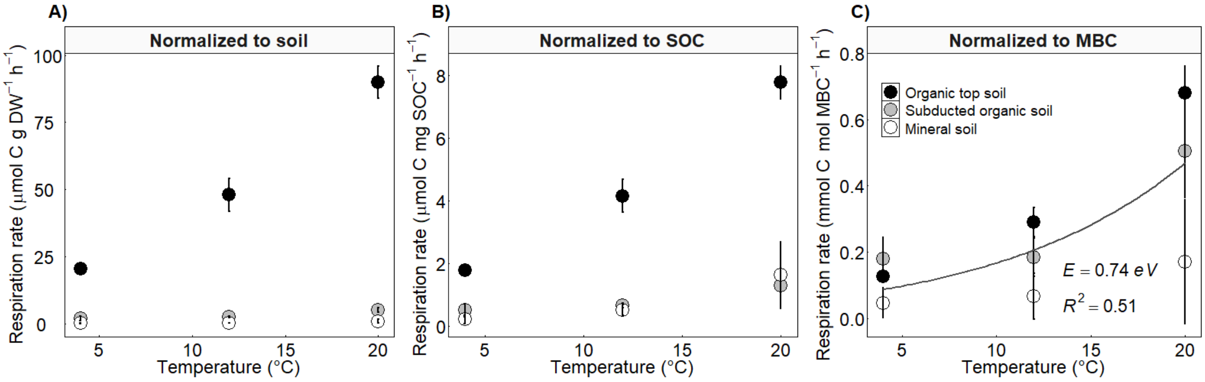


Figure 3: *Mean respiration rate expressed (A) per gram of dry weight of soil, (B) per mg of soil organic carbon (SOC) and (C) per mol of microbial biomass carbon (MBC) in organic (black points), subducted organic (grey points) and mineral (empty circles) soil horizon. Grey line shows temperature response of respiration rate predicted by eq. 1 with E being 0.74 eV (see tab. 1). Error bars represent standard deviation of the mean.*

When we incubated the three very different soil horizons, each at three different temperatures (4, 12 and 20°C) the respiration rate varied widely when expressed per gram of dry soil (fig. 3A) or milligram of SOC (fig. 3B). This variability, however, narrowed down once the respiration rate was normalized to the unit of microbial biomass quantified as microbial biomass C (MBC) using a fumigation-extraction method, whose accuracy will be discussed in the last section. If eq. 1 is used to predict the biomass specific respiration rate assuming scaling coefficient a is 1 (since mean body mass of microbial cells cannot be determined), and activation energy E is 0.74 eV (as reported in Table 1), 51% of entire variability in measured data was explained by estimating a single equation parameter (i.e.

normalization constant). When variability in E among horizons with different SOC quality was tested statistically, no significant difference was found **(Čapek et al., 2015)**. The SOC quantity and quality clearly do not always matter as much as was originally suggested. Microbial biomass is not always in equilibrium with SOC (compare figs. 3B and C), and the SOC quality does not affect temperature sensitivity of soil respiration consistently. Unfortunately, we do not fully understand what exactly causes the differences in MBC to SOC ratio among different soil horizons (Serna-Chavez et al., 2013). As long as this knowledge is lacking, the most reasonable course of action is to keep measuring microbial biomass to improve prediction of heterotrophic respiration because knowing microbial biomass itself could do a reasonable job. It is important to state, however, that large fraction of variability remains unexplained.

Counting independent (and thus rapid) methods of microbial biomass assessment are available since late 70’s (Jenkinson, 1976) but the available microbial biomass data in context of temperature gradients are surprisingly rare. To check how microbial biomass performs as a predictor of soil respiration across temperatures world-wide, I performed the meta-analysis of available studies that reported respiration rate and MBC at different temperatures. In 2019, I was able to find only 27 original studies **(Čapek et al., 2019)** while the combination of keywords “heterotrophic soil respiration” and “temperature” yielded more than thousand published studies registered on Web of Science. Most of the published studies simply assumed that microbial biomass can be neglected and attempted to explain the variability in respiration rates across temperatures by other soil variables (including SOC content and quality), often reaching contradictory conclusions (see Gershenson et al., 2009 as a nice example). The most debated issue was and still is the variability in temperature sensitivity of respiration, which I will keep assuming to be invariable for now, at least as long as **the respiration is normalized to unit of microbial biomass**. The logical implication of such assumption is that the variability in temperature sensitivity observed among different studies may simply result from temperature related changes in MBC, which were not detected because biomass was not measured in majority of studies. To be rigorous, the variability in temperature sensitivity has one more aspect of a technical nature, which needs to be addressed to allow straightforward comparison among published studies.

To define temperature sensitivity quantitatively as a single value, which soil scientists can refer to, Q_{10} parameter was introduced. Q_{10} is defined by following equation:

$$Q_{10} = \left(\frac{R_{T_2}}{R_{T_1}}\right)^{\frac{10}{T_2-T_1}} \qquad \text{(eq. 2).}$$

In eq. 2, R_{T_1} and R_{T_2} denote respiration rates at two different temperatures T_1 and T_2 ($T_2 > T_1$). Q_{10} expresses relative change of respiration with 10°C temperature increase. If Q_{10} is 2, respiration doubles when temperature increases by 10°C (theoretically from any reference temperature T_1). This definition of temperature sensitivity is straightforward and easy to understand so most studies use it. Unfortunately, this definition is misleading because it implicitly assumes that the temperature response is strictly exponential, which may be sometimes true, but only in certain temperature range. If the Q_{10} is defined in terms of an eq. 1, assuming that microbial biomass does not change with temperature, we get following function:

$$Q_{10} = e^{\frac{-E}{k}\times\left(\frac{1}{T+283.15}-\frac{1}{T+273.15}\right)} \qquad \text{(eq. 3),}$$

which is paradoxically temperature dependent. If E is 0.74 eV, Q_{10} decreases from 3.03 within 0 – 10°C temperature range to 2.47 within 20 – 30°C. Some variability in temperature sensitivities reported in different studies as Q_{10} can thus result from different temperature ranges used in these studies (Davidson and Janssens, 2006). To harmonize published data, temperature sensitivity needs to be defined using some response function, which can be considered general enough (i.e. not the exponential one). According to Tuomi et al. (2008), Gaussian model (eq. 4) is that function from statistical point of view. According to Schipper et al. (2014), Macromolecular Rate Theory equation (eq. 5) is that function from theoretical point of view. The two equations are defined by the following formulas:

$$R = R_0 \times e^{a\times(T+273.15)+b\times(T+273.15)^2} \qquad \text{(eq. 4),}$$

$$R = \frac{k\times(T+273.15)}{h}\times e^{-\frac{\Delta H^{\ddagger}_{T_0}+\Delta C^{\ddagger}_{P}\times(T-T_0)}{R\times T}+\frac{\Delta S^{\ddagger}_{T_0}+\Delta C^{\ddagger}_{P}\times[ln(T)-ln(T_0)]}{R}} \quad \text{(eq. 5),}$$

in which R_0, a and b are some parameters without physiological meaning, h is Plank's constant, R is universal gas constant and $\Delta H^{\ddagger}_{T_0}$, $\Delta S^{\ddagger}_{T_0}$ and $\Delta C^{\ddagger}_{P}$ represent enthalpy, entropy and heat capacity of a macromolecular process, respectively. Unlike eq. 1, both functions have a maximum at certain temperature above which the respiration starts to decrease with temperature, very intuitive unimodal pattern. Both functions, however, have more equation parameters than eq. 1 as a result and, as evident from eqs. 4 and 5, not Tuomi et al. (2008) nor Schipper et al. (2014) consider important to scale respiration rate to microbial biomass.

Our meta-analysis showed exactly the opposite, scaling respiration to microbial biomass is essential. It is so essential that unimodal response function is not required. Anytime biomass increases with temperature, the temperature sensitivity of respiration increases and vice versa **(Čapek et al., 2019)**. The decreasing trend of respiration with temperature above optimum (determined to be approx. 25°C), can be explained by the decrease of microbial biomass (fig. 4) without the necessity to apply unimodal response functions defined by Tuomi et al. (2008) and Schipper et al. (2014). Our meta-analysis showed that microbial biomass performs well as a predictor of soil respiration world-wide. Its variability across temperatures could explain some differences in reported temperature sensitivities of respiration. Since laboratory and field experiments were included in the meta-analysis, the importance of microbial biomass cannot be simply interpreted as an artefact of laboratory incubations. However, these results imply another important but troublesome thing. No general response function describing the effect of temperature on respiration rate exists. In case of soil microbial biomass, there is no such thing as a metabolic activity, which expresses itself as a rate of respiration and could be simply described by some equation, such as eq. 1. The vaguely defined "metabolic rate" needs to be dissected into specific microbial processes, each of which probably has a different temperature response. Our meta-analysis indicated the growth and death (sometimes referred to as turnover) rates of microbial biomass as two important processes. The importance of microbial death rate was also indicated to be of considerable importance by Hagerty et al. (2014). Eq. 1 therefore needs to be abandoned because it is too simplifying and the microbial biomass needs to be treated as a pool (or two pools as explained later), whose rate of change is determined by several processes, respiration rate being a result of their combinations. To extend this line of evidence, one more process that is typical for soil microorganisms needs to be highlighted, the production of extracellular enzymes.

