# Microbial Resource Limitation and Terrestrial Ecosystem Ecology

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#### DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Science at Lund University to be publicly defended on 2<sup>nd</sup> of February 2024 at 09.00 in Blue Hall, Department of Biology, Sölvegatan 37, Lund

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Netherlands Institute of Ecology (NIOO-KNAW), Netherlands

#### Organization: LUND UNIVERSITY

**Document name:** Department of Biology

Author(s): Mingyue Yuan

#### Date of issue 2024-02-02 Sponsoring organization:

Title and subtitle: Microbial Resource Limitation and Terrestrial Ecosystem Ecology

#### Abstract:

Soil microbes function as decomposers in terrestrial ecosystems. They break down the organic matter, which plays a significant role in supplying nutrients to plants, but also in regulating biogeochemical cycling which feeds back to climate change. Like other organisms, soil microbes can be limited by the availability of essential resources, which could consequently affect their ability to decompose organic matter. Besides, climate change events could change the resource regime of an ecosystem, which could alter microbial resource limitation and trigger microbial mining of the limiting resource from organic matter. This PhD aimed to better understand these mechanisms. By using limiting factor assay, I found that microbial growth in both arctic and tropical soil was primarily limited by carbon (C) and secondarily limited by phosphorus (P). I found that climate change events could alter microbial resource limitation. In the Arctic, C enrichment due to arctic greening or shrubification alleviated microbial C limitation. Conversely, nitrogen (N) enrichment in the Arctic due to warming-accelerated decomposition enhanced microbial C limitation, which stimulated microbial use of new plant litter input. In the tropics, elevated atmospheric CO<sub>2</sub> (eCO<sub>2</sub>) increased the C availability of the ecosystem. This enhanced the microbial C limitation, which might be explained by a more active community under eCO2 demanding more C. This enhanced microbial C limitation was found to stimulate microbial use of new plant litter input. In summary, soil microbial growth was primarily limited by C in all studied environments. The microbial C limitation could be altered by climate changes events, and the direction would be determined by both the change of resource availability in the environment and the status of the microbial community.

Key words: soil microbes, microbial growth, resource lir	mitation, ecosystem ecology
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Classification system and/or index terms (if any)

Supplementary bibliographical information

Language English

ISSN and key title:

Number of pages: 63

Security classification

ISBN: 978-91-8039-918-0 (print). 978-91-8039-919-7 (electronic)

Recipient's notes

Price

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Faculty of Science Department of Biology

ISBN 978-91-8039-918-0 (print) ISBN 978-91-8039-919-7 (electronic)

Tryckt i Sverige av Media-Tryck, Lunds universitet Lund 2023



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"Our questions and our experiments intersect numberless curved lines the straight line that leads to truth."

Justus Freiherr von Liebig, 1842

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### Abstract

Soil microbes function as decomposers in terrestrial ecosystems. They break down the organic matter, which plays a significant role in supplying nutrients to plants, but also in regulating biogeochemical cycling which feeds back to climate change. Like other organisms, soil microbes can be limited by the availability of essential resources, which could consequently affect their ability to decompose organic matter. Besides, climate change events could change the resource regime of an ecosystem, which could alter microbial resource limitation and trigger microbial mining of the limiting resource from organic matter. This PhD aimed to better understand these mechanisms. By using limiting factor assay, I found that microbial growth in both arctic and tropical soil was primarily limited by carbon (C) and secondarily limited by phosphorus (P). I found that climate change events could alter microbial resource limitation. In the Arctic, C enrichment due to arctic greening or shrubification alleviated microbial C limitation. Conversely, nitrogen (N) enrichment in the Arctic due to warming-accelerated decomposition enhanced microbial C limitation, which stimulated microbial use of new plant litter input. In the tropics, elevated atmospheric  $CO_2$  (eCO<sub>2</sub>) increased the C availability of the ecosystem. This enhanced the microbial C limitation, which might be explained by a more active community under eCO<sub>2</sub> demanding more C. This enhanced microbial C limitation was found to stimulate microbial use of new plant litter input. In summary, soil microbial growth was primarily limited by C in all studied environments. The microbial C limitation could be altered by climate changes events, and the direction would be determined by both the change of resource availability in the environment and the status of the microbial community.

### Popular science summary

Now I would ask you to take a deep breath, then image that you step into a tranquil forest, where trees stand firmly rooted, their branches reaching skyward while leaves swaving gently in the breeze. Sunshine streams through the canopy, painting the forest floor in a mosaic of hues. Mosses and lush grass interweave like a plush carpet on the ground, sometimes exposed rocks and soil add contrasting textures to the landscape. To the eyes of ecologists, this tranquil scene is a hub of natural processes related to element cycling. Plants are busy with capturing carbon dioxide (CO<sub>2</sub>) from the air and extracting vital nutrients such as nitrogen and phosphorus from the soil. They convert these compounds with the sun's energy into organic molecules, that form building blocks for leaves, branches, and stems. If plants were the only residents in the forest, one day they would have consumed all  $CO_2$  and all nutrients from the soil, and the forest would end up covered by dead plants and their fallen leaves. Fortunately, there are other inhabitants in this forest. These inhabitants work in the opposite way of plants, they break down complex organic molecules into simpler forms, including inorganic compounds, which can be absorbed by the plants again (Now we don't need to worry about the forest will die!). When these inhabitants break down organic carbon, they respire some of the carbon as CO<sub>2</sub> back to the air. Here are two hints if you would like to guess who they are: they are invisible for the naked eye and they live below ground.

Yes, they are soil microbes, the main characters of this thesis!

As all other organisms on Earth, soil microbes need food for their growth, like carbon, nitrogen or phosphorus. During my PhD, I detected what the limiting resource for soil microbial growth was in an arctic soil and in a tropical soil. I found that soil microbial growth in soils from both ecosystems was primarily limited by carbon and phosphorus, which inferred that microbes were looking for these elements when they decomposed soil organic matter.

Climate change is a force that can alter the resource availability for soil microbes. In the Arctic, when the temperature increases, there is a notable shift in plant community from mosses and sedges to more robust shrubs like dwarf birch. These shrubs produce more leaves, contributing additional carbon to the soil when they eventually drop those leaves. Meanwhile, belowground decomposition also speeds up in a warming Arctic, leading to a faster breakdown of organic matter and, consequently, the release of nitrogen into the soil. All these events can potentially alter microbial resource limitation. To test it, I measured microbial resource limitation in soil samples from a climate change simulation experiment in the North of Sweden, where plant litter and N was added to the soil to simulate the abovementioned changes. I discovered that adding more N to the soil intensifies the microbial carbon limitation, while adding more carbon-rich plant litter and roots weakens it.

Elevated concentrations of atmospheric  $CO_2$  (eCO<sub>2</sub>) are another severe climate change event that we are all confronting now. As  $CO_2$  is the main source for plant photosynthesis, plants are expected to thrive under eCO<sub>2</sub>, produce more carbon-rich substrates from which a portion is transferred to soils. Hence, I expected a reduction in microbial carbon limitation when I started measuring soil samples from a free-air  $CO_2$  enrichment experiment in Australia. Surprisingly, I found that eCO<sub>2</sub> instead enhanced microbial carbon limitation. One possible explanation for this result is that the eCO<sub>2</sub> provided a carbon-rich feast for the microbes, which made the community more active, and thus led to a higher carbon-needs than before.

During my PhD, I was also interested to see how the changes in resource limitation affected microbial use of resources, - can a change in food make them malfunction? To test that, I added plant leaf litter to soil samples from both arctic and tropical soils, and measured the microbial use of it. I found that microbial use of plant leaf litter was stimulated when microbial carbon limitation was enhanced. This means that when microbes are getting hungrier for carbon, they break down fallen leaves to gain carbon!

### List of Papers

### Paper I

Hicks, L.C., **Yuan, M.**, Brangarí, A., Rousk, K., & Rousk, J. (2022) Increased Above- and Belowground Plant Input Can Both Trigger Microbial Nitrogen Mining in Subarctic Tundra Soils. Ecosystems 25, 105–121.

### Paper II

**Yuan, M.**, Na, M., Hicks, L.C. & Rousk, J. (2023) Limiting resource for soil microbial growth in climate change simulation treatments in the Subarctic. Ecology e4210.

#### Paper III

**Yuan, M.**, Na, M., Hicks, L.C. & Rousk, J. (2022) Will a legacy of enhanced resource availability accelerate the soil microbial response to future climate change? Soil Biology and Biochemistry, 165, 108492

#### Paper IV

Na, M., **Yuan**, M., Hicks, L.C. & Rousk, J. (2022) Testing the environmental controls of microbial nitrogen-mining induced by semi-continuous labile carbon additions in the subarctic. Soil Biology and Biochemistry, 166, 108562.

#### Paper V

Neurauter, M\*., **Yuan, M.**\*, Hicks, L.C.& Rousk, J. (2023) Soil microbial resource limitation along a subarctic ectone from birch forest to tundra heath. Soil Biology and Biochemistry, 177, 108919. \*shared first author.

#### Paper VI

**Yuan, M.**, Macdonald, C., Hicks, L.C. & Rousk, J. Will elevated atmospheric CO<sub>2</sub> slow ecosystem nutrient cycling via induced aboveground-belowground competition for P? manuscript

### Author contributions

### Paper I

LCH, KR and JR conceived of the study, implemented the field experiment, and conducted the vegetation survey; LCH, MY and AB sampled soils; AB conducted the NDVI measurements; MY analysed the soil samples, with help from LCH; LCH analysed the data and led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

### Paper II

MY, JR and LCH conceived the ideas and designed the methodology; LCH and JR sampled soils; MY and MNa performed the experiment and data analysis under the supervision of LCH and JR; MY led the writing of the manuscript supervised by JR and LCH. All authors contributed critically to the drafts and gave final approval for publication.

