STOICHIOMETRIC IMBALANCE IN TERRESTRIAL ECOLOGY

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The First Law of Ecology: Everything is connected to everything else. The Second Law of Ecology: Everything must go somewhere. The Third Law of Ecology: Nature knows best. The Fourth Law of Ecology: There is no such thing as a free lunch.

Barry Commoner

Life is like riding a bicycle. To keep your balance, you must keep moving.

Albert Einstein

Contents (short)

Contents

Abstract

In this thesis, we investigated stoichiometric imbalance due to changes in carbon (C), nitrogen (N), and phosphorus (P) availability in different terrestrial scenarios. In the first study, we investigated the effect on biomass of a two-way N and P fertilisation gradient, ranging from deficient to sufficient supply of nutrients, in barley (*hordeum vulgare*). In particular, shoot-root biomass allocation and synergistic effects were investigated when availability of both N and P was varied. In the second study, we moved our attention to soil as we investigated the accrual of nutrients into soil microbial biomass along a soil organic matter (SOM) gradient formed from a post-mining chronosequence. In a third study, we investigated stoichiometric imbalance in soil due to changes in soil and microbial stoichiometry after a freeze-thaw (FT) event. In the first two studies, we found, as expected, that accrual of nutrients into biomass (plant and soil microbial) increased with increasing nutrient availability. Unexpectedly however, these responses were not linear but instead exhibited critical values that determined the response of the variables in question. In the first study, we could show that there was a synergistic response of barley biomass to N and P fertilisation. This effect, however, was only observed above a critical P level, when P was not limiting growth. Furthermore, we could show that balanced supply of nutrients had a greater effect when P was limiting. From this we further hypothesise that the plants were not able to cope with increases in stoichiometric imbalance due to widening N:P ratio when P was limiting, yet they were more able to do so when not limited by P.

Similarly, in the second study of the recultivated soils of the post-mining chronosequence, we could show that the accrual of carbon and nitrogen into microbial biomass and relative respiratory carbon losses shifted around a 1% soil organic carbon (SOC) threshold. We interpreted this result to mean that when the soils contained less than 1% SOC, soil microbes were extremely carbon limited and in a stoichiometric inefficient state due to stoichiometric imbalance. Whereas when SOC increased above this critical value, stoichiometric imbalance was alleviated and the microbes shifted to a more efficient stoichiometric state. Lastly, in the third study, we could show that, in the few hours after a freeze-thaw event, there was an enrichment of nitrogen into soil microbial biomass. This was due to a coupled effect of increased N uptake by the surviving microbial biomass and disproportionate C losses via respiration. We hereby could present evidence that stoichiometric imbalance due to enriched microbial N, in the first freeze-thaw cycle, may be the first step that leads to significant N losses in subsequent freeze-thaw cycles in agricultural soils. In summary, we present two main findings in this thesis: Firstly, we provide evidence, for the first time, that critical thresholds between stoichiometric states may exist that are dependent on the absolute nutrient status of the system, rather than relative stoichiometric ratios. Secondly, we present a mechanism for microbial derived N_2O emissions after freeze-thaw events due to short-term stoichiometric imbalance in soil microbial biomass. These results contribute to a better understanding of stoichiometric transformations in ecology and may lead to a more complete understanding of nutrient cycling and nutrient limitations in terrestrial ecosystems.

1 Introduction and aims

1.1 Introduction

In chemistry, stoichiometry simply refers to the ratios and balance of reactants in a chemical reaction. Ecological stoichiometry, thus, deals with the balancing of biologically relevant chemical elements in ecological processes and interactions (Sterner and Elser, 2008). It predominantly deals with relative abundances of carbon (C), nitrogen (N) and phosphorus (P), as these are the 3 most growth-limiting nutrients.