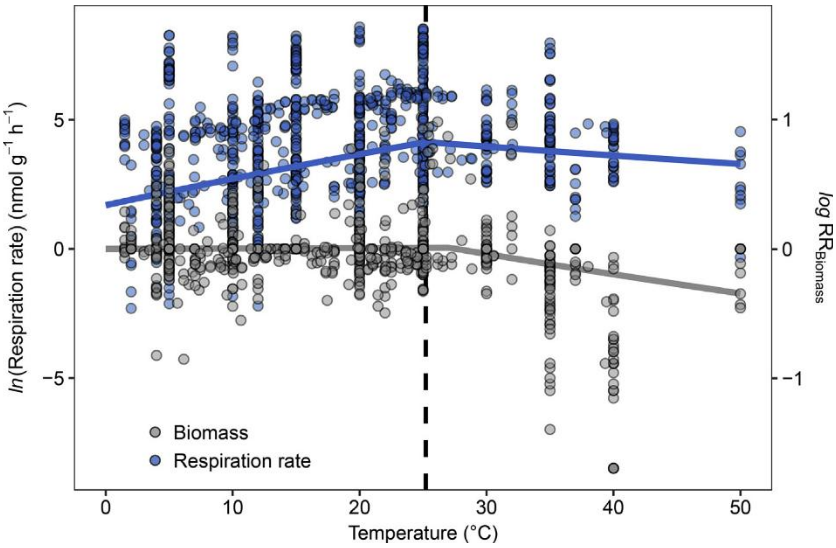


Figure 4: *Relationship between temperature and the natural logarithm of heterotrophic soil respiration rate (blue points) or decadic logarithm of soil microbial biomass response ratio (logRR, grey points, right y-axis). logRR defines the magnitude of change of the microbial biomass at the given temperature relative to control, which is defined as the MBC of a respective observation (i.e. data set) at 15 °C or the closest temperature. A logRR value of zero indicates no change of microbial biomass relative to control, while logRR less than zero indicates a decrease of microbial biomass relative to control. The vertical dashed line represents the breakpoint temperature (25.2 ± 2.4 °C) at which the temperature sensitivity of soil respiration rate changes. The blue and grey solid lines represent fits of best statistical models.*

As mentioned in the Introduction, soil microorganisms must produce extracellular enzymes. Without this production, they cannot guarantee the access to assimilable food. Unfortunately, this production, unlike e.g. growth, cannot be measured. We can measure an ambiguous variable (see critical review of Nannipieri et al., 2018), which has been defined as "potential enzyme activity", by a method, whose accuracy will be discussed in the last section. It is assumed that this potential activity is, to some extent, proportional to the concentration of extracellular enzymes in soil (e.g. Calabrese et al., 2022). Applying this assumption, the increase of potential enzyme activity over time may suggest that enzymes were produced. Similarly to all assumptions stated so far, this one may be true only in specific cases. It happened to us that we measured no change of potential enzyme activity over time at three different temperatures 4, 12 and 20°C in some soils from arctic **(Čapek et al., 2015)** and increase of potential enzyme activity over time in other arctic soils **(Diáková et al., 2016)** incubated at same three temperatures. It also happened that the magnitude of this increase differed between temperatures **(Diáková et al., 2016)**. When we calculated Q_{10} of heterotrophic respiration and Q_{10} of potential enzyme activity of all classes of extracellular enzymes that we can measure (that is β-glucosidase, cellobiosidase, phosphatase, chitinase and leucine-aminopeptidase) across all three temperatures, we found an interesting linear relationship (fig. 5), which may suggest that the more extracellular enzyme microbial biomass produces, the more CO_2 is lost from soil via respiration **(Diáková et al., 2016).** In theory, such relationship is meaningful. When microbial biomass doesn't change with temperature, but pool of extracellular enzymes does, there should be greater CO_2 production at higher temperatures because the enzyme production of heterotrophic microorganisms requires energy whose gain is associated with formation of CO_2. Even though logical, high degree of scepticism needs to be applied due to methodological and conceptual limitations of potential enzyme activity measurements (Nannipieri et al., 2018).

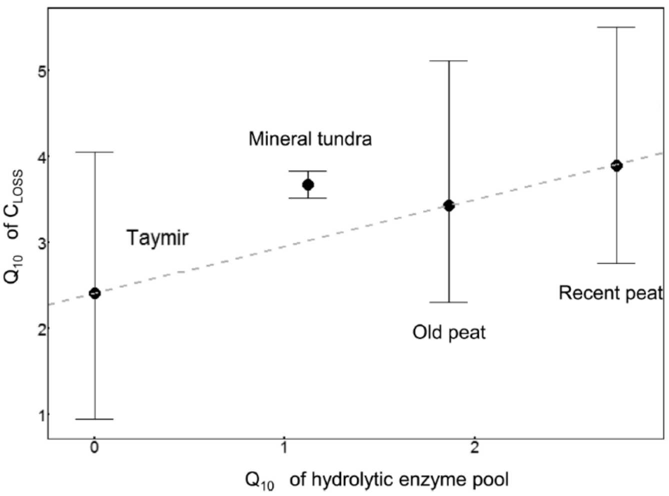


Figure 5: *Relationship between temperature sensitivity (Q_{10}) of C_{LOSS} and pool of hydrolytic enzymes. The relationship was described by linear regression with following parameters: slope = 0.49, intercept = 2.65 and adjusted R^2 = 0.64. Full circles show mean values, and vertical bars represent standard errors.*

As much as the microbial biomass itself is important, treating it as a static equation variable that is independent of soil conditions is sufficient to explain only a certain fraction of variability in collected data. To improve explanatory power, a detailed treatment of microbial physiology is required. I will do so using a concept of ecological stoichiometry (Sterner and Elser, 2002) in the following chapter because I will hereafter assume that the microbial physiology is significantly affected by soil nutrients.

Transformation of organic forms of nutrients into inorganic ones

Focussing closely on microbial physiology inevitably increases the complexity of the description. However, the benefit of doing so increases much more. It allows us to expand the list of important consequences of the presence and activity of microorganisms that can be treated at once. The other important consequence I will focus on is the mineralisation of nutrients, i.e. the transformation of organic forms of nutrients into inorganic forms. The inorganic forms could be used by plants or leached from soil to water bodies and thus, affect their chemistry. This is a very important ecosystem process, which has been estimated to cover approx. 58% and 98% of all nitrogen (N) and phosphorus (P) demand of terrestrial vegetation (Cleveland et al., 2013). I will assume that soil microorganisms are solely responsible for mineralization first and define exceptions to this assumption later.

The third statement of the Introduction section on heterotrophic soil microorganisms that transform organic material to survive and eventually grow makes these microorganisms dependent on the chemistry of their food source. To grow, all essential nutrients must be extracted from organic material. It becomes insufficient to only know SOC and microbial biomass in units of C per e.g. gram of dry soil. The content of other nutrients in the organic material and microbial biomass must be known as well. This content can be specified in relative terms as various ratios. The content of any nutrient, for example N, can be expressed with respect to any other nutrient/element. Nevertheless, the most convenient approach is to express it with respect to C for now. We have C to N ratio of organic substrate that the microorganisms feed on (denoted as $C{:}\,N_S$) and microbial C to N ratio (denoted as $C{:}\,N_B$). $C{:}\,N_S$ depends on chemical composition of organic substrate (e.g. different amino-acids have different N contents). Soil microorganisms need both, C and N from that substrate unless they live in association with plants that feed them to certain extent. If the flux of organic C from soil to microbial biomass is specified as uptake U_C, the consumption of N by microorganisms feeding on that substrate can be simply calculated as $U_C \times \frac{1}{C:N_S}$. Notice that the identity of substrate is not specified (see later). It is assumed that the organic compounds dissolved in soil solution have approximately the same C to N ratio as the entire organic material (which is not always true). It is also possible to define how much N soil microorganisms need to grow at certain rate using similar rationale defined for uptake. Defining growth rate in units of C per e.g. gram of dry soil and time as G_C, the N demand required to achieve such growth rate is $G_C \times \frac{1}{C:N_B}$. Comparing the two calculated N fluxes, N uptake and demand, allows to guess, for example, if the substrate contains enough N to make soil microorganisms growing at the rate G_C. This very simple comparison represents an essential ingredient of prediction of nutrient mineralization in soil (Manzoni et al., 2008). It is important to stress that the term **"mineralization" is defined here in respect to nutrients only**. Therefore, this term should not be confused with "C mineralization", another term which is sometimes used to refer to respiration in soil literature.

Mineralization (change in concentration over time in more precise terms) **of any nutrient** denoted as E can be defined by an equation (Manzoni et al., 2008):

$$\Delta E = U_C \times \left(\frac{1}{C:E_S} - \frac{CUE}{C:E_B}\right) \qquad \text{(eq. 6)},$$

in which the yet undefined variable CUE represents C use efficiency, intrinsic microbial characteristic, which was already specified in fig. 2 without explanation. Paradoxically, there is a high ambiguity in literature about what exactly CUE represents and how it could be estimated. This incredible ambiguity made us to collect all possible definitions and select the most convenient one from this list **(Manzoni et al., 2018)**. Convenient in terms of eq. 6 is to define CUE as the fraction of U_C, which is converted to microbial biomass (i.e. $\mathrm{U_C \times CUE = G_C}$; **Manzoni et al., 2018)**. The other fraction (i.e. $1 - CUE$) is respired as CO_2. U_C is a hyperbolic or simply multiplicative function of microbial biomass and substrate concentration (e.g. Allison et al., 2010; Manzoni and Porporato, 2009; Menkel and Knights, 1995). Knowing microbial biomass and substrate concentration therefore makes theoretically possible to predict mineralization of any nutrient as well as respiration rate using two variables that characterize chemical composition of the biomass and substrate ($C{:}\,E_S$ and $C{:}\,E_B$), and one variable, which indirectly characterizes energetics of microbial growth (CUE). It is important to stress that net change in concentration of inorganic forms of nutrients (ΔE) could be positive as well as negative. When substrate contains very little nutrients (lot of carbohydrates or lipids, so $C{:}\,E_S \gg C{:}\,E_B$), ΔE is negative and microbial biomass needs to compensate deficiency of nutrients in organic substrate by consumption inorganic forms of nutrients, a process, which is referred to as nutrient immobilization in soil literature. It logically follows that inorganic nutrients must be present in soil solution to make this possible (see later). After abandonment of eq. 1, eq. 6 will serve as the next level of complexity, which will be dissected later. Before that, I will document the usefulness of this simple equation to explain conflicting patterns in effects of fertilization on plant production across terrestrial ecosystems, knowledge, which could be considered important for human population.