### Paper III

MY, JR and LCH conceived the ideas and designed the methodology; LCH, MY sampled soils. MY and MNa performed the experiment and data analysis under the supervision of LCH and JR; MY led the writing of the manuscript supervised by JR and LCH. All authors contributed critically to the drafts and gave final approval for publication.

#### Paper IV

MNa, JR and LCH conceived the ideas and designed the methodology; LCH and JR sampled soils; MNa and MY performed the experiment and data analysis under the supervision of LCH and JR; MNa led the writing of the manuscript supervised by JR and LCH. All authors contributed critically to the drafts and gave final approval for publication.

#### Paper V

MNe, MY, JR and LCH conceived the ideas and designed the methodology; LCH and JR collected soil samples. MNe and MY performed the experiment and data analysis under the supervision of LCH and JR; MNe and MY co-led the writing of the manuscript and revision of the manuscript, supervised by JR and LCH. All authors contributed critically to the drafts and gave final approval for publication.

Paper VI

MY, JR and LCH conceived the ideas and designed the methodology; CM sampled soils and provided data collected from the field experiment. MY performed the experiment and data analysis under the supervision of LCH and JR; MY led the writing of the manuscript, supervised by JR and LCH. All authors contributed critically to the manuscript.

Lettice C. Hicks – LCH Mingyue Yuan – MY Albert Brangarí – AB Kathrin Rousk – KR Johannes Rousk – JR Meng Na – MNa Markus Neurauter – MNe Catriona Macdonald – CM

### Introduction

All living organisms constantly interact with their surrounding environment, and vice versa. A variety of environment factors can limit the survival, growth, and reproduction of organisms. This interaction is conferred by the "limiting factor" in studies of ecology (Smith and Smith, 2009). The limiting factor can refer to the environmental conditions conducible for life, which could be categorized by light, temperature, moisture, or pH etc. The availability of essential resources (i.e., chemical elements) can also be a limiting factor that constrains organisms' growth, development and metabolism (Smith and Smith, 2009). The availability of essential resources e.g., carbon (C), nitrogen (N) and phosphorus (P), demonstrably shapes the ecology of organisms in all ecosystems (Vitousek, 2004). In terrestrial ecosystems, microbes function as decomposers, whose activity controls the second largest flux in global organic matter cycling (Sterner and Elser, 2002). Decomposition provides mineral nutrients to primary production (which is the largest flux in global organic matter cycling) and thus determines ecosystem productivity (Wardle et al., 2004). It therefore also modulates the carbon balance between atmosphere and soil which is of great significance under climate change (Bardgett et al., 2008).

This thesis aims to contribute to better understanding resource limitation for soil decomposer microbes. By conducting several empirical studies during my PhD, I have attempted to answer the following questions:

- 1. What is the limiting resource for soil microbial growth?
- 2. How do climate change events alter resource limitation for soil microbes?
- 3. What's the link between microbial resource limitation and decomposition?

Before stepping into soil microbial ecology, let's first take a detour to see how this subject has been developed in plant ecology and phytoplankton ecology.

# **1.1 Nutrient limitation in plant ecology: from crop yield in a field to the C sequestration under climate change**

The concept of nutrient limitation originated from agricultural science, where the botanist Carl Sprengel found that plant growth was limited not by the total nutrients available but rather by the nutrient in scarcest supply. Later, this statement was popularised by the chemist Justus von Liebig and became well-known as the "Law of the Minimum" or "Law of Limiting Factors", in which the scarcest nutrient was termed the "limiting factor" (Fig.1). Plant growth is generally limited by N or/and P availability in soil, which explains why N and P are widely used as fertilizer to sustain crop yield in agriculture (Smil, 2000; Vitousek et al., 2009).



Figure 1. Justus von Liebig and the paradigmatical barrel to explain the Law of Minimum (The capacity of a barrel is determined by the shortest plank of it) (Credit: Wikipedia)

Apart from practical applications in agriculture, the study of nutrient limitation for plants has also deepened our understanding of ecosystem ecology. One classic example is that Walker and Syers (1976) found that P fractions in soil declined with soil age, i.e., a lack of P in highly-weathered soil (i.e., old soil). Later, Vitousek and Farrington (1997) tested this theory along a chronosequence in Hawai'i and found that tree growth was limited by N in the young soil and limited by P in the old soil, which suggested different nutrient limitations for primary production during soil and ecosystem development.

Recently, many studies have recognized the significance of nutrient limitation to terrestrial ecosystem responses to global climate change. One case is about the elevated atmospheric carbon dioxide (eCO<sub>2</sub>). As eCO<sub>2</sub> leads to climate warming, the sequestration of C by plants is expected to mitigate this detrimental effect, as in general, plant photosynthesis is stimulated by eCO<sub>2</sub> (DeLucia et al., 1999), which could lead to an increase in ecosystem C uptake. However, in most cases, this anticipated increase in plant biomass accumulation doesn't happen under eCO<sub>2</sub> because plant growth is constrained by availability of N or P (Norby et al., 2010; Reich and Hobbie, 2013; Ellsworth et al., 2017; Terrer et al., 2019), which suggests that the nutrient limitation is an important constraint on plants-associated mitigation of climate change.

# **1.2** Nutrient limitation in phytoplankton ecology: from the resource competition theory to eutrophication control

Like plants in terrestrial ecosystems, phytoplankton are the primary producers in aquatic and marine ecosystems, and their growth is also limited by resource availability. P, N and at times silicon (Si) have been demonstrated as main limiting nutrients for phytoplankton (Tilman et al., 1982; Howarth, 1988). Studies of nutrient limitation has great importance in determining the community structure and biomass in phytoplankton ecology. One of the classic implications of nutrient limitation in phytoplankton ecology might be the resource competition theory, which posits that the species which can use the limiting resource to the lowest concentration is the better competitor (Tilman et al., 1982). The theory was tested and supported by results from many empirical studies. For example, one study about growth of four algal species under different Si-limited and P-limited conditions found that one algal species which has relative higher requirements of both Si and P was competitively displaced by the three other species (Tilman, 1981). Another study found the abundance of green algae was higher than the abundance of diatoms in Si-limited conditions, which was consistent with that green algae had relative low requirement of Si (Kilham, 1986). In addition, co-limitation, defined as the community being simultaneously limited by two different resources has been observed and wellstudied in phytoplankton ecology. Candidates for interpreting co-limitation are different species within a community might be limited by different resources, or the acquisition of one nutrient depends on another nutrient (Saito et al., 2008).

The knowledge of nutrient limitation for phytoplankton also provided an important guide for eutrophication control (Conley et al., 2009). Due to anthropogenic activities e.g., the application of fertilizers, N or P levels in aquatic or marine ecosystems has significantly increased, which caused toxic algal blooms. P has been identified as the limiting nutrient for phytoplankton in most freshwaters, which led to control of P loading to lakes as a key management strategy to improve the water quality (Schindler, 1974). Moreover, N has been identified as limiting nutrient for phytoplankton productivity in most marine ecosystems, which suggests that reducing N inputs could be an effective way to reduce the eutrophication in the ocean (Howarth and Marino, 2006).

#### 1.3 Resource limitation in soil microbial ecology

All organisms rely on nutrients to survive and reproduce, and soil microbes are no exception. Like studies in plant ecology (Chapin III et al., 1986; Vitousek, 2004), studying the elemental limiting factors for soil microbes is also applicable in microbial ecology. Soil microbes are well-known decomposers, which break apart soil organic matter (SOM) step-by-step into simpler forms (including e.g., mineral nutrients) in terrestrial ecosystems (Berg and Laskowski, 2006). The decomposition

process is the main determinant of the nutrient supply to primary producers (e.g., plants), but also is crucial in the regulation of global biogeochemical processes. During decomposition, microbial respiration can directly release  $CO_2$  to the atmosphere (Rustad et al., 2000). At the same time, microbial growth, which combines elements to build up biomass, is the primary input of C with long residence times to soil (Liang et al., 2017). Thus, microbial use of C for respiration and growth contributes to C fluxes in global element cycling, and therefore contributes to exacerbating or mitigating against climate change. Clearly, the resource limitation for soil microbes can provide necessary information to improve the understanding of the microbial community and their function. However, compared to studies assessing nutrient limitation for plants and phytoplankton, studies assessing resource limitation for soil microbes have a shorter history and are less systematic.

Microbial growth has been found to be generally limited by C availability (Nordgren, 1992; Joergensen and Scheu, 1999; Aldén et al., 2001). N or P availability have been also reported to be limiting for microbes in some ecosystems. For instance, soil microbial biomass and respiration were limited by N in some arctic tundra soils (Nordin et al., 2004; Sistla et al., 2012), while in some tropical soils microbial activities were P-limited (Cleveland et al., 2002; Camenzind et al., 2018). Many approaches have been developed and applied to determine the resource limitation. There were growth rate-based approaches (Aldén et al., 2001), biomassbased approaches (Sistla et al., 2012), respiration-based approaches (Nordin et al., 2004) used in the above cases. Besides, enzyme activity-based approaches (Sinsabaugh and Follstad Shah, 2012) and stoichiometry-based approaches (Mooshammer et al., 2014) are also commonly-used to determine limiting resources for soil microbes. The variety of approaches brings up a methodological issue: does the determination of limiting factor depends on the approach used? This would hinder the interpretation and prediction of how microbial resource limitation affects decomposition. I will review and discuss this issue in Section 4.2. In this thesis, the growth rate-based approach, i.e., limiting factor assay (LFA) was used to determined limiting resource for microbial growth.

In addition to evidence for a single limiting factor, co-limitation has also been found to exist in many ecosystems. Co-limitation is identified when an increase of microbial growth or activity occurs in response to the combined addition of different nutrients, rather than a single nutrient addition (Davidson and Howarth, 2007). For instance, microbial respiration in a taiga forest was co-limited by C and N (Vance and Chapin III, 2001); while in another study the soil microbial community in a rainforest was co-limited by C and P (Fanin et al., 2015). It is contrasting with the implication of Liebig's Law of Minimum, that there is always single limitation. I will discuss about this conflict in Section 4.4.

## **Tools and Approaches**

#### 2.1 Soil microbial growth, respiration, biomass and enzymatic activities

In this thesis, I define microbial growth as the rate of growth, i.e., the rate of microbial biosynthesis. Growth rates can be estimated by incorporating tracers into constituents of cells. Herein, bacterial growth was estimated by <sup>3</sup>H-leucine (Leu) incorporation into proteins and <sup>3</sup>H thymidine (TdR) incorporation into DNA (Bååth, 1992, 1994). Thymidine is a precursor for DNA synthesis, and leucine is a precursor for protein synthesis, and thus the estimates use either DNA synthesis or protein synthesis as a proxy to estimate rates of bacterial growth. Fungal growth was estimated using the <sup>14</sup>C-acetate incorporation into ergosterol method (Newell and Fallon, 1991) adapted for soil (Bååth, 2001; Rousk et al., 2009). Acetate is a precursor for lipid synthesis including ergosterol, which can be used to estimate the rate of ergosterol synthesis. Ergosterol is a widely used biomarker for soil fungi, and therefore, rates of ergosterol synthesis can be used to estimate fungal growth. There is a commonly intuitive concern that as leucine, thymidine and acetate are substrates for microbes, methods using these could induce microbial growth and thus lead to estimates of microbial use of added substrates, rather than to growth rates under "natural conditions". However, the concentrations of added radioactive compounds are far lower compared to the concentration of these substrates in natural soils. For example, the concentration of  ${}^{3}$ H leu used in this thesis is 275 nM, while the total dissolved amino acid concentration in soil water is approximate 23 µM (Hobbie and Hobbie, 2013). Besides, the incubation time is too short for the microbial adapting to the disturbance of the growth condition. Taken together, the addition of these compounds acts as tracers rather than substrates from microbial growth.

The rates of bacterial growth and fungal growth were needed to be converted to rates of microbial C production to estimate the total microbial growth. To convert bacterial growth rates from pmol of incorporated Leu to growth rates in  $\mu$ g of C, we first established a conversion factor between the rate of Leu incorporation and rate of TdR incorporation. Although theoretically both Leu and TdR incorporation can be used to estimate bacterial growth rate, Leu is preferentially used since the Leu incorporation technique is tracing protein synthesis, which means it can capture changes in both the size (e.g., bigger cells with more protein per DNA) and the number of individuals (e.g., more cells), whereas TdR incorporation to DNA only reflects the latter. After converting the rate of Leu incorporation to the rate of TdR incorporation, the bacterial and fungal growth was converted in units of C by applying the conversion factors established by Soares and Rousk (2019), where 1 mg bacterial biomass-C corresponds to 5.5 pmol incorporated TdR and 1 mg fungal biomass-C corresponds to 2.4 pmol incorporated acetate.

Soil respiration was measured using 1.0 g fwt soil in 20-mL glass vials. The headspace was immediately purged with pressurised air and vials were closed with crimp lids. Samples were incubated for 24 hours before the headspace concentration of  $CO_2$  for each sample was determined using a gas chromatograph.

Microbial biomass was estimated by measuring substrate induced respiration (SIR) (Anderson and Domsch, 1978). Briefly, 12 mg glucose/talcum (1:4 w:w) was vigorously mixed into 1.0 g fwt soil. After 15 min, vials were purged with pressurized air and incubated at 22 °C for 2h. Then concentration of CO<sub>2</sub> was determined using a gas chromatograph. Microbial biomass was estimated where 1  $\mu$ g CO<sub>2</sub> per hour at 22 °C corresponded to 20  $\mu$ g microbial biomass C (Anderson and Domsch 1978). In paper I, microbial phospholipid fatty acid (PLFA) was also measured to estimate microbial biomass and relative abundance of functional groups (e.g., bacteria, fungi) (Frostegård and Bååth, 1996).

The potential activities of four extracellular enzymes were quantified using fluorometric assays using the high-throughput fluorometric method, following the protocol developed by (Bell et al., 2013). These enzymes included: β-1,4glucosidase (BG), which is thought to be related to microbial C acquisition;  $\beta$ -1,4-N-acetyl-glucosaminidase (NAG) and L-leucine aminopeptidase (LAP), which are thought to be related to microbial N acquisition; acid phosphatase (AP), which is thought to be related to microbial P acquisition (Sinsabaugh and Follstad Shah, 2012). According to the theory of ecological enzymatic stoichiometry, when enzymes associated with the acquisition of a certain resource have relatively higher potential activity compared to other enzymes, the ratio of these two enzymes is used to proxy the relative microbial limitation by those two resources (Sinsabaugh and Follstad Shah, 2012). For example, a ratio of enzyme associated with acquisition of C and enzymes associated with acquisition of N being above 1 would suggest that microbes are more C than N limited. Hence, I calculated BG/(NAG + LAP), BG/AP, and (NAG + LAP)/AP, to infer limitations related to C versus N, C versus P, and N versus P, respectively.

### 2.2 Limiting factor assay

To determine limiting resources for soil microbes, one classic way that draws on the original conception of limiting factors for plant productivity is factorially adding different resources to soil samples and then measuring the microbial response (limiting factor assay, "LFA", Aldén et al., 2001). An increase of the chosen biological endpoint used to estimate microbes will occur only after the addition of the limiting resource, while all other additions will not affect it.

To identify the optimal concentrations for the LFA-additions and a suitable incubation time, pilot experiments were conducted (details see Paper II, Paper V and Paper VI). Briefly, solutions of C (as  $C_6H_{12}O_6$ ), N (as  $NH_4NO_3$ ) and P (as KH<sub>2</sub>PO<sub>4</sub>/K<sub>2</sub>HPO<sub>4</sub>, to maintain soil pH) were added to soil. Glucose was provided at two different concentrations. Responses to combined CNP addition were also determined, where C was supplied together with N and P to achieve a C:N:P mass ratio of ca. 20:1:1. This addition ratio was chosen based on the average microbial biomass C:N:P of 60:7:1 (Cleveland and Liptzin, 2007), and considering a microbial carbon use efficiency of 0.5 (Sinsabaugh et al., 2013), such that half of added C was expected to be used for respiration and the other half being available for growth. Therefore, the C:N ratio of LFA addition matched the anticipated microbial C:N demand of approximately 20:1. P was added in excess because considering P sorption might make a portion of the added P unavailable. Microbial growth rates were determined at several timepoints after the resource additions. The concentrations of addition and incubation time were determined when both bacterial growth and fungal growth were clearly stimulated.

To determine the limiting resource for microbial growth in soils from an subarctic tundra ecosystem (Paper II), a subarctic ecotone from tundra to forest (Paper V) and a subtropical woodland (Paper VI), microbial growth rate was measured after the factorial additions of C, N and P, the expectation was that microbial growth would increase in response to addition of limiting resource (Fig.2).



Figure 2. Flow diagram of the limiting factor assay, adjusted from Aldén et al., 2001.

### 2.3 Climate change simulations in the field

Climate change is one external force which can alter the resource limitation for soil microbes. On Earth, climate warming is amplified in the Arctic (Budyko, 1969), which leads to more-pronounced responses happening in these ecosystems (Post et al., 2019). The increasing temperature in the Artic has led to increased plant productivity (Hudson and Henry, 2009; Myers-Smith et al., 2020), a shift of vegetation composition towards shrubs (Van Wijk et al., 2003; McManus et al., 2012; Frost and Epstein, 2014), and treeline advancement (Kulmala, 2018). These changes in plant productivity and composition will increase the input of C-rich litter to the soil (Walker et al., 2006; Sistla et al., 2013) and also the belowground rhizosphere inputs. In addition to the influence on plant communities, warming can also accelerate nutrient cycling (Xue et al., 2016; Sarneel et al., 2020), leading to increased soil N availability (Salazar et al., 2020). To detect how these phenomena affected the microbial resource limitation, changes in resource availability due to climate change were simulated in a tundra health in northern Sweden (Abisko, 68.19N, 18.50E) (Paper I).