The comparison between resource stoichiometry and nutrient demand of organisms can be performed with respect to any two elements of interest and any individual organism or population of organisms. In the following case study, the two elements of interest are N and P, and two populations of organisms are soil microorganisms and plants. Almost two hundred years ago, in 1840, Justus von Liebig defined his Law of the minimum. This law can be derived using similar considerations that lead to formulation of eq. 6. The simple comparison of the N to P ratio of plant nutrient demand (defined as N to P of leaves; **Čapek et al., 2018)**, with N to P ratio of soil resources allows to identify which of the two nutrients is the limiting in respect to plant demand assuming that only these two nutrients may be limiting. Adding the limiting nutrient, not the other one, to the soil is expected to improve plant growth/yield. There are thousands of fertilization experiments all over the world whose meta-analysis, however, suggests something else. In majority of fertilization experiments, the addition of both N and P increases plant growth roughly to the same extent (Elser et al., 2007). In several cases, the addition of N or P decreases plant yield (Elser et al., 2007; Harpole et al., 2011). The empirical data thus put the validity of Liebig's Law of minimum and the rationale of eq. 6 into question. There have been several attempts to explain the empirical observations (e.g. Elser et al., 2007; Farrior et al., 2013) but none of these explanations consider soil nor soil microorganisms explicitly. The reason has probably the same

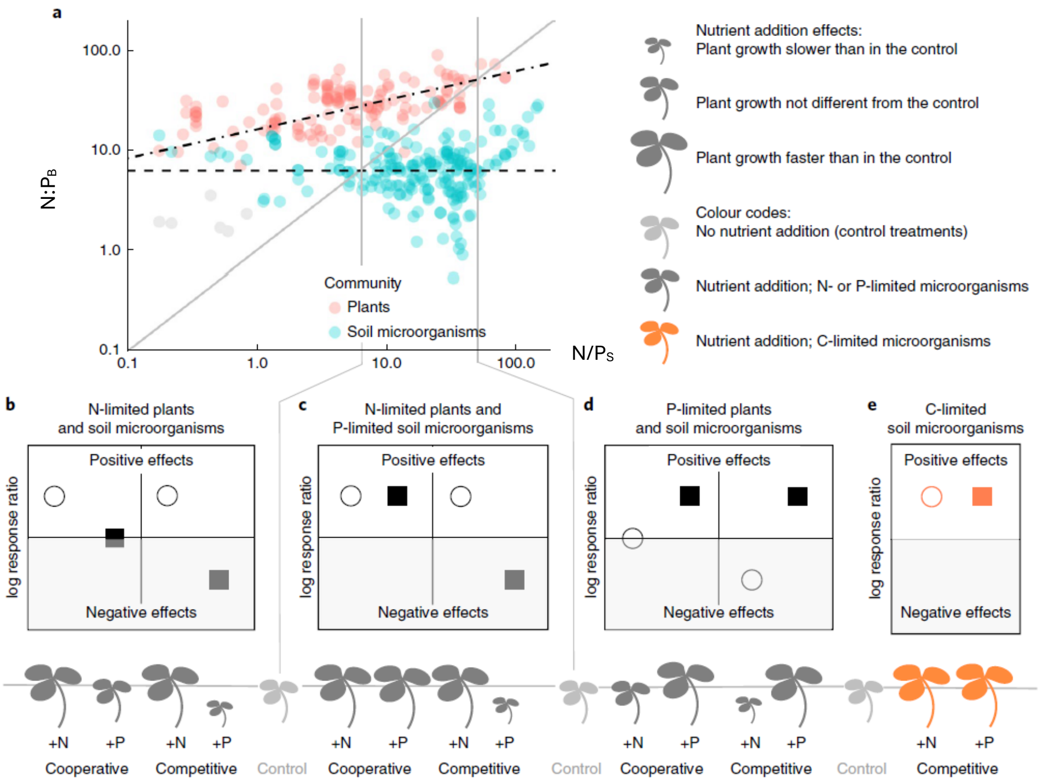


Figure 6: Consequences of differential plant and microbial nutrient limitations. a) The relationship between nutrient demand (biomass N:P ratio, $N:P_B$) of plants (red points, $n = 134$) and soil microbial communities (blue points, $n = 199$), and soil N:P ratio ($N:P_S$). The grey line represents the one-to-one relationship; the black dashed line represents the mean microbial $N:P_B = 6.3$ and the black dot–dashed line represents linear relationship between $N:P_S$ and plant $N:P_B$. Grey points designate six outlier values of plant $N:P_B$ (including them would not alter our conclusions). Note that both axes have a logarithmic scale. b–e) Expected responses of primary production (calculated as log response ratios, logRR) to N (open circles) and P (closed squares) fertilization within seven categories of different soil conditions. Orange symbols illustrate the expected response ratios when competitive soil microorganisms are C-limited. The horizontal line indicates a logRR of 0, which denotes no change; logRR lower than 0 indicates a net decrease (negative effects), whereas logRR greater than 0 indicates a net increase (positive effects).

root as the temperature sensitivity of soil respiration discussed above. The concentration of nutrients in soil and soil microbial biomass is not measured in majority of fertilization experiments. In 2018, I have been able to identify 51 studies out of thousands, which report some meaningful data on soil and soil microbial biomass, and which could be used to test the following theoretical framework **(Čapek et al., 2018)**. The framework depicted on fig. 6 considers plants and soil microorganisms as the two most important populations of organisms in terrestrial ecosystems that can either compete or cooperate. The nutrient demand of these populations is different due to different body/cell compositions (fig. 6a). Microorganisms need more P in respect to N than plants, i.e. their $N\colon P_B$ is lower. Along the gradient of stoichiometry of soil nutrients ($N\colon P_S$), plants and microorganisms can be limited by the same nutrient (fig. 6b and d) or plants can be N limited while microorganisms are P limited (fig. 6c). If content of organic material in soil is low, microorganisms could be eventually limited by C (fig. 6e). There are two modes of interaction between plants and soil microorganisms, they either form symbiotic relationships and cooperate, or compete for the same pool of nutrients in soil. Importantly, soil microorganisms always outcompete plants. The role of soil microorganisms as particularly strong competitors has been suggested by experimental studies (Schmidt et al., 1997a; Schmidt et al., 1997b), one meta-analysis (Kuzyakov and Xu, 2013), and our research of arctic soils **(Wild et al., 2018)**. As shown on fig. 6 b-d, the effect of fertilization can be explained by Libeig's Law of the minimum anytime the nutrient that is limiting plant growth is added. If the non-limiting nutrient is added, the effect of fertilization depends on soil microorganisms and that effect could be guessed using rationale of eq. 6.

If soil microorganisms are N limited, $N\colon P_S < N\colon P_B$. It implies that soil microorganisms will tend to immobilize N from the soil. Thus, when plants and microorganisms are both N-limited (fig. 6b), plants competing with soil microorganisms should not perform very well. The situation gets worse for plants when P is added to soil (fig. 6b). $N\colon P_S$ becomes much lower than microbial $N\colon P_B$ ($N\colon P_S \ll N\colon P_B$) and that makes the tendency of microorganisms to immobilize N greater. Adding P is thus expected to decrease plant growth, causing the negative effect of fertilization (fig. 6b, right half of the plot). No effect of P addition is expected when plants and soil microorganisms cooperate because microorganisms have no reason to harm their symbionts (fig. 6b, left half of the plot). If the microorganisms are P limited while plants are N limited, P addition increases microbial growth. This could affect plant growth in opposite directions depending on the type of relationship the two populations have (fig. 6c). The increased growth of plant symbionts is expected to improve growth of plants as well (fig. 6c, left half of the plot). The similar increase of plant growth/yield following N as well as P addition observed in meta-analysis of Elser et al. (2007) does not need to strictly imply that Liebig's Law of the minimum is violated. It may imply instead that two populations of organisms, each having different $N\colon P_B$, are present in a terrestrial ecosystem and their reaction to fertilization affects each other. If the two populations compete and not cooperate, improved growth of soil microorganisms following P addition causes decrease of plant growth simply because P limitation of microorganisms is switched to N limitation, i.e. $N\colon P_S > N\colon P_B$ changes to $N\colon P_S < N\colon P_B$ (fig. 6c, right half of the plot) so microorganisms start to immobilize N that limits plant growth. The situation at $N\colon P_S$ being higher than microbial as well as plant $N\colon P_B$ (fig. 6d) is mirror to that when $N\colon P_S$ is lower than microbial and plant $N\colon P_B$ (fig. 6b). At last, when microorganisms are C limited, any nutrient addition will improve plant growth because mining of limiting C from organic material by microbes causes mineralization of both N and P (fig. 6e). So, all possible effects of fertilization on plant growth are conceivable by acknowledging that soil microorganism may positively (as a result of cooperation) or negatively (as a result of competition) affect plant growth depending on initial $N\colon P_S$.