Photo of the field. (Credit: Mingyue Yuan)

The field experiment was established in June 2017, with treatments including (i) control, (ii) chronic litter addition, (iii) chronic N addition, and (iv) combined chronic litter and N addition. Later, in June 2019, two additional treatments were established, including (v) extreme litter addition, and (vi) extreme N addition (Fig. 3). For the chronic litter treatment, Birch litter was selected because Betula is one of the most dominant plant families responding to Arctic warming, and contributing to treeline advance and shrubification of Arctic ecosystems (Chapin III and Shaver, 1996; Rundqvist et al., 2011; Mekonnen et al., 2021). The chronic N treatment was conducted to simulate the inorganic N release (i.e., increased N mineralization) from high-latitude soils expected in response to a moderate < 3 °C warming scenario (Mack et al., 2004). For the extreme treatments, three-times the annual additions of N or litter were added as a single addition in June 2019. These extreme additions represented a pulse of litter input or inorganic N resulting from e.g., a summer heatwave (Marchand et al., 2005; Zona et al., 2014) (Fig. 3).



Figure 3. Schematic overview of the field experiment and measurements conducted, from Paper I

"Space-for-time" substitution is an approach for studying ecological changes at different sites that are assumed to be at different stages of ecosystem development (Parker et al., 2015). This approach was employed in Paper V, to infer the changes in vegetation composition due to warming in the Arctic. A subarctic ecotone extending from lower-altitude birch forest to higher-altitude tundra heath near Abisko, Sweden was chosen to investigate changes in resource limitation for soil microbes. Four different sites were established along the ecotone in 2021, including forest, treeline, shrub and tundra heath. At each site, a factorial fertilization experiment where N (10 g m<sup>-2</sup> year<sup>-1</sup>) and P (10 g m<sup>-2</sup> year<sup>-1</sup>) were established to test if nutrient loading affected the limiting factor for microbial growth.



Figure 4. Hypothesized changes in C, N and O availability along the ecotone from tundra heath to birch forest. From Paper V.



Four studied sites along the ecotone. Top left corner: tundra; top right corner: shrub; bottom left corner: treeline; bottom right corner: forest (Credit: Mingyue Yuan)

Elevated atmospheric carbon dioxide (eCO<sub>2</sub>) is one of the most profound consequences of anthropogenic activities which is leading to climate warming (Vitousek, 1994). eCO<sub>2</sub> may also alter environmental resource availability to soil microbes, as eCO<sub>2</sub> can stimulate plant photosynthesis (Ainsworth and Long, 2005; Lee et al., 2011). In particular, eCO<sub>2</sub> have been found to stimulate plant C allocation to leaves and roots. Such responses should provide more C-rich substrates belowground via litterfall or root deposition (Finzi et al., 2001; King et al., 2001; Iversen et al., 2012). Free-air CO<sub>2</sub> enrichment (FACE) has been widely-used to learn how ecosystems respond to eCO<sub>2</sub> (Norby and Zak, 2011). To investigate how microbial resource limitation was affected by eCO<sub>2</sub>, we sampled soil from a mature Eucalyptus-dominated woodland in Richmond, Australia (EucFACE, 33°37'S, 150°44'E). The facility was established in 2012, with where six rings built up in the forest. Three of them acted as controls, which were under ambient CO<sub>2</sub> (400 ppm), and another three released CO<sub>2</sub> into 25m diameter circular plots, creating a CO<sub>2</sub> concentration of 550 ppm (Drake et al., 2016).



Photo of EucFACE facility. (Credit: EucFACE facility).

#### 2.4 Microcosm experiment in the laboratory

In ecology, the term "legacy effect" has been used to describe effects of the historical conditions on the current process (de Vries et al., 2012; Crawford and Hawkes, 2020). Microcosm experiments were conducted in Paper III, and Paper VI to study the legacy effect of changes in environmental availability affects microbial use of new resource inputs. To do so, litter and N were added into soil samples from the subarctic tundra (Paper I) following a factorial design, including non-amened control, litter addition, N addition, combined N and litter addition (Paper III). After the additions, the microcosms were incubated for two months, microbial growth and respiration were monitored. For soil samples from EucFACE facility, litter was added solely or combined with N and/or P (Paper VI), therefore the design included non-amended control, litter addition, combined litter and N addition, combined litter and P addition, combined litter, N and P addition. The microcosms were incubated for six weeks, and microbial growth, respiration and enzymatic activities were monitored during the incubation.

Both litter enrichment in the field (Paper I) and litter addition in the lab (Paper III and Paper VI) simulated the increase in aboveground C inputs. However, rhizosphere input is also a substantial source of increasing C availability in soil (Iversen et al., 2015; Moore et al., 2020). Root exudates are thought to provide more labile C compared to leaf-litter inputs. Additionally, it has been found that labile C inputs can trigger microbes to start decomposing more native SOM: a phenomenon termed the "priming effect" (Kuzyakov, 2010), and one of the proposed explanations for the priming effect is that microbes decompose native SOM to mine the limiting resource (Kuzyakov et al., 2000). To test if enhanced rhizosphere inputs affected soil priming, a semi-continuous labile C addition experiment was conducted in Paper IV: Labile C was added into the subarctic tundra soil in a form or glucose or alanine, with or without inorganic N. The substrates were added semi-continuously every 2 days, to simulate the root exudates input.

### **Empirical** work

#### 3.1 What's the limiting resource for soil microbial growth?

Plant productivity at our studied subarctic tundra was found to be stimulated by N fertilization (Paper I), while the plant productivity at our studied subtropical woodland was found to be limited by P (Ellsworth et al., 2017). These findings are inconsistent with the global pattern of nutrient limitation, that plant productivity is found to be typically limited by N in the arctic, boreal or temperate ecosystems (geologically younger) and more likely limited by P in the tropical ecosystems (geologically older) (LeBauer and Treseder, 2008; Vitousek et al., 2010; Du et al., 2020). We therefore hypothesized that microbial growth would be limited by N in the subarctic tundra soil while it would be limited by P in the subtropical woodland soil. In contrast, we found that microbial growth in these two sites were both primarily limited by C, and secondarily limited by P (Fig. 5). In addition, we found that microbial growth was co-limited by CNP along the studied subarctic ecotone from tundra to heath (Paper V).



Figure 5. Relative microbial growth in response to LFA-addition. Reproduced from Paper II and Paper VI.

It was not surprising that we found microbes were limited by C and P in the tropical soil, which was consistent with other findings from tropical soil (e.g., Cleveland et al., 2002). However, finding a primary C and secondary P limitation in arctic soil was inconsistent with the traditional view that arctic ecosystems are generally Nlimited (Shaver and Chapin, 1980), as well as findings of microbes being N-limited in other arctic tundra ecosystems (e.g., Alaskan tundra sites) (Mack et al., 2004; Sistla et al., 2012). However, the finding of microbial C limitation was partly in line with studies conducted in tundra soils near to our field site, where C addition had been found to increase microbial biomass (Jonasson and Michelsen, 1996; Schmidt et al., 2000; Haugwitz et al., 2011), and where previous studies near to our field site using combined N, P and potassium (K) addition suggested that microbes could be P-limited (Ruess et al., 1999; Rinnan et al., 2007). One possible explanation of the contrasting findings could be the difference of vegetations in these two regions: that the abundance of shrub in the Alaskan tundra is greater compared to in the Swedish tundra, which may result in both a higher uptake of N from soil by plants and also a higher plant-derived C inputs to soil in the Alaskan tundra (Shaver and Jonasson, 1999: Van Wijk et al., 2003), which might result in a microbial N limitation observed in the Alaskan tundra soil and microbial C limitation observed in the Swedish tundra soil.

### 3.2 How do climate change events alter resource limitation for soil microbes?

We hypothesized that litter addition treatment would exacerbate the putative microbial N limitation, and that N fertilization would alleviate the microbial N limitation in the subarctic tundra soil. However, microbial growth was found to be primarily limited by C and secondarily limited by P (Paper II). The degree of microbial C limitation was found to be alleviated by the addition of C-rich birch litter and strengthened by N-fertilization in the field (Fig. 6).



Figure 6. Relative microbial growth in the LFA. From Paper II.

The space-for-time substitution was used to simulate the change of vegetation composition due to arctic warming (Paper V), to test if the changes in vegetation altered soil microbial resource limitation. We hypothesized that microbes would be relatively more C and N limited in tundra compared to in forest, while would be relatively more P limited in forest compared to in tundra, due to that higher plant derived-C inputs and more fixed N in the forest, and generally P declining during the ecological succession (Fig. 4). Moreover, the field N or P fertilization was expected to shift the resource limitation for microbial growth, resulting in weaker N limitation in N-fertilized plot and weaker P limitation in P-fertilized plot. However, the results varied (Fig. 7). First, bacterial growth increased only in response to LFA-CNP addition, while fungal growth increased in response to both LFA-CN addition and LFA-CNP addition. These responses suggested that bacterial growth was multilimited by CNP, while fungal growth was co-limited by C and N. Second, there was no shift in either bacterial or fungal resource limitations along the ecotone. Third, fungal responses to LFA were strongest in the forest soils and the magnitudes declined from forest to tundra, whereas the bacterial responses to LFA were strongest in the tundra soils and magnitudes declined from tundra to forest. These results together suggested that vegetation changes due to arctic warming might not alter resource limitation for growth, but rather affect degrees of fungal-to-bacterial dominance where fungi were more dominant decomposers in the forest soil and bacteria were more dominant in the tundra (McLaren et al., 2017).



Figure 7. Relative bacterial (blue), and fungal (red) growth in response to LFA-additions in soils across a subarctic ecotone. From Paper V.

Another consequence of climate change which might alter microbial resource limitation is eCO<sub>2</sub>. We hypothesized that microbial growth in a mature woodland would be limited by P, and that eCO<sub>2</sub> treatment in the field would enhance microbial P limitation (Paper VI). Contrary to the expectation, microbial growth was found to be primarily limited by C and secondarily limited by P. eCO<sub>2</sub> was found to be enhanced the microbial C limitation (Fig. 8), which was surprising because eCO<sub>2</sub> was expected to lead to more plant-derived C inputs into soil which would consequently alleviate the microbial C limitation. One possible explanation for this enhanced microbial C limitation may be the increase in soil N and P availability observed in the field due to eCO<sub>2</sub> (Hasegawa et al., 2016; Ochoa-Hueso et al., 2017). These increased in N and P availability might be disproportionally higher than the increase in belowground C input, which thus enhanced microbial demand for C. An alternative and more likely explanation could be that the eCO<sub>2</sub> led to more labile C inputs belowground, which resulted in a more metabolically active decomposer community, e.g., the microbial community shifted to a faster-turnover community under eCO<sub>2</sub>, which increased the C demand of the whole community.



Figure 8. Relative microbial growth in response to LFA-additions. From Paper VI.

# **3.3** What's the link between microbial resource limitation and decomposition?

Microcosm experiments were conducted to detect the link between microbial resource limitation and decomposition (Paper III, Paper VI). In subarctic soil, a legacy of enhanced N input stimulated microbial use of new litter input in the laboratory, with a growth response that was dominated by the bacterial community (Fig. 9). The most likely explanation for this legacy effect was that the field N addition increased the microbial C limitation (Paper II) (Kamble et al., 2013). However, it was also possible that the N addition in the field increased plant-C derived inputs shift the bacterial community being better at using C (Campbell et al., 2010; Eilers et al., 2010; Ramirez et al., 2012; Leff et al., 2015).

Microbes have been found to decompose native SOM to mine the limiting resource, which has been used to explain the "priming effect" (Kuzyakov et al., 2000). In nature, one common trigger for the priming effect is thought to be rhizosphere deposition. In Paper IV, the root inputs were simulated by semi-continuously adding C or/and N (in a form of glucose,  $NH_4NO_3$  or alanine) to the soils from the same site as in Paper I, II and III. According to the results from LFA (Fig. 5), that microbes were primarily limited by C, the addition of N was expected to induce microbial mineralization of C. Contrary to this expectation, the addition of N didn't impact on priming of soil organic carbon (SOC) mineralization (Fig.10), suggesting that microbes might target other resources rather than C in the soil. Considering the finding of P being the secondary limiting resource for the microbial growth (Paper II), it is fair to postulate that soil microbes in this tundra ecosystem might actually mineralize native SOM to target P, rather than C or N. Moreover, litter addition was found to alleviate microbial C limitation (Fig 6), but the priming SOC mineralization by labile C was more pronounced in the soil from litter-amended plot, and there were no changes in priming of SOM mineralization (Fig. 10), which also suggested that microbes might target P in this soil, while SOC mineralization was coupled with mineralization of P.

In the mature woodland soil, we found that microbial C limitation tended to strengthen under  $eCO_2$  (Fig.5). When microbes were provided with new litter with or without N and P, microbial growth was stimulated by the C-rich litter addition, with a more pronounced increase occurring in soils from the  $eCO_2$  field-treatments (Fig.11). These results together suggested that the microbial C limitation was enhanced by  $eCO_2$ , which stimulated microbial use of new litter input. The finding was also in line with previous findings from our study site, where  $eCO_2$  stimulated turnover of SOM, as inferred by both increased soil heterotrophic respiration and higher root mass loss under  $eCO_2$  (Castañeda-Gómez et al., 2020; Jiang et al., 2020).



Figure 9. Cumulative microbial growth, fungal-to-bacterial growth ratio and microbial respiration in 2-month incubation after resource additions. Data from Paper III.



**Figure 10**. Cumulative priming of bacterial growth, fungal growth and SOC mineralization. From Paper IV. C: control; N: chronic N; L:chronic litter; NL: chronic litter and N; XN: extreme N; XL: extreme litter.



Figure 11. Cumulative microbial growth, fungal-to-bacterial growth ratio and cumulative microbial respiration in a 6-week incubation after resource additions, Data from Paper VI.

# **Conclusion and Discussion**

### 4.1 Remarks

Surprisingly, microbial growth in both arctic and tropical soils was primarily limited by C and secondarily limited by P. Litter input and N fertilization had contrasting effects on plant productivity and microbial resource limitation in the studied arctic ecosystem. Fertilization with N increased plant productivity (Paper I), and exacerbated the microbial C limitation (Paper II), which consequently enhanced microbial growth response to new litter inputs (Paper III). Litter input had no effect on plant productivity (Paper I), but alleviated microbial C limitation (Paper II), the latter effect explained why microbial growth responses to new litter addition did not differ in litter-amended soil and non-amended soil (Paper III). Moreover, litter amendment enhanced the priming of labile C addition on SOC mineralization, but not priming of soil organic N (SON) mineralization (Paper IV), this suggested that microbes might actually mineralize native SOM to target P, which was found as the secondary limiting resource for microbial growth in this soil. In the studied tropical ecosystem, eCO<sub>2</sub> increased the ecosystem C availability, but did not stimulate plant productivity due to the P deficiency in the field (Ellsworth et al., 2017). eCO<sub>2</sub> enhanced the microbial C limitation, which consequently enhanced microbial growth response to new litter input (Paper VI), which might contribute to the increased C flux observed under eCO<sub>2</sub> (Jiang et al., 2020).

Back to the three questions listed in the Introduction. From this study, I have learnt that microbial growth is primarily limited by C and secondarily limited by P (Question 1). Climate change event can alter microbial resource limitation (Question 2), but the direction varies. In the Arctic, C enrichment alleviated microbial C limitation, while C enrichment in the tropic enhanced microbial C limitation. Microbial C limitation enhanced by C enrichment was probably related to a more metabolically-active community which demanded more C, suggesting that the changes in microbial resource limitation was not only driven by the resource inputs into soil but also by how it shaped the microbial community. Moreover, results suggest that microbial resource limitation will trigger microbial mining of the limiting resource from organic matter, which will accelerate decomposition (Q3).



Figure 12. Consequence of changes in environmental resource availability to plant productivity, microbial resource limitation and microbial growth responses to new resource inputs in the arctic site and the tropical site. (+) represented an increase in the observed component, while "no change" represented the field treatment or laboratory additions did not change a component as expected.

### 4.2 Methods matter

There are plenty of approaches that have been applied to determine limiting factors for soil microbes, I have categorized commonly-used ones as growth-based approaches, respiration-based approaches, biomass-based approaches, enzyme-based approaches and stoichiometry-based approaches (Table 1).

Approaches	Assumption and references	Examples and references
Growth-based approach	An increase in growth occurs when the limiting resource is added.	This current thesis
(LFA)		
Respiration- based approach	Microbial respiration will increase up to a maximum rate after the limiting nutrient is added (Scheu, 1992, 1993).	C and N co-limitation in a taiga forest soil (Vance and Chapin III, 2001); C and N limitation in a tropical plantation soil (Ilstedt and Singh, 2005);
Biomass-based approach	An increase in biomass occurs when the limiting resource is added.	N limitation in a tussock tundra soil (Sistla et al., 2012); C and P colimitation in two rainforest soils (Krashevska et al., 2010; Fanin et al., 2015).
Enzyme-based approach (EEA)	Four enzymes including $\beta$ -1,4-glucosidase (BG), $\beta$ -1,4-N-acetylgucosaminidase (NAG), leucine aminopeptidase (LAP) and acid/alkaline phosphatase (AP) are used as indicators for the total microbial need for C(BG). N (LAP+NAG), and P (AP). The relative activities of these four enzymes are used to reflect the microbial demand for different resources and, thus the soil microbial resource limitation (Sinsabaugh and Follstad Shah, 2012).	P limitation in high-weathered soil (Deng et al., 2019); C and P limitation in a temperate forest soil (Bai et al., 2021); P limitation in peatland (Hill et al., 2014).
Stoichiometry- based approach	Heterotrophic microbes can be strictly homeostatic, indicating that they acquire different nutrient in fixed proportions to maintain their chemical composition. The imbalance between the stoichiometry (C:N:P molar ratio) of resources and soil microbial communities can indicate the microbial resource limitation.	The C:N ratio of plant litter, SOM and microbial biomass was 71:1, 17:1 and 7:1 respectively, it suggested that soil microbes were relatively more N-limited in topsoil and more C-limited in subsoil (Mooshammer et al., 2014).

 Table 1. Approaches being employed to determine limiting resource for soil microbes.

The variety of approaches brings up a methodological issue: the determination of limiting factor might depend on the approach used. For example, in Paper II, I used LFA to detect limiting resource for microbial growth in a tundra soil. The results suggested microbial growth was primarily limited by C and secondarily limited by P (Fig. 5). In another tundra ecosystem which located on the same latitude, microbial

biomass was found to be limited by N (Sistla et al., 2012). Although this inconsistency can be explained by different vegetation communities at these two sites (see Section 3.1), different approaches being used in these two studies, i.e., growth-based approach and biomass-based approach, could also cause the different diagnosis about microbial resource limitation. Moreover, in Paper VI, I used both LFA and EEA to determine limiting resource for microbes in soil from the mature woodland. Results of LFA suggested that microbial growth was primarily limited by C and secondarily limited by P. The ratio of BG/(NAG+LAP) was lower than 1 while the ratio of (NAG+LAP)/AP was higher than 1 in the soil, suggesting that microbes were limited by N, and which was alleviated by eCO<sub>2</sub> (Table 2 in Paper VI). This was alarmingly inconsistent with the conclusion drawn from LFA.