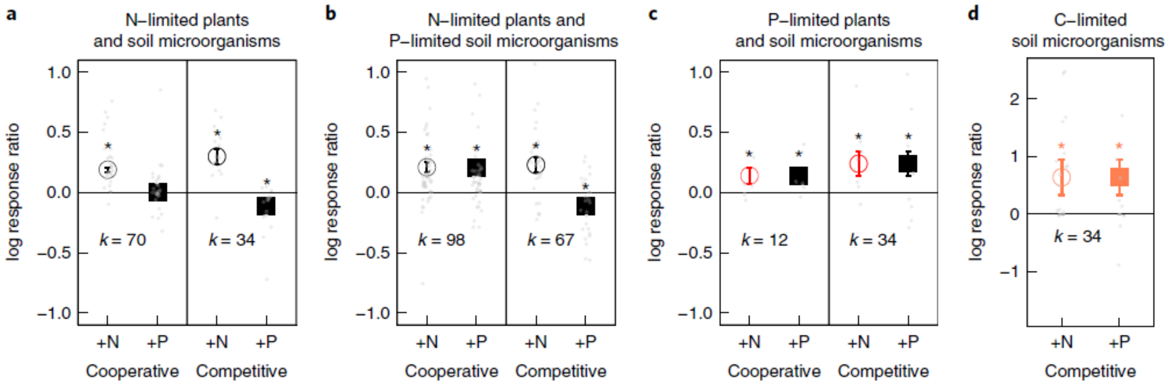


Figure 7: *Correspondence between the theory and published data. Observed response of primary production (calculated as logRR) to N (open circles) and P (closed squares) fertilization within seven categories of different soil conditions. Orange symbols represent the response ratios when competitive soil microorganisms are C-limited. The horizontal line indicates logRR of 0, which denotes no change. logRR lower than 0 indicates a net decrease, whereas logRR greater than 0 indicates a net increase. The number of observations (k) are indicated for each category. All estimates marked by * are significantly different from zero at P ≤ 0.05. Error bars represent standard errors. Red points indicate when observations are not in agreement with our expectations (fig. 6). Grey dots represent the original response ratios.*

The meta-analysis of the 51 fertilisation experiments largely confirms the validity of described framework (fig. 7). It confirms that, in general, soil microorganisms could affect the concentration of soil nutrients and thus plant growth, following the rationale of eq. 6. Although logical, the theoretical framework represents a very rough simplification. Sunlight, temperature, soil moisture, and all other nutrients are assumed to be at their optimum. None of these assumptions could be proved unequivocally in most studies. The magnitude of simplification is so high that some of the co-authors of our study remain very sceptical about the validity of the framework. The framework performs reasonably well across the large gradient of $N{:}P_S$ very likely because such a large gradient dominates the multidimensional variability of soil conditions across studies. Closer look on fig. 7 reveals several inconsistencies between the theory and observations. It is thus, necessary to conclude that even though microbial biomass importantly affects nutrient concentration in soil, and that the rationale of eq. 6 can explain several patterns of fertilization effect on plant growth thanks to soil microorganisms, a lot of residual variability remains unexplained. In next section, I will attempt to explain some of this residual variability by various microbial adaptations to nutrient scarcity. I will inspect variables of eq. 6 one by one and define how each of them could vary and why. Next, I will acknowledge the methodological limitations as a significant source of uncertainty that could cause deviations of observations from the theory.

Microbial adaptations to nutrient scarcity

Carbon use efficiency (CUE)

CUE is probably the most studied and controversial characteristic of soil microbial biomass (Geyer et al., 2019; Hagerty et al., 2018; He et al., 2024; **Manzoni et al., 2018**; Tao et al., 2023; Xiao et al., 2024). It is known that CUE varies considerably for various reasons (e.g. Geyer et al., 2019; **Manzoni et al., 2018**). Apart from the technical/methodological reasons (see later), one axis of this variability is undoubtedly associated with adaptation of microbial community to nutrient scarcity **(Manzoni et al., 2017)**. In previous section, growth of soil microbial biomass per C basis was defined as $U_C \times CUE = G_C$. The nutrient demand was then defined as $G_C \times \frac{1}{C{:}E_B}$ and it was assumed that deficiency of nutrients in organic substrate could be compensated for by consumption of inorganic forms of nutrients (i.e. $-\Delta E$). It is, however, not entirely reasonable to assume that any soil at any depth contains enough inorganic nutrients to sustain certain growth rate of nutrient deficient microbial biomass. It may happen that some soils do not contain enough nutrients so the $-\Delta E$ calculated by eq. 6 is not feasible to attain.

Saying by other words, defining G_C as $U_C \times CUE$ is reasonable only as long as soil microbial community is C limited, not nutrient limited. It is necessary to acknowledge possible effect of nutrients on G_C using some response function φ (G_C becoming defined as $U_C \times CUE \times \varphi$), which could, in its simplest form, look like this **(Manzoni et al., 2017)**:

$$\varphi = min\left[1, \frac{-I_E}{U_C \times \left(\frac{1}{C:E_S} - \frac{CUE}{C:E_B}\right)}\right] \qquad \text{(eq. 7).}$$

In eq. 5, I_E represents flux of inorganic nutrient E from soil solution to microbial biomass, i.e. inorganic nutrient uptake. This inorganic nutrient uptake is divided by the theoretical inorganic nutrient demand defined in eq. 6. The value of the response function is either 1 or less than 1 depending on how much of the nutrient demand can be covered by I_E. The growth of the of microbial community could now be limited by C or a nutrient according to the equation:

$$G_C = CUE \times \min\left[U_C, I_E \times \left(\frac{CUE}{C:E_B} - \frac{1}{C:E_S}\right)^{-1}\right] \qquad \text{(eq. 8).}$$

The last term of eq. 8 in square brackets has one important implication. The demand of microbial biomass for inorganic nutrients is in the denominator. The greater the denominator is, the lower the nutrient limited growth rate. It makes no sense to have an enormous demand for inorganic nutrients in conditions of nutrient scarcity. Looking on eqs. 7 and 8, it is possible to identify three theoretical mechanisms how to maximize φ (being ideally 1). The nutrient content of microbial biomass could be reduced ($C: E_B$ increases) so the nutrient demand gets lower (Butler et al., 2025), microorganisms could pick a dissolved organic substrate with high nutrient content somehow ($C: E_S$ decreases; **Čapek et al., 2021a**) or they could decrease their CUE. All three possibilities are feasible, but I will focus on the last one because this section is dedicated to CUE and CUE is known to vary a lot. If $C: E_S$ and $C: E_B$ are constant, the optimal CUE ($CUE_{Optimal}$) can be derived from eq. 8 (**Manzoni et al., 2017**):

$$CUE_{Optimal} = \min\left[CUE_{Maximal}, C: E_B \times \left(\frac{1}{C:E_S} + \frac{I_E}{U_C}\right)\right] \qquad \text{(eq. 9).}$$

In eq. 9, $CUE_{Maximal}$ is some thermodynamic maximum, frequently assumed to be around 0.8 (Dijkstra et al., 2015; Roels, 1980). $CUE_{Optimal}$ (i.e. optimal at nutrient limited conditions) is expected to decrease from this maximum value when $C: E_B/C: E_s$ decreases (or its reciprocal $C: E_S/C: E_B$ increases as plotted in fig. 8). Physiologically, this can be achieved for example by changing the resistance of membrane across which the proton-motive force driving ATP production (under aerobic conditions) is generated

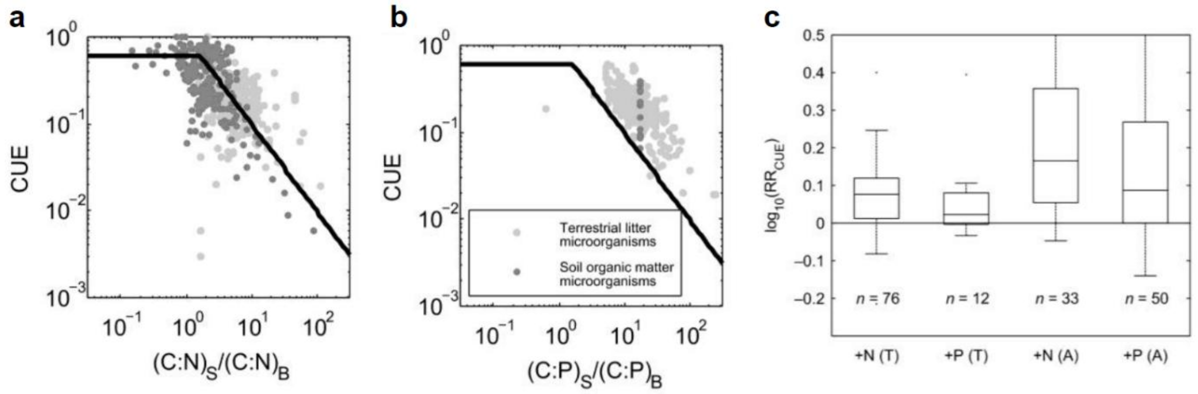


Figure 8: *C use efficiency (CUE) estimates for terrestrial microorganisms as a function of the substrate C:N (a) and C:P ratio (b) normalised by the organism elemental composition (i.e. biomass C:N and C:P, respectively). (c) log-response ratios of CUE for inorganic nutrient amendment (RR_{CUE} = CUE in fertilised samples over CUE in control samples; log-response ratio expected to be larger than 0 in nutrient-limited conditions) to terrestrial ecosystems (T). The central mark represents the median, the edges are the quartiles, and the whiskers span the data range (excluding outliers, indicated by dots). Scales on the y-axis differ among panels.*

(Cook and Russell, 1994). Eq. 9 also somewhat logically expects that nutrient addition should increase $CUE_{Optimal}$ at the conditions of nutrient limitation. Both predictions can be directly tested thanks to the fact that CUE is so distinguished physiological parameter that a lot of soil scientists measure it.

The meta-analysis of available data proves both predictions of eq. 9 to be right (fig. 8). Changing the CUE with concentration of nutrients in soils (both their organic and inorganic forms), it is possible to finally start explaining some of the residual variability identified earlier. Rates of respiration, nutrients mineralization and immobilization per unit of microbial biomass can all vary as function of CUE. At constant microbial biomass and substrate concentration, respiration rate defined as $U_C \times (1 - CUE)$ may increase when nutrient concentration decreases or inorganic nutrient uptake decreases (as shown by e.g. Mormile et al., 2013; Spohn and Chodak, 2015). If this decrease occurs along the gradient of temperature, the temperature sensitivity of soil respiration increases and vice versa. The term "optimal" suggests that microorganisms modify CUE so that their nutrient demand adapts to concentration of nutrients in soil. This may further suggest that microorganisms do not need to always compete with plants for nutrients as strongly as anticipated or that nutrient addition unambiguously relieves nutrient limitation of microorganisms. CUE has been shown to vary between approx. 0.05 and 0.8 (**Manzoni et al., 2018**) so microorganisms with $C\colon N_B$ around 5 can theoretically adapt to $C\colon N_S$ between 12.5 and 200. It is, however, important to note that low CUE may deplete organic C, i.e. source of C and energy for growth, because the loss of C from soil is disproportionately high (as experimentally shown by Walker et al., 2018). Nevertheless, soil microorganisms have more convenient adaptations, that can avoid this to happen. They can modify the $C\colon E_S$ to better suit their needs.

Nutrient content of organic substrate ($C\colon E_S$)

The insoluble organic debris that soil microorganisms feed on contains some nutrients. This content was defined as $C\colon E_S$ in previous sections and it was assumed that the dissolved organic substrates roughly retain nutrient content of the organic debris, which they are derived from. Such an assumption is convenient, but the reality is very different. As stated in the Introduction, the solubilization of organic debris is, among others, performed by extracellular enzymes. There is no magical universal enzyme, which would cut organic debris into same soluble pieces with $C\colon E_S$ of original organic material. There are many enzymes with specialized function simply because the organic debris is composed of many different chemical compounds with different characteristics (Burns and Dick, 2002). The most abundant polymer in nature, cellulose, is depolymerized to glucose dimers by cellobioses or to glucose monomers by β-glucosidases. Another abundant polymer of β-1,4-N-acetylglucosamine known as chitin is depolymerized by N-acetylglucosaminidases (Cohen-Kupiec and Chet, 1998). Lignin and other polyphenolic compounds are oxidized and unevenly broken into smaller units by phenoloxidases and peroxidases (Sinsabaugh, 2010). Ester bonds between C and P are subject of hydrolytic attack by phosphomonoesterases (Nannipieri et al., 2011). There are also several aminopeptidases, which depolymerize proteins into monomeric amino acids (e.g. Suzuki et al., 2001). Focusing on extracellular enzymes increases the complexity of the discussion to such an extent that it becomes impossible to cover it exhaustively (we just started touching the complexity of soil environment). Moreover, there are many misconceptions, controversies and misunderstandings about the soil enzymes in literature (e.g. Moorhead et al., 2023; Mori, 2020; Mori et al., 2021; Nannipieri et al., 2018; Schimel et al., 2022; Sinsabaugh et al., 2013, 2009). For that reason, I will not attempt to go into detail. I will only state some theoretical considerations, which make the eq. 6 to not perform adequately leaving the issue open for future research:

1. Combined effect of activity of various enzymes causes $C\colon E_S$ of dissolved organic substrate to deviate strongly from $C\colon E_S$ of organic debris. In respect to eq. 6, it is thus reasonable to consider $C\colon E_S$ of dissolved or extractable forms of organic material (**Čapek et al., 2021a**) instead of entire organic material as done in earlier studies (Manzoni et al., 2008).
2. Production of extracellular enzymes requires energy, C and N (enzymes are proteins) so it affects CUE as well as N demand of microorganisms to an extent, which no one can even guess regardless the few apparently reasonable attempts that have been made (Calabrese et al., 2022; Kaiser et al., 2015; Malik et al., 2019). At the moment, the frequently applied and probably very inaccurate simplification is that the enzyme production makes approx. 5% of G_C (Allison et al., 2010).

3. Several enzymes make inorganic forms of nutrients to be released from organic material directly into soil solution disobeying the mechanism of eq. 6. This has traditionally been considered critical for P, which is released as PO_4^{-3} from organic material to soil solution due to phosphomonoesterases (**Manzoni et al., 2017**; McGill and Cole, 1981). Nevertheless, the same issue can be documented in respect to N (Barraclough, 1997) even though majority of soil scientists ignore it ever since the publication of Schimel and Bennett (2004), which is rather unconvincing to my opinion. To acknowledge this issue, we tend to argue that the production of specific extracellular enzymes is dictated by the microbial nutrient demand (Allison and Vitousek, 2005). In addition, the organic compounds with specific terminal functional groups that are attacked by enzymes are wrapped in moiety of other organic compounds and these compounds have to be depolymerized at the same time so the difference between $C{:}E_S$ of soluble and insoluble organic material has its limits. Thus, eq. 6 is obeyed to certain extent but there is some shift as shown on fig. 8a, b for example.
4. Extracellular enzymes are not produced by microorganisms but also by plants (e.g. Marklein and Houlton, 2012). It is therefore not entirely true that soil microorganisms are solely responsible for mineralization of nutrients nor that plants are ultimately dependent on that process. Also, certain fraction of soluble organics and/or nutrients can be released abiotically as already mentioned in the introduction section.

The theoretical concept developed so far performed fairly well in several cases, so it is reasonable to ask how important all theoretical inaccuracies listed above are in quantitative terms. I will document this on fig. 7c. Our theoretical concept of plant-microbe interaction along the gradient of $N{:}P_S$ did not perform as expected at conditions of P limitation. At these conditions, I expected that N addition should either decrease plant growth due to increasing $N{:}P_S$ or cause no change. The data, however, shows significant positive effect of N addition as if it does not increase the microbial demand for additional P. In our study, we explained the obvious discrepancy between theory and data by an extracellular enzymes production (**Čapek et al., 2018)**. We argued that N addition could cause the production of extracellular phosphomonoesterases by soil microorganisms and plants. These enzymes mediated the release of P from organic material relieving the P limitation as suggested by (e.g. Marklein and Houlton, 2012). Although reviewers and editor accepted our explanation, it is worth noting that it is a pure speculation. Other soil scientist could come up with alternative, similarly credible but similarly speculative explanation. We made 14 different predictions for 14 different categories (figs. 6 and 7), and the theory did not correspond with the data only in 2 cases (fig. 7) thus, in 14%.

To extend the theory further, I will focus on soluble organic compounds and their N content. As explained above, these compounds derive from insoluble organic material by a mechanism(s), which are impossible to describe with reasonable accuracy, so I will make a shortcut. It is possible to measure soluble organic compounds (similarly as microbial biomass) and quantify their N content without exactly knowing the mechanisms of their origin and thus, we may study their effect on microbial biomass physiology. There could be several different soluble organic compounds with different N contents in soil solution and all of them could be theoretically consumed by microorganisms. U_C represents the sum of all C atoms of all compounds that have been taken up. $C{:}N_S$ is then dictated by relative contributions (denoted by symbol f) of those compounds to U_C and their specific N contents. Of course, some compounds, such as glucose, have no N so their $C{:}N$ is undefined. Therefore, I will refer to categories of compounds with some N content rather than to specific well defined chemical compounds. This approach is analogical to categorization of organic C pools according to inherent turnover rates in classical models. If there are n different categories with different $C{:}N$, supply of organic N to microorganisms is $C{:}N_S = e^{\sum_{i=1}^{n} f_i \times ln(C{:}N_i)}$ (see for explanation of bias of untransformed ratios; Isles, 2020). In theory, soil microorganisms may optimize their N supply by changing relative contributions (f) of organic compounds in the mix of compounds they consume. It could be achieved by either production of extracellular enzymes whose activity increases abundance of a category of compounds with high N content in soil solution (Reuter et al., 2020) or by selective uptake of organic compounds with high N content (causing uptake being independent of compound abundance in soil solution) (Zhang et al., 2015). Such microbial adaptation would be convenient because CUE does not need to decrease that much (as depicted on fig. 8). To prove an existence of such adaptation is, however, difficult. Even though compound specific analyses of soil solution as well as enzyme essays are available, identification of relative contributions of different organic compounds to microbial uptake is challenging (Chakrawal et al., 2025). Our research suggests that another simplification circumventing the methodological/technical issues associated with quantitative estimation of organic compounds contribution to microbial uptake could be made. We showed that the two broad categories of organic compounds with different $C:N$ can be defined and their f directly measured. One category is represented by all soluble organic compounds in soil solution, and the second is represented by microbial biomass **(Čapek et al., 2021a).** In soil, microbial biomass is constantly growing and dying (Chapman and Gray, 1986; Van Veen et al., 1987). The dying microbial biomass can become substrate for growing microbial biomass. This so called "cryptic growth" (Chapman and Gray, 1986) causes the microbial N to be reused all over again (Kaiser et al., 2014). The relative contribution of both categories in the mix of compounds that active microbial biomass use could be quantified using an analysis of stable isotopes (fig. 9). There is a consistent difference in $\delta^{13}C$ of soil organic C and microbial biomass C (fig. 9, see also Coyle et al., 2009). Assuming negligible isotope fractionation (Ekblad et al., 2002) and difference in use efficiency of both categories of substrates for simplicity, the closer the $\delta^{13}C$ of respired CO_2 to $\delta^{13}C$ of microbial biomass C is, the greater the contribution of microbial biomass denoted as f_{MBC} in legend to fig. 9. The contribution of the other fraction, i.e. soluble organic substrate is then $1 - f_{MBC}$. When f_{MBC} is zero (microorganisms consume only soluble organic substrate and not dead microbial biomass), we get eq. 6. We developed this theory using soils from Czech glacial lakes' catchments in Bohemian forest - Plešné and Čertovo. The soils were sampled from two different soil horizons (litter and organic topsoil horizon) and then mixed in different ratios (fig. 9). The soil selection and subsequent soil mixing formed a gradient of initial soluble organic $C:N$ and inorganic N while microbial biomass C and its $C:N$ was nearly invariable across horizons and mixing treatments. Ideal conditions for testing validity of eq. 6 at different initial concentrations of inorganic N. We found that eq. 6 can predict the change in concentration of inorganic N in soil solution well anytime there was at least some inorganic N in soil solution at the beginning of 48 hours long incubation (**Čapek et al., 2021a**). When inorganic N concentration was initially very low and $C:N$ of soluble organic compounds in soil solution high at the same time, eq. 6 did not performed well unless measured f_{MBC} was explicitly acknowledged **(Čapek et al., 2021a)**.