The diagnosis of microbial resource limitation is not only targeting the soil microbes *per se*, but also aims to elucidate how microbial resource limitation affects decomposition. I.e., it can be used to answer questions like: does microbes decompose organic matter to obtain the limiting resource? how will microbes use the limiting resource when it is available? My point of view is that both the detection of microbial resource limitation and its ecological relevance should be considered when selecting the approach to determine limiting factor for soil microbes. Based on this, I think the growth-based approach (LFA) is relatively more appropriate compared to other approaches. My reasoning is, as follows:

1) Growth is able to both reflect instantaneous microbial responses to the factorial resource addition and detect microbial use of limiting resource.

Three of above listed approaches determine the limiting resource by measuring microbial responses to a factorial resource addition: growth-based approaches, respiration-based approaches and biomass-based approaches. The shortcoming of biomass-based approaches is that it requires relatively longer incubation time compared to growth-based approaches, which leads to a risk that the changes in biomass due to addition of the limiting resource might be eliminated by other factors, like predation. For example, in Paper II, C was found as primary limiting resource for microbial growth, however, in Paper I, microbial biomass was not found to increase in response to C-rich litter addition in the field. And one possible explanation of this inconsistence was predators might consume the increase part of biomass in the litter-amended plot, or cause for a fast community turnover.

Respiration-based approaches are able to reflect the instantaneous microbial response to the factorial resource addition. However, the response is the status of the whole metabolism which could include anabolism (e.g., microbes uptake resources to grow) and catabolism (e.g., microbes uptake resource to gain energy), of which for catabolism microbes prefer labile C-rich resource, it means that an addition of labile C will generally induce a microbial respiration response. I observed that in Paper II, Paper V and Paper VI, that microbial respiration responses

to LFA additions did not occur in parallel with the growth responses, and always respond to additions containing C.

*2) The potential bias from enzyme-based approaches and stoichiometry-based approaches.* 

The assumption of EEA is based on that microbes produce different enzymes to obtain different elements from the SOM, and that the relative activity between different enzymes can reflect the microbial demand of certain resources, i.e., microbes are limited by a certain element. There are four enzymes being widelyused to resolve microbial C. N. P limitation (see Table 2). My first concern is that decomposition of organic matter is a quite complex process which many enzymes are involved. Do only four enzymes can reflect the microbial acquisition and thus demand of specific resource? Besides, if we zoom into these four enzymes, the sum of NAG and LAP is used to refer N limitation. However, it has been found that when chitin, peptidoglycan, or protein are more abundant compared to cellulose in the environment, microbes may also use NAG and LAP to gain C (Mori, 2020). In this case, an increase in NAG and LAP will not reflect the microbial N limitation. My second concern is that enzyme synthesis prerequisite sufficient supplies of resources (Allison and Vitousek, 2005), if the production of one enzyme is limited by another resources, how this enzyme (or the ratio of this enzyme to other enzymes) can reflect the resource limitation? My third concern is that microbial enzymatic activities respond to changes in environmental resource availability in different ways, which leads to a difficulty to use EEA to test if microbial resource limitation is altered by changes in environmental resource availability. For example, NAG and LAP are used to reflect relative microbial N limitation, however, in Paper IV, neither addition of inorganic N in the field or in the laboratory decreased LAP (Fig. S4 in Paper IV). In paper VI, combined litter and N addition increased NAG and LAP. Taken together, EEA might not be appropriate approach when resolving microbial resource limitation.

Stoichiometry-based approaches have been used to infer resource limitation in relatively large scale, which is the strength of these approaches. For instance, it has been used to infer that the primary production in both aquatic and terrestrial ecosystems are commonly limited by N and P (Sterner and Elser, 2002; Elser et al., 2007), and soil microbes are generally limited by C (Soong et al., 2019). However, the strength of these approaches is also its weakness. It treats the environment has a static stoichiometry and the microbial community as an undifferentiated "homeostatic" whole, e.g., microbes within the community grow but the stoichiometry of the community will not change. It indicated that these approaches are not appropriate when microbes behave in a non-homeostatic way, e.g., fungal mycelia have very flexible stoichiometry (Camenzind et al., 2020), and the shifts in microbial community composition may alter microbial biomass stoichiometry (Mooshammer et al., 2014).

3) Available methods of measuring growth rate, e.g., Leu or TdR technique and Ace-in-erg technique, can resolve bacterial growth and fungal growth separately, thus enabling the understanding of microbial resource limitation.

The opportunity to assess resource limitation for bacteria and fungi separately provides two distinguishing advantages of LFA. First, it helps understand the how these two groups affect the microbial resource limitation for the whole community. In Paper II, the synchronous increase of both C and N availability resulted in a progressive P limitation, and bacterial growth was found to be closer to P limitation compared to fungal growth, suggesting that the progressive P limitation was probably driven by the increase P demand of the bacterial community. Second, the difference in bacterial resource limitation and fungal limitation could be corelated with the turnover of the SOM. Generally, fast turnover of labile components of SOM is regulated by the bacterial community, and slow turnover of more persistent fractions of the SOM is regulated by the fungal community (Liang et al., 2017). In paper VI, eCO<sub>2</sub> was found to enhance microbial C limitation, and microbial response to LFA-C addition was dominated by bacteria, suggesting that when there are new organic labile C inputs into the soil, a faster turnover of it will be regulated by bacteria.

#### 4.3 The interpretation and implication for microbial C limitation

Microbial C limitation was found in both arctic soil and tropical soil (Paper II, Paper VI), suggesting a general C limitation for soil microbes. Microbes not only use C as a source to build biomass, but also a fuel to gain energy, which means that an observation of microbial C limitation could indicate both resource and energy limitation (Hobbie and Hobbie, 2013; Gunina and Kuzyakov, 2022). Many studies have interpreted C limitation as energy limitation (Soong et al., 2019; Cui et al., 2021), and a study states that microbes use most of SOM as energy rather than as a source of C (Gunina and Kuzyakov, 2022). It is hard to distinguish if C is as a limiting factor for growth or as energy limitation. I still want to highlight that C limitation could include energy limitation because when energy limitation occurs, it can constrain the microbial use of other resources (e.g., N, P). For instance, one study in a tropical soil found that adding cellulose, as a source of both growth and energy, enhanced microbial biomass in response to P fertilization (Fanin et al., 2012). In Paper II and Paper VI, I found that C combined with P addition stimulated microbial growth more than the microbial growth stimulation induced by C, or P alone (Fig.6, Fig. 8), suggesting that P limitation of microbial growth was probably accompanied by an energy limitation caused by lack of C.

My initial expectation regarding to the findings of microbial C limitation was that microbes would allocate more C into growth when they received new C input, which would add to the ecosystem C sequestration via microbes. However, when litter was

added into these two soils, both microbial growth rates and respiration rates increased (Paper II, Paper VI). This suggested a double-edged sword effect of C input on soil microbial C sequestration. Subsequently, I estimated carbon use efficiency (CUE), which was defined by the ratio of C used to growth to total C assimilation (Manzoni et al., 2012). By definition, high CUE indicates that relatively more C is allocated to growth which can promote C stabilization in soils. I found that microbial CUE either had no change or decreased in response to new C-rich litter additions (Fig. 13), which suggested that alleviating microbial C limitation merely didn't lead to C stabilization. The LFA-CNP addition was found to tend to increase microbial CUE (Paper II, Paper VI). This suggested a stoichiometrically balanced resource supply (the addition of resources matched the elemental composition needed by microbes) rather than alleviating microbial resource limitation, plays an important role in an efficient growth (Roller and Schmidt, 2015), and the consequent C sequestration.



Figure 13. Microbial CUE in response to lab additions. Data from Paper III and Paper VI.

### 4.4 Primary/secondary limitation, co-limitation, and multi-limitation

The classic way to test the limiting resource for growth is according to an increase after factorial addition of resources. According to the Law of Minimum, there is only one scarcest resource, which is the limiting resource (There is only one shortest plank of the barrel, Fig. 1). However, many empirical studies showed that growth not only responded to one resource, which expand the types of resource limitation to "co-limitation" and "multi-limitation". There are plenty types of limitation in the study of resource limitation (Table 2, Fig. 14).

Туре	Definition
Primary/secondary limitation	Growth is limited by only one resource at any given time (von Liebig and Gregory, 1842). When the primary limitation is alleviated, the secondary one will appear.
Simultaneous co-limitation	Growth is limiting by more than one resource simultaneously (only the combination of resources can induce increased growth) (Harpole et al., 2011)
Independent co-limitation	Growth is limited by more than one resource, and different resource can individually increase growth (Harpole et al., 2011)
Multi-limitation	Growth is limited by several resources simultaneously (Ågren et al., 2012).

 Table 2. Definitions of different types of resource limitation.



Figure 14. Diagram of growth response to factorial resource additions, in a manner of different types of limitation.

In Paper II and Paper VI, I drew the conclusion that microbes were primarily limited by C and secondarily limited by P, because the patterns of growth response were consistent with the pattern of primarily/secondary limitation (Fig. 14). In Paper V, microbial growth was triple-limited by C, N and P ("multi-limitation"). Distinguishing different types of limitation is important because of the underlying ecology they are implied to. The primary C limitation and secondary P limitation suggested that sufficient C could be a prerequisite for microbial use of P. In another words, when C availability increased, the availability of P may greatly influence the overall microbial growth. While in Paper V, microbial growth was multi-limited by C, N and P, suggesting that microbial use of these three resources might be interdependent.