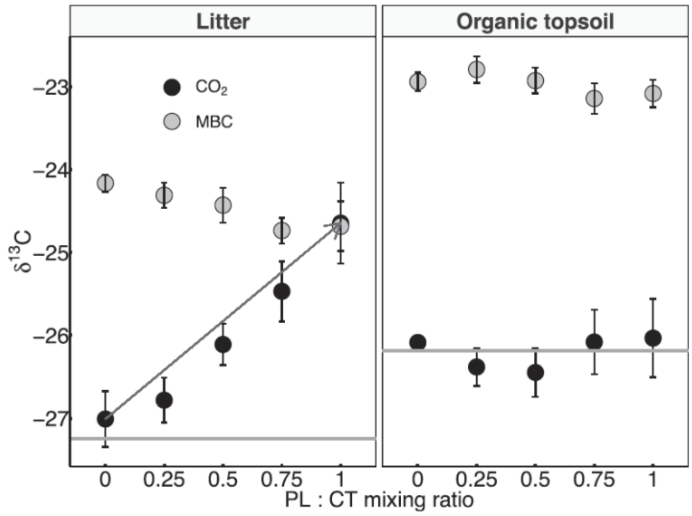


Figure 9: *Isotopic signals of respired CO_2 (black symbols) and microbial biomass carbon (MBC, grey symbols) in the litter and topsoil organic horizons of two spruce forest soils (PL - Plešné and CT – Čertovo catchments) mixed at five different ratios (i.e., 0:1, 0.25:0.75, 0.5:0.5, 0.75:0.25, and 1:0 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. The solid arrow shows the change in the isotopic signal of respired CO_2 across litter mixtures that was used to calculate f_{MBC}.*

Reuse of N allows soil microorganisms to become partially independent of soil N supply (both organic and inorganic). In theory, optimal f_{MBC} could be derived similarly as optimal CUE (eq. 9) assuming no net change of microbial biomass (i.e. net increase would require additional N). That leads to an important question. Why would soil microorganisms optimize CUE to avoid nutrient limitation when this strategy causes the decrease in effectivity of their energetic metabolism in short term and amount of their food (i.e. organic C) in long term. N could be reused instead. This is an interesting challenge for future research. I believe that there are four phenomena, which may limit the possibility of N reuse. The first phenomenon is a spatial distribution. Microbial biomass and their food are distributed unevenly in soil so the paradox of physical disconnection is frequently highlighted as a being very important (e.g. Schmidt et al., 2011; Shi et al., 2020). Dying part of microbial biomass could be physically separated from the growing part making the possibility of N reuse limited. Second, organic N is located behind the wall of structural organic compounds, i.e. cellulose and/or lignin, in fresh plant litter (Berg and McClaugherty, 2020b). The wall needs to be removed first, and the removal necessarily causes temporal increase in concentration of soluble organic compounds without any N. At these conditions, the CUE must decrease due to excess of organic C in microbial metabolism and no net growth of the microbial biomass. In fact, most of the variability in $C{:}E_S/C{:}E_B$ visualized in fig. 8 is associated with changing chemistry of plant litter across the ecosystems and time (i.e. age of the litter). Third, not all microbial N can be reused in the short term. Some fraction of microbial N is composed of amino sugars that form microbial cell walls. These compounds are known to have a long turnover time (Joergensen and Wichern, 2025) and their accumulation is hypothesized to cause build-up of insoluble, almost recalcitrant N in soil over time (Kaiser et al., 2015). Thus, every time the microbial biomass turns over, certain fraction of N is lost so not all the microbial N can be reused indefinitely. Fourth, there could be confounding effect of soil fauna, specifically the microbial feeders. It has been shown experimentally that the presence of microbial feeders significantly decreases "CUE estimates", which we tend to interpret as a microbial-specific CUE (Fry et al., 2019). The pattern of decreasing CUE along the gradient of increasing $C{:}E_S/C{:}E_B$ could be partially driven by the confounding gradient in abundance and activity of microbial feeders. However, the microbial feeders are expected to increase the nutrient mineralization at the same time (e.g. Ekelund et al., 2009). The trend of decreasing CUE should be therefore accompanied by an increase in mineralization, but that is usually not observed (Manzoni et al., 2010).

Microbial biomass stoichiometry ($C{:}E_B$)

Eq. 6 assumes that soil microorganisms are homeostatic with respect to their nutrient content. Their $C{:}E_B$ is expected to be constant and reflect their nutrient demand. This assumption is violated when soil microorganisms form and utilize reserve compounds such as polyhydroxybutyrate (C storage) and polyphosphate (P storage), or their growth rate changes. The metabolism of storage compounds (Dawes and Senior, 1973) as well as changes in microbial biomass chemistry along the gradient of growth rate (Herbert, 1961) have been thoroughly studied in pure cultures. Changes in chemical composition of soil microbial biomass are frequently not considered even though they significantly impact the microbial physiology **(Čapek et al., 2024, 2023)** and associated soil processes (Manzoni et al., 2021). In last two decades, a lot of soil scientists rather focused on changes in species composition of microbial biomass, which should affect $C{:}E_B$ too (e.g. Mouginot et al., 2014). However, this research was not very rewarding so far because information about species composition does not seem to provide anticipated extra benefits for quantitative prediction of soil processes (especially in respect to effectivity/cost ratio) (Bruni et al., 2025; Khurana et al., 2023). Targeted research of reserves metabolism of soil microorganisms seems to be more promising in this respect (Manzoni et al., 2021; Mason-Jones et al., 2021). Unfortunately, the research did not get too far yet.

In theory, the reserves form some sort of buffer. They could be formed in periods of excess and utilized when needed. They make soil microorganisms to be less dependent on changes in $C{:}E_S$. Their presence, however, makes $C{:}E_B$ (especially $C{:}P_B$ due to the fact that polyphosphates can make up to 20% of biomass dry mass) to vary lot (Godwin and Cotner, 2015a, 2015b). This is very unfortunate because measured $C{:}P_B$ then lacks informativeness. It does not reflect P demand of microbial biomass correctly because the P demand is defined in respect to growth. For that reason, the presence of polyphosphates in microbial biomass makes eq. 6 to completely fail to predict changes in concentration of inorganic P over time (**Čapek et al., 2021a**). These changes were, instead, simply predicted by adsorption of P to soil minerals although the microbial component should dominate (Olander and Vitousek, 2005).

Our research suggests that the intracellular polyphosphates can be found in soil almost everywhere. We have seen that several microbial communities sampled from all over the world grow quickly on organic substrate without any external source of P probably due to utilization of polyphosphates (**Čapek et al., 2016**). When we attempted to indirectly analyse presence of polyphosphates in soil samples from Czech glacial lakes' catchments in Bohemian forest, we found that they may constitute significant fraction of entire microbial P (up to 70%; **Čapek et al., 2024**). Because of polyphosphates, we miss a simple quantitative theoretical framework that could quantify/predict microbial contribution to P cycling in soil with a reasonable accuracy (the chemical contribution is well established). In this respect, the P is more problematic than N (**Čapek et al., 2021a)**. It is a challenge I would like to focus on in future.

Significance of methodological bias

Almost all the evidence discussed so far identify soil microorganisms as the most important drivers of organic material transformation in soil. The explicit representation of microbial biomass in biogeochemical models is a reasonable choice from that point of view. As argued by Allison et al. (2010), these models are, unlike the classical ones, flexible enough to simulate almost anything. But this is their greatest limitation at the same time. In the previous section, I showed that microorganisms employ several different strategies to deal with nutrient scarcity, and some of them overlap. These adaptations affect the rates of biomass specific respiration and nutrient mineralization/immobilization. Due to the high physiological flexibility of soil microorganisms, the microbially-explicit models can undoubtedly simulate almost anything, but it is very difficult to determine if the model fits the data well for good reasons. There is a high co-linearity between model parameters, so equally good fit to data could be reached by very different model settings (Sierra et al., 2015). When the model fits the data well for wrong reasons, the predictions of that model could be severely biased (see communication of for example He et al., 2024; Tao et al., 2023; Xiao et al., 2024). At this point, we are leaving the comfort of academic debate. In 2015, Wieder et al. (2015b) showed that implementation of ecological stoichiometry to global land models dramatically changes predictions of future land C storage. It turned the land from sink of atmospheric CO_2 to source (Wieder et al., 2015b). The anticipated sink strength of the land counterbalancing anthropogenic CO_2 emissions to large extent turned completely upside down. The extent of the controversy was so high that critical voices were raised soon after (Brovkin and Goll, 2015; Wieder et al., 2015c). To be sure that models fit the data for good reasons, a lot of different data products of high quality are required (Sierra et al., 2015). That should force all soil scientists to get back to the fundamentals of accurate data collection. In the following three sections, I will document that the fundamentals are, unfortunately, very often inaccurate.

Heterotrophic soil respiration

It seems nearly impossible to measure the rate of CO_2 production incorrectly. The soil is simply closed in a gas tight container, and the change of CO_2 concentration is determined. Thanks to gas chromatography, a tedious titration of residual NaOH, which remained unreacted with CO_2 in a closed container (Sparling and West, 1990), was abandoned and that allowed to analyse a lot of soil samples at once. However, the presence of a cup with NaOH made possible to estimate all forms of inorganic C inside the closed container at the same time while gas chromatography only determines the gaseous CO_2. The other forms of inorganic C, particularly H_2CO_3 and HCO_3^- dissolved in soil solution need to be calculated from gaseous CO_2 applying known equilibrium constants. Even though this issue was clearly articulated by Sparling and West in 1990, it has been ignored in many studies until now **(Čapek and Šantrůčková, 2024)**. As we showed in our response to one such study, ignoring CO_2 dissolution may significantly impact the estimation of our key microbial parameter, CUE **(Čapek and Šantrůčková, 2024**).

Extracellular enzymes

Extracellular enzymes cannot be measured at all, at least not quantitatively. Instead, soil scientists measure the potential enzyme activity. The principle is relatively simple. We assume that there are some free enzymes in the soil solution. The soil with free enzymes is turned into slurry, i.e. relatively homogenous substance, and artificial substrate degradable by some specific class of enzymes is added. The product concentration of an enzymatic reaction is measured and the rate of change of the product over time is reported as a potential enzyme activity. For example, if we intend to measure the potential activity of soil phosphomonoesterases, 4-methylumbelliferyl phosphate is added to the soil and the fluorescent product 4-methylumbelliferyl is determined (German et al., 2011). In this case, the phosphate and the fluorescent product are produced in 1:1 allowing us to determine the mass balance. Also, it is known that the abiotic degradation of substrate is negligible. In other cases, the situation is not that simple. To determine activity of phenoloxidases and, eventually, peroxidases, L-DOPA or ABTS are used as substrates (German et al., 2011). As the name of enzyme classes suggests, the reaction is oxidative. Unfortunately, there are many oxidizing agents in soil, and therefore, non-enzymatic degradation of the substrate occurs (Bach et al., 2013). In addition, products of enzyme degradation of L-DOPA or ABTS are not available. Therefore, there is no straightforward way to calibrate the assay and recalculate raw data to the concentrations of a reaction product. Soil scientists could either use some literature derived conversion factors (extinction coefficients) or define their own experimentally. However, the conversion factors are relatively variable (unpublished results), and it is very likely that the reaction stoichiometry is not fixed but stochastic to some extent. When another class of enzymes, leucine-aminopeptidases, are measured, L-Leucine-7-amido-4-methylcoumarin hydrochloride is used as a substrate. This substrate decays very fast (unpublished results) so some of the reaction product is added to the soil slurry together with the substrate.

Even if the reaction stoichiometry is fixed, a commercially available reaction product is purchased, and the substrate is degraded predominantly by enzymes, the accurate and easily interpretable results are not guaranteed. It is assumed that the enzyme reaction follows Michaelis-Menten kinetic defined by equation:

$$\frac{dP}{dt} = \frac{V_{max} \times S}{K_m + S} \qquad \text{(eq. 10),}$$

in which P is product concentration, S is substrate concentration, V_{max} is a maximum reaction velocity (defining the asymptote of hyperbolic function) and K_m is affinity constant. Technically, $\frac{dP}{dt}$ or $\frac{\Delta P}{\Delta t}$ is measured in soil slurry and reported. As eq. 10 suggests, $\frac{dP}{dt}$ is not always a unique characteristic of an enzyme because it depends on substrate, V_{max} and K_m. Any comparison between soils or treatments would be therefore useless without knowing assay conditions accurately. To allow more straightforward comparison of data, $\frac{dP}{dt}$ should be measured in such an excess of S that the eq. 10 could be simplified to $\frac{dP}{dt} = V_{max}$. At such conditions, zero order kinetic applies and the concentration of product at any time t ($P(t)$) is defined as $V_{max} \times t$ thus, slope of linear regression without the intercept between the product concentration and time is V_{max}. The reported potential enzyme activity should represent V_{max}. Since V_{max} is theoretically function of enzyme concentration and its catalytic efficiency, it is reasonable to treat V_{max} as being proportional to enzyme concentration (Calabrese et al., 2022; Malik et al., 2019). Unfortunately, insufficient S is often added so the reported $\frac{dP}{dt}$ is not always V_{max} (Margenot et al., 2018). Many soil scientists also simplify their enzyme assay dramatically. They add a substrate and measure product concentration only once assuming the linearity and absence of the intercept without controlling it directly. Especially the linearity could be, however, violated for several reasons.

One reason, which we directly identified, is the presence of biochemical inhibition **(Čapek et al., 2021b)**. Acid phosphomonoesterases in Bohemian Forest soils are inhibited by their own product (i.e. phosphate) and organic P in soil solution **(Čapek et al., 2021b)**. The change in concentration of inhibitors over time significantly affects the reaction rate causing the increase in product concentration over time to be non-linear. We also found the same issue with leucine-aminopeptidase, β-glucosidase and cellobiosidase (unpublished results). In theory, biochemical inhibition could be minimized by extra addition of substrate because the form of inhibition is usually competitive. But the theoretical concentration of this extra substrate is so high that it exceeds the solubility of 4-methylumbelliferyl based substrates. Also, the substrate excess may not work well because phosphomonoesterases could be additionally inhibited by high substrate concentration (Steen and Ziervogel, 2012).

When all of this is taken together, the only method we have to get some information about extracellular enzymes is rarely informative. The reported potential enzyme activities are vague numbers, which sometimes reflect V_{max}, sometimes V_{max}, K_m and S in different proportions and sometimes, some additional effect of biochemical inhibition. It all affects the interpretation of the data. One good example is the frequently reported relationship between concentration/addition of inorganic P and activity of phosphomonoesterases (e.g. Marklein and Houlton, 2012; Olander and Vitousek, 2000). The relationship is interpreted as evidence of constitutive production of phosphomonoesterases. The enzymes are produced when the inorganic P is low to get more P, and it stops (or reduces) once the inorganic P increases/is added. Using this concept, it is theoretically possible to apply another optimality principle, in respect to production of extracellular phosphomonoesterases, to better simulate changes in concentration of inorganic P in soil solution. However, the relationship between the enzyme activity and inorganic P concentration may equally likely reflect the presence of biochemical inhibition of phosphomonoesterases measured under non optimal reaction conditions. Our optimality principle would be wrong because the roughly constant enzyme concentration in soil may simply generate different activity towards added artificial substrate depending on background concentration of inorganic P. The two interpretations thus, generate two models with different structures and model parameters. Both models would very likely fit the experimental data reasonably well but for wrong reasons in one or the other case.

To make the enzyme assay more informative, we argued that all kinetic constants of a reaction should be always determined (including inhibition constants), and we provided general recommendations on how to do it **(Čapek et al., 2021b)**. Of course, that would make the assay more tedious so fewer samples could be analysed at once. The reviewers as well as the editor, which handled our study, expressed a high degree of scepticism. None of them was convinced that anyone would every conduct enzyme assay according to our recommendations. However, I do not think that we can move forward without that. As I attempted to document in previous sections, the enzyme part of the microbial component in soil is the least established.

Microbial biomass C, N and P

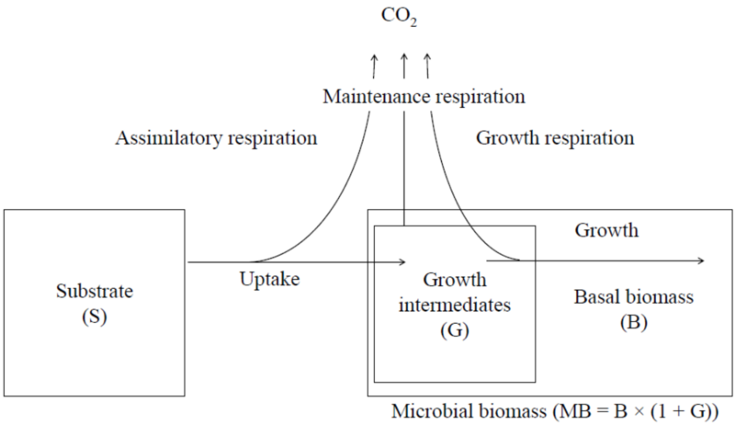


Figure 10: *Schematic representation of the mathematical model treating soil microbial biomass as two different pools. Different boxes represent different carbon pools. Arrows represent carbon fluxes.*

As much paradoxical as it may sound, there is no method to determine soil microbial biomass accurately. Most of the microorganisms is firmly attached to soil particles so they cannot be physically separated from soil matrix quantitatively. For that reason, specific parts of microbial biomass denoted as proxy-parameters are quantified, and the total is calculated using conversion factors. Microbial biomass C is most often measured by fumigation-extraction method (Vance et al., 1987). Soil is exposed to chloroform vapours, and the flush of extractable organic C is assumed to derive from lysed microbial cells. This chloroform labile organic C (CLC) represents fraction of total microbial biomass C. This fraction is represented by conversion factor. Two most often used conversion factors are 0.38 (Vance et al., 1987) and 0.45 (Wu et al., 1990). The microbial biomass C could be also measured as DNA, ATP or PLFA applying different conversion factors **(Čapek et al., 2023).** None of the conversion factors, however, is constant but changes with chemical composition of microbial biomass and that causes problems with accurate quantification (**Čapek et al., 2023)**. To improve accuracy of microbial biomass estimates, the chemical composition must be known and there is a simple way to estimate it. However, it requires redefining microbial biomass according to scheme depicted on fig. 10, i.e. to acknowledge that microbial biomass is not a single chemically homogeneous pool.

To allow the chemical composition of the microbial biomass to change, it must be divided into two (at least) different functional pools – basal microbial biomass (B) and growth intermediates (G). G is a temporary pool that serves as a buffer between the soil and B. It accumulates organic C from soil and gets transformed into B (fig. 10). Since G is normalized to a unit of B, total microbial biomass is $MB = B \times (1 + G)$. The two pools have different chemical compositions so the composition of the entire microbial biomass changes with G. The trick is to define contribution of measured proxy-parameters to pools B and G. We did that using published data applying strict criteria for data inclusion.

Using this data, we managed to define two different functional relationships between G and the conversion factors specific to CLC (k_{ec}) and DNA (k_{DNA}):

$$k_{ec} = \frac{0.41 \times G + 0.24}{1+G} \qquad \text{(eq. 11)},$$

$$k_{DNA} = \frac{0.06}{1+G} \qquad \text{(eq. 12)}.$$

When CLC and DNA are measured in soil at the same time, G could be simply calculated as:

$$G = \frac{{}^{CLC}/_{DNA} \times 0.06 - 0.24}{0.41} \qquad \text{(eq. 13)}.$$

Once G is known, it is possible to calculate conversion factors k_{ec} and k_{DNA} according to eqs. 11 and 12 and thus, total microbial biomass C using either of measured proxy-parameters (CLC or DNA). Improving accuracy of microbial biomass estimates only requires measurement of two microbial proxy-parameters at the same time. This is usually done anyway because CLC is measured as a microbial proxy-parameter and DNA is extracted from soil in order to analyse species composition of microbial biomass. No extra effort is therefore required.