Another question arises here: why does co-limitation or multi-limitation occur? These two phenomena have been well reported in plant or phytoplankton ecology. and there are several candidate explanations. One explanation is that different species within one community can be limited by different resources but the community will show being co-limited (Danger et al., 2008). Another explanation is the utilization of one resource depends on the availability of another nutrient (Ågren et al., 2012). One example for this is that the production of plant extracellular phosphatase is found to be limited by N availability at the site (Treseder and Vitousek, 2001). Besides, co-limitation by two resources can be explained as a transition regime between two regimes each with single limitation factors, which is suggested by a study where three regimes of limitation were observed including Climited, C and N co-limited, and N-limited in a culture of bacteria and yeasts (Egli and Zinn, 2003). All these three explanations can contribute to multi-limited microbes by C, N and P in the subarctic ecotone (Paper V). However, more empirical work is needed to test these explanations. To test if there are different groups being limited by different resource, quantitative stable isotope probing (qSIP) can be combined with LFA. qSIP method sequences bacterial DNA fractions of multiple density after exposing bacteria with isotope tracers and a subsequent isopycnic centrifugation (Hungate et al., 2015), which can reveal the relative contribution of individual bacterial taxa to the whole growth and thus growth rates of individual taxa. Comparing this information in the control to one after LFA additions could reveal if different groups contribute differently to different LFA addition, which will indicate different resource limitation existing within the microbial community. Testing if the co-limitation/multi-limitation is due to the utilization of one resource depending on the availability of another nutrient is more complicated, because there are many metabolic processes that can be entangled. For example, energy limitation caused by lack of C can limit microbial use of P (see Section 4.3), and microbial enzyme synthesis was found to be limited by availability of C and N (Allison and Vitousek, 2005). Testing different regimes of microbial resource limitation in natural ecosystem is less feasible compared to abovementioned tests, which probably requires years-long monitoring of limiting resource for microbes, and meanwhile it requires a consideration that how other environmental factors for example, extreme weather, can affect the results.

## Merits and drawbacks

In this chapter, I want to discuss the merits and drawbacks of this PhD thesis in the bigger picture of soil microbial ecology. I accidently learned there were seven outstanding problems in soil microbiology, proposed by Selman A. Walksman in 1913. After one century, this questions still constitute the challenges in the modern soil microbiology (Elsas et al., 2019). It is so fascinating and intriguing, that I intend to reflect this thesis by that the merits could contribute to the complex answers of these seven problems, and the drawbacks will be when it cannot contribute to the answers.

The seven grand questions are reworded by McLaren (1977) as following:

#### 1. What organisms are active under field conditions in what ways?

First, which metric can be an appropriate indicator for "active organisms" under field conditions? Respiration, biomass, or growth? I think it is microbial growth. Biomass can indicate organisms are alive but it doesn't necessarily mean they are active. Exocellular enzymes can persist in the environment for quite a while, therefore it hardly can reflect the current status of the microbial community. Respiration is able to indicate "physiologically active" but sometimes respiration can take place without the changes of the microbial community (e.g., no growth). When microbial growth rate is used to be indicator of "active organisms", it is plausible to assume that a higher growth rate will positively correlate with a higher rate of microbial growth was limited primarily by C and secondarily by P in both arctic soil and tropical soil, as well as limited by C, N and P in along a subarctic ecotone, which suggests microbial resource limitation is ubiquitous in the nature. Therefore, I would infer that microbes are active under field conditions in a way incorporating resource for growth, but which is commonly-limited by the resource availability.

# 2. What associative and antagonistic influence existed among soil microflora and fauna?

Although soil fauna was not the objective in this study, they might influence the results of the field treatment. Microbial growth in the arctic soil was determined to be primarily limited by C (Paper II), however, the C-rich litter addition in the field didn't result in either an increase in microbial growth (Paper I) or an increase in

microbial biomass (Paper II), one possible explanation could be that fauna in the field constrained the microbial community, that the predation might eliminate the increase in biomass, and when the size of community was constrained, the growth rate might be constrained as well. Therefore, I would infer that soil fauna might affect soil microbes in an antagonistic manner, that fauna can constrain the growth and size of microbial community even when limiting resource is available.

I also noticed that the antagonistic relationship between soil fungi and bacteria related to the resources. In Paper IV, microbial responses to resource additions were dominated by fungal growth in forest soil and dominated by bacterial growth in tundra soil. In Paper VI, fungi dominated the growth response to new combined litter and N addition, while bacteria dominated the growth response to new combined litter and P addition. Taken together, it suggests that exploitation competition for resources commonly exists between fungi and bacteria.

# 3. What relationships existed between soil organic matter transformations and soil fertility?

Traditional view intrinsically links soil fertility to the soil organic matter (Elsas et al., 2019), and most studies elaborate soil fertility from a plant perspective, that high-fertility soil is the one provides sufficient nutrients such as N, P, K which sustain plant productivity. However, from a soil microbial perspective, high-fertility soil might be soil provides sufficient resources for microbes, i.e., low degree of resource limitation. This thesis is lacking power to provide a comprehensive picture of how soil fertility can affect soil microbial activity and thus soil organic matter transformation. However, I think it can contribute a piece to the puzzle. I found that enhanced microbial C limitation stimulated microbial use of C-rich litter, i.e., microbes uptake more C from litter and incorporated to their growth. It suggested enhanced microbial C limitation might trigger plant-associated C being transformed to microbial-associated C in soil.

## 4. What is the meaning and significance of energy balance in soil, in particular with reference to C and N?

From my point of view, balance from a soil microbial perspective might mean a balance of availability of all essential resources is sufficient for maintenance and growth. From the perspective of the ecosystem functions of soil microbes, i.e., nutrient supplies for primary production but also the C balance of ecosystem, energy balance could be an optimal point between these two functions. In this work, a supply of CNP with a C:N:P mass ratio of 20:1:1 led to an increase in microbial CUE (Paper II, Paper VI), which indicated a promoting C stabilization via microbes. However, it is unclear how the availability of essential nutrients for plants (e.g., N, P) changes under this circumstance.

#### 5. How do cultivated plants influence soil transformations?

Studies included in this thesis were conducted in pristine ecosystems, therefore I would expand this question as how plants influence soil transformations. This thesis shows a mechanism that plants can influence soil transformations due to altering microbial resource limitation. That an increase in plant-derived C inputs into soil could either alleviate microbial C limitation and consequently reduce microbial decomposing of new C-rich organic matter, or shape the microbial community in a direction of having higher demand of C (Paper II, Paper III), which consequently stimulates microbial decomposition of new C-rich organic matter (Paper VI). It is plausible to assume that when the latter take place, microbes would preferentially incorporate the limiting resource, i.e., C from organic matter into their biomass.

#### 6. How can one modify soil populations and to what ends?

It has been stated that microbes are often highly resistant to changes, however they may be slowly shifted when the environment changes. Powerful environmental factors include e.g., resource availability, pH level, water regime and soil management (Elsas. et al., 2019). By comparing the microbial resource limitation in arctic soil (geologically younger) and the microbial resource limitation in tropical soil (geologically older), we could see that soil microbes' populations tended to be more C and P limited along the development of soil. However, it is a very rough comparison, there must be other environmental factors modifying the microbial resource limitation, for example, different climates and vegetation of these two sites (Also see question 7).

The resolution of the composition of microbial populations in this thesis is the two main decomposer groups: bacteria and fungi. In this thesis, bacteria were found to be closer to P limitation compared to fungi (Paper II, Paper VI). It suggests that when resource availability is the main determinant of composition of soil population, declining P availability, which generally takes place during ecosystem succession, might have negative effect on bacterial community.

## 7. What interrelationships exist between physicochemical conditions in soil and microbial activities?

There are plenty of environmental factors which can regulate microbial activities together with resource limitation, such as pH and water regime. Water regime can affect microbial resource limitation: a drought condition might be able to limit the supply of substrates, and a rewetting event might be able to alleviate the resource limitation. pH is also a strong regulator for microbial activities, especially for bacteria, which could affect the fungal-to-bacterial dominance when the community

responds to resource inputs. In Paper V, microbial responses to LFA-additions were dominated by fungi in the forest soil, while were dominated by bacteria in the tundra soil, which could be driven by the type and composition of the resource (plant-litter and high C:N ratio SOM favour fungal community), but also could be caused by the pH that soil pH is lower in forest compared to in tundra which favoured fungi.

#### The final point

This thesis does not answer all of the above seven grand questions, or indeed any of them completely, -rather it contributes to answering some of them. I am satisfied with this for now, because I have realized that the relation between resource limitation and soil microbes is more complicated than it appears. It actually makes the study of microbial resource limitation fascinating: I began my PhD with the arctic ecosystem. It was no surprise to find that N increased plant productivity there, as well as C was the primary limiting resource for soil microbes. However, it was very surprising for me that P was the secondary limiting resource. When I saw the soil samples from Australia, I was so confident to postulate that soil microbes living there was limited by P, and  $eCO_2$  would enhance the microbial P limitation due to more plant-derived C into soil. However, I found C was the primary limiting resource for soil microbial growth, and microbial C limitation exacerbated under  $eCO_2$ . Besides, I had believed that microbial resource limitation was mainly driven by the resource availability, but it turned out that composition of microbial community also contributed to it.