Unfortunately, our analysis indicated several issues in previously published microbial data. First, the most frequently applied conversion factors 0.45 or 0.38 (converting CLC to microbial biomass C) do not reflect chemical composition of most soil microorganisms correctly because they are too high. The most representative conversion factor is 0.24, which applies to microbial biomass with G being very close to zero (slowly growing microorganisms; Blagodatskaya and Kuzyakov, 2013). All microbial biomass reports are very likely underestimated by ~ 58 to 88%. Let's get back to our eq. 1 and close the circle. In the introduction section, quantification of relative contribution of microbial biomass to metabolic rate of entire soil community have been criticized to ignore presence of dormant soil microorganisms, which could make roughly 60% of total microbial biomass (fig. 1). "Luckily", microbial biomass estimates are underestimated by 60% or more for the same reason thus, the ignorance of dormancy is counterbalanced and calculations of Johnston and Sibly (2018) match independent data sets well.

Second, microbial biomass C is used to estimate key physiological parameters CUE and microbial death rate. Due to variable chemical composition of microbial biomass, these estimates may be biased. The extent of bias depends on selection of microbial proxy-parameter that is measured. It is possible that parametrization of microbially-explicit models, which are calibrated against these data, could be biased too.

Fumigation-extraction is a very convenient method because any analyte/element can be measured in chloroform flush and thus, its content in microbial biomass estimated. That's how $C{:}E_B$ defining nutrient demand of microbial community in eqs. 6, 7, 8 and 9 is calculated. Analogically to CLC and k_{ec}, we can define chloroform labile N (CLN) and reactive P (CLP). The respective conversion factors are then k_{en} and k_{ep}. The possible methodological bias associated with $C{:}E_B$ calculation can be acknowledged explicitly as:

$$C{:}N_B = \frac{k_{en}}{k_{ec}} \frac{CLC}{CLN} \qquad \text{(eq. 14)},$$

and

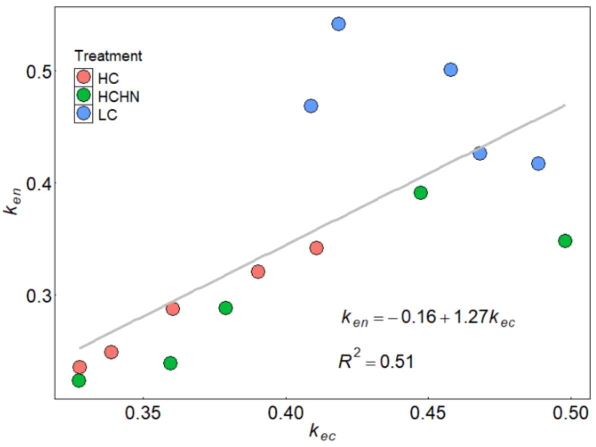


Figure 11: *Relationship between two conversion factors k_{ec} and k_{en} determined in study of Bremer and van Kessel (1990). The conversion factors were determined at three different treatments, following addition of low amount of organic C (LC), high amount of organic C alone (HC) or in combination with inorganic N (HCHN). The solid grey line represents the linear regression between the conversion factors. Formula of the regression and its R^2 is reported.*

$$C:P_B = \frac{k_{ep}}{k_{ec}}\frac{CLC}{CLP} \qquad \text{(eq. 15).}$$

As shown above, k_{ec} is not constant so it could affect $C:E_B$. On the other hand, k_{en} and k_{ep} could compensate for k_{ec} changes if their variability is somehow related to k_{ec}. According to study of Bremer and van Kessel (1990), there is some relationship between k_{ec} and k_{en} (fig. 11). Unfortunately, this relationship is not 1:1. The regression between the two suggests that $\frac{k_{en}}{k_{ec}}$, which multiplies measured $\frac{CLC}{CLN}$, increases with increasing k_{ec}. When k_{ec} is between approx. 0.24 and 0.5, $\frac{k_{en}}{k_{ec}}$ is expected to be between 0.59 and 0.94. Measured $\frac{CLC}{CLN} = 10$ could results from $C:N_B$ either 5.9 or 9.4. Even though this difference seems to be small, it exceeds the relative difference in $C:N_B$ of two major categories of soil microorganisms, i.e. bacteria (6.12) and fungi (8.30) (Mouginot et al., 2014), which were believed to cause variability in measured $C:N_B$ across soils (Strickland and Rousk, 2010).

The methodological bias increases a lot when we consider microbial biomass P and k_{ep}. Soluble reactive P (SRP) is measured in chloroform flush as an analyte (Brookes et al., 1982) although there is no phosphate in microbial biomass. The organic P released from microbial biomass is expected to hydrolyse to a certain degree in acidic conditions over 24 hours incubation of extracts from fumigated soils. The conversion factor k_{ep} therefore reflects susceptibility of different forms of organic P to hydrolysis in acidic conditions. We showed that intracellular polyphosphates are particularly susceptible to hydrolysis and thus, k_{ep} depends on relative contribution of polyphosphates to total microbial biomass P (**Čapek et al., 2024)**. The contribution of polyphosphates cannot be related to G similarly as k_{ec} so the variability in k_{ep} follows largely independent trajectory. k_{ep} cannot compensate changes in k_{ec} and the entire variability in the multiplication factor $\frac{k_{ep}}{k_{ec}}$ increases. Assuming that k_{ep} could vary between 0.43 and 0.90 as a function of polyphosphate content **(Čapek et al., 2024)**, the minimum and maximum $\frac{k_{ep}}{k_{ec}}$ could be 0.68 and 3.75, respectively. Measured $\frac{CLC}{CLP} = 100$ could results from $C:P_B$ 68 or 3750. This range covers almost entire variability in $C:P_B$ of different consortia of aquatic heterotrophic bacteria reported by Godwin and Cotner (2015b)(28 - 8500) and even exceeds the entire variability in measured $C:P_B$ of soil microbial communities (Cleveland and Liptzin, 2007). Nevertheless, the relative contribution of polyphosphates to the microbial biomass P can be estimated and k_{ep} corrected. To do that, CLC and ideally DNA must be measured along with CLP **(Čapek et al., 2024)**.

The purpose of this section was to document the degree of methodological bias associated with quantification of different microbial biomass related variables. It was not, however, intended to cause state of depression. The methodological bias is not stochastic. It is always a result of some unaccounted biological, biochemical, or chemical principle. The data are not all wrong, they only need to be interpreted correctly in view of these principles. I believe that microbially-explicit models need to acknowledge these principles directly to accommodate measured variables, not their recalculated derivatives such as potential enzyme activity.

Future perspectives

Microorganisms represent one of the most important members of the soil community. Understanding their abundance and physiology could help us to understand and predict important ecosystem processes such as heterotrophic respiration and nutrient mineralization/immobilization. The respective research could be fruitful, but it must be conducted with particular care to avoid methodological bias and results misinterpretation. I would like to go on and disentangle all the residual variability, which remains to be explained. The contribution of unexplained variability is still significant as documented in figures 3, 4, 7 and 8. I am, however, aware that this is not possible. There are too many confounding factors, which deserve to be studied. Based on my experience acquired so far, I defined four most important avenues of my eventual future research:

1. Specific microbial death rate. As suggested above, the death rate impacts production of CO_2, reuse of microbial N and thus, N mineralization/immobilization. Moreover, it is believed that microbial necromass partially causes build-up of organic material with long turnover rate in soil (Angst et al., 2021; Deng and Liang, 2021; B. Wang et al., 2021; C. Wang et al., 2021) improving

soil health as a result. The specific death rate constant is applied in most current microbially-explicit models. All microorganisms are assumed to die at the same rate independently of soil conditions, microbial physiology or grazing. It is a simplification, which is very likely too simplifying. Unfortunately, the microbial death rate is very difficult to measure because its estimation is confounded by changes in the chemical composition of microbial biomass (**Čapek et al., 2023)**. The hypothesis driven specific research could disentangle some of the variability in reported estimates. Most ecologically important, in my opinion, is the effect of grazing because effects of soil conditions such as pH, moisture and temperature could be withstood by microorganisms to certain extent.

2. Polyphosphates. Our research suggests that they are critical to understanding microbial physiology and impact of microorganisms on P cycling in soil. The potential research is, however, limited by the absence of accurate and high throughput method of their estimation. Designing the accurate method is an aim of one master thesis under my supervision. Once the method is designed and thoroughly tested, the target research of polyphosphates could be theoretically initiated.
3. Chemical diversity of soluble organic material. The chemical identity of organic compounds that soil microorganisms are exposed to undoubtedly affects their physiology (e.g. Malik et al., 2016). There are two focal points, which deserve to be studied. The direct effect on microbial physiology, and the functional relationship between the chemical identity of soluble organic material, chemical composition of organic debris, and activity of extracellular enzymes. The effect of chemical identity of the soluble organic material on microbial physiology is good to know but it is largely useless when the origin of its variability is not fully understood.
4. Non-homogeneity of the soil. Throughout the entire document, I largely assumed that soil is a homogenous substrate. None of the other simplifications is further from reality. To upscale the identified concepts to entire ecosystems, the spatial distribution of all key variables in the soil must be acknowledged. Without it, it is impossible to predict, for example, leaching of nutrients or soluble organic material from soils to adjacent water bodies accurately.

All items in the list are, in my opinion, equally important. I am not sure what topic would become the focal point of my research as this will probably depend on several circumstances that I cannot anticipate at this moment. I would personally wish the research defined in points 1, 3 and 4 to be successful because it would make simulation/prediction of the distribution of microbial biomass in soil finally possible. We could then not only imply the importance of microbial biomass from biased measurements performed in different soils from different ecosystems exposed to different treatments but quantify it more accurately than I was able to do in this document.

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Appendixes