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# Acknowledgements

Johannes, before I started my PhD, my thought of a supervisor was someone who gave students tasks and ensured they would finish their PhD on time. You have completely changed my perception. You have shown me a supervisor can be a person who illuminates the wonders of Science, supports students in their explorations, and stands as a constant backup for them. Thanks for bringing me into the world of microbial ecology, I am really grateful for the space you have created—a space where my curiosity isn't just allowed but encouraged to run wild. Thanks for all understand and empathy towards my life and thanks for sharing your life philosophy with me.

Lettice, You have been always very patient with me. I still remember how lost I was when I wrote my first manuscript, then you suggested that I could write it piece by piece. You always make time for me when I get stuck or my thoughts become tangled threads. You have this amazing ability to understand me all the time, even when I can't understand myself sometimes! I could not have accomplished my PhD without your help and support! Also, thanks for sharing many cosy entertainment options for my spare time: cake, ice cream, Skåneleden and nice café places!

**Meng**, we were the best team in the lab! I still don't know how, but we could literally function as each other's substitutes when we worked on projects together (although somehow, we were equally bad at fieldwork). Thanks for all the company in the lab, in the field, on the way to the train station, and during our explorations of Skåne. **Markus**, I trusted you more than myself when it came to things related to chemistry. Together, we achieved a perfect work-life balance. I can't count how many times we spontaneously jumped on the train to Svedala after work, just as I can't count how many apple strudels I had when you were here.

**Kathi**, thank you for being my teacher of moss, cyanobacteria and N fixation. I greatly admire your enthusiasm about them! **Albert**, thanks for encouraging my occasional interests in mathematics. And your modelling course is the best I have ever participated in. **Catriona**, thanks for allowing me use the soil from EucFACE

facility, I have had so much fun working on the Australian soil (everything is different from soil samples from northern hemisphere!). This project has significantly broadened my horizons.

**Haifeng** and **Yuqian**, you are not my co-authors, but you do contribute a lot to my thesis. Thank for always being so patient for all my questions. **Haifeng**, thanks for being my teacher in enzyme activity. I know there must have been moments when you did want to block me, but thanks for not doing that. **Yuqian**, thanks for explaining things about N and pH to me, though it is high likely I will ask you the same questions again (and again).

**Pål Axel**, thanks for being my examiner, thanks for not only conducting the meetings for individual study plan, but also always reminding me the plans for vacation.

Thanks **Dr. Peter Vitousek**, for your spectacular book "*Nutrient Cycling and Limitation: Hawai'i as a Model System*". It has been the textbook for my PhD (and I will definitely use it for my postdoc)! I have learnt so much and gotten so many inspirations from the book.

**Dimitri**, thanks for your friendship, I know can share not only happiness but also frustrations with you anytime. I will miss our "morning-coffee" conversations a lot, which has been a wonderful lecture about plant, mycorrhiza and evolution. Thanks for always taking time to ask how I am doing, no matter how crazy your schedule is.

**Micaela**, my favourite officemate ever. I failed to come up with words that can beat "we are like day and night", which you wrote down in your thesis, and I cannot agree more. We do have some magic in this office. yes, I am referring to the synchronicity and telepathy. It is so nice to have you around to share all the happy and frustrating moments (thanks for not kicking me out of our office when I screamed to my R code).

A big thanks to members and alumni of Microbial Ecology, for creating such a nice work environment here. I enjoy all our fika time! **Sara**, you always bring this bright spirit. When I feel I am almost turning into a zombie, I know I can turn to you for some energy. and I am very proud of ice cream club! **Anna**, thanks for consistently

helping me out with the lab things and encouraging my Swedish learning. Kristin, vou are the sunshine in the corridor! and I cherish our time together. Håkan, thanks for being the opponent for my half-time and for your constructive feedback. Also, thanks for being my teacher about mycorrhiza, forest, N cycling, and chronosequence. Erland, thanks for sharing your insights about techniques and ecology with me, and special thanks for mentioning the seven grand questions during fika! Edith, thanks for your constant compliments for my work. François, I will never forget the way you showed me how mycorrhiza live in the roots (you brought me out of the Ecology Building and just started digging roots out from the soil). Carlos, thanks for being my kött (och bok) vän, I feel I could be myself in front of you. Ainara, Margarida, Katja and Lokesh, thanks for sharing me the tips on navigating the PhD journey. Juan Pablo, thanks for sharing your insights about P with me and for teaching me measure enzymatic activities, and I do enjoy our endless argument about which is better: conifer forest or deciduous forest? **Yingi** and **Oinmei**, you are wonderful chemists, that means I don't fully understand your projects, but meeting you here brought me so much happiness! Tao and Sivuan, it was wonderful to have field work with you, I will never forget the "Abisko spicy". Cristina, Molly, Blandine and Casey, thanks for keeping me company during the "dark" time, the strawberry farm and the crepe night are two of my fondest memories from my PhD life. Michiel, Zhaomo and Bowen, we have different interests in science, but I do enjoy every conversation with you. Vanesa, thanks for all helps in the lab!

To all my PhD fellows: Ashish, Agnieszka, Ana, Daniel T, David (thank for all friendly "how's life?"), Emma Kä, Kalle, Qinyang, Violeta. It has been fantastic getting to know you here. To PhD fellows from Kalmar: Yelena and Danilo, I am very happy to have met you in GENECO. To people from the Department of Biology: Staffan and Emma Kr, thanks for all the friendly chats when I encounter you in the department or on the bus. Nathalie, thanks for including me in the evolution dinner! Nils, thanks for sharing the story about moss and volcanic islands, it is fascinating! Erling, thanks for helping with the shipment of my soil samples. Anders and Inger, thanks for helping translate my work into less nerdy language and thanks for the photograghs! Thanks to all library staff, for helping me find books and downloading very old papers!

I want to express my thanks to all coordinators of the following courses, which I have participated in during my PhD: **Daniel C** and **Lettice**, thanks for the Global element cycling course (Lund University); **Petra**, thanks for the Root, soil, microbe interactions course (Swedish University of Agricultural Science ); **Kathi** and **Carsten**, thanks for the Interactions between plants, microorganisms and soil course (University of Copenhagen); **Torben** and **Efrén**, thanks for the BEFLUX course

(Aarhus University). I have learnt a lot for the courses! **Karin**, **Bengt** and **Emily**, thanks for organising the GENECO graduate school (Lund University), genetic ecology is cool! **Olof**, thanks for organising Finish on Time workshop, it helps me a lot!

I also want to express my thanks to all people who have inspired me during my PhD. **Karina**, I still have the notes from our first meeting, where you drew a triangle to illustrate the three factors that can affect soil organic matter. And I was so happy when crossing you in Abisko this summer. **Lars**, thanks for showing the wonderful common garden experiment, and I appreciate the comprehensive email you sent when Haifeng and I had questions about the experiment. **Wenxin**, thanks for answering my "large scale" questions. **Jia**, thanks for being my teaching in remote sensing. **Shangshi**, thanks for sharing your knowledge of microbial biomass and shrub encroachment in grassland ecosystems. **Riku**, thanks for hosting us at the wonderful Oulanka Research Station. **Duncan** and **Kevin**, I am grateful for your support of my passion for Arctic research and for helping me navigate the Toolik Field Station, it was an incredible experience! **Amanda** and **Haley**, thanks for helping me all the paperwork and fieldwork at Toolik! **Ruby**, I feel so lucky to have met you during the BEFLUX course, thanks for all help for the Alaska project.

Xueying, Qijiang and Li. You are my family in Sweden, thanks for helping me establish my life here, I know I can rely on you anytime for practical things. Meiyuan, Peter, Feng, Huadong, thanks for inviting me to all festival gatherings, which made me feel at home! Veronika and Nicolai, thanks for all the warm company! Noor and Qichao, you have the magic that always makes me believe I am a superwoman. H and D. Thanks for all the deep care and unconditional support.

### List of Papers

- Hicks, L.C., Yuan, M., Brangarí, A., Rousk, K., & Rousk, J. (2022) Increased Above- and Belowground Plant Input Can Both Trigger Microbial Nitrogen Mining in Subarctic Tundra Soils. *Ecosystems 25*, 105–121.
- Yuan, M., Na, M., Hicks, L.C. & Rousk, J. (2023) Limiting resource for soil microbial growth in climate change simulation treatments in the Subarctic. *Ecology*, e4210.
- III. Yuan, M., Na, M., Hicks, L.C. & Rousk, J. (2022) Will a legacy of enhanced resource availability accelerate the soil microbial response to future climate change? *Soil Biology and Biochemistry*, 165, 108492.
- IV. Na, M., Yuan, M., Hicks, L.C. & Rousk, J. (2022) Testing the environmental controls of microbial nitrogen-mining induced by semicontinuous labile carbon additions in the subarctic. *Soil Biology and Biochemistry*, 166, 108562.
- V. Neurauter, M\*., Yuan, M.\*, Hicks, L.C.& Rousk, J. (2023) Soil microbial resource limitation along a subarctic ectone from birch forest to tundra heath. *Soil Biology and Biochemistry*, 177, 108919.
- VI. **Yuan, M.**, Macdonald, C., Hicks, L.C. & Rousk, J. Will elevated atmospheric CO2 slow ecosystem nutrient cycling via induced aboveground-belowground competition for P? *Manuscript*



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