The field of ecological stoichiometry is most often traced back to a key finding from oceanographer Alfred C. Redfield (1958), wherein the average C:N:P ratio of phytoplankton biomass was found to be fixed at 106:16:1 (the famous Redfield ratio) and matched that of the surrounding water. This result prompted great thought into how the biotic and abiotic environments interact and how matter and energy are interchanged between these boundaries. The Redfield ratio is still used as a reference from which to predict nutrient limitation and imbalance in aquatic systems (Hall et al., 2011). Based on these same principles, stoichiometric ratios of biomass C:N:P have since been derived for terrestrial ecosystems and taxa, for example, in terrestrial plants (Kerkhoff et al., 2005; McGroddy et al., 2004), soil microbial biomass (Chen et al., 2016; Cleveland and Liptzin, 2007; Hartman and Richardson, 2013; Zechmeister-Boltenstern et al., 2015), and soil fungi (Zhang and Elser, 2017). Whilst demonstrating varying degrees of inter- and intraspecific variation, these derived ratios are considered to be the optimum elemental ratios required for these organisms to function (Sterner and Elser, 2017b).

While it has been shown that the stoichiometry of specific taxa or ecosystems may be constrained to varying degrees, this is not to say that the stoichiometry of living things is fixed (Sistla and Schimel, 2012). Matter and energy are constantly exchanged in food web and trophic interactions (Hessen et al., 2013), in global nutrient cycling and in ecosystem processes (Lovett et al., 2006; Welti et al., 2017). Moreover, organisms may exhibit changes in their elemental ratios according to life history (Acharya et al., 2004). Stoichiometric flexibility can be observed at both the organism level and community level (Chen et al., 2016; Sistla and Schimel, 2012), i.e., when the different organisms that make up a community have different stoichiometric requirements, changes in proportions of significant/functional groups may change the overall stoichiometric requirements of the community. Stoichiometry of living matter can more accurately be viewed as a dynamic equilibrium with a constant flux of nutrients between levels of biological hierarchy and the abiotic environment (Sistla and Schimel, 2012; Zechmeister-Boltenstern et al., 2015).

Like chemical stoichiometry, ecological stoichiometry is bound by physical laws; namely, the first law of thermodynamics: energy and matter cannot be created or destroyed. This leads us to one of the core principles of ecological stoichiometry; homeostasis and plasticity of consumer resource stoichiometry vs resource stoichiometry. Organisms cannot ”create” elements. Therefore, their elemental make-up is determined by the nutritional resources available to them. The relationship of the organism’s stoichiometry to the resource can be classed as either plastic (biomass stoichiometry changes proportionally with changes in resource stoichiometry (Fig. 1.1a)), or homeostatic (biomass stoichiometry is fixed despite changes in resource stoichiometry (Fig. 1.1b)) (Sterner and Elser, 2017b). These are, of course, extremes of the model and the reality is that most organisms neither maintain strict homeostasis nor are completely plastic 100% of the time. Rather, they fall somewhere between the two extremes in varying degrees (Fig. 1.1c). Yet, it serves a purpose to explore these extremes to understand the effect of deviations in biomass stoichiometry to that of the resource.

Stoichiometric imbalance (or stoichiometric mismatch) is the degree to which an organism’s stoichiometry differs from the resource stoichiometry (Sterner and Elser, 2017b). The greater the deviation, the greater the imbalance. When an organism exhibits stoichiometric mismatch, there must be strategies that allow it to maintain this difference. When an element is in excess, this must be excreted. When an element is deficient in the resource, the organism must be able to store and enrich its own stock of said nutrient.

An organism or a system may also be deemed to be in stoichiometric imbalance when C:nutrient and N:P ratios are high and are often classed as nutrient limited. The specific ratios of C:nutrient and N:P, which are characteristic of nutrient limitation, are dependent on a multitude of factors, including ecosystem type, taxa, ambient nutrient conditions etc.

Responses to stoichiometric imbalance in terrestrial ecosystems

In this thesis, we explore responses to stoichiometric imbalance in terrestrial ecosystems and focus on plant and soil microbial stoichiometry, as these are the two main drivers of terrestrial systems. Plants typically show large inter- and intraspecific variation and flexibility in biomass stoichiometry (Elser et al., 2010). This is likely due to their ability to respond to variation in available nutrient and carbon supply (Sistla and Schimel, 2012; Sterner and Elser, 2017b), e.g., through nutrient storage in vacuoles (Marty, 1999), and nutrient resorption (Aerts, 1996). A major source of plant interspecific variation is the differential investment into structural carbohydrates. For example, lignin and cellulose (with high biomass C:N) are characteristic of higher terrestrial plants, whereas these are lacking in non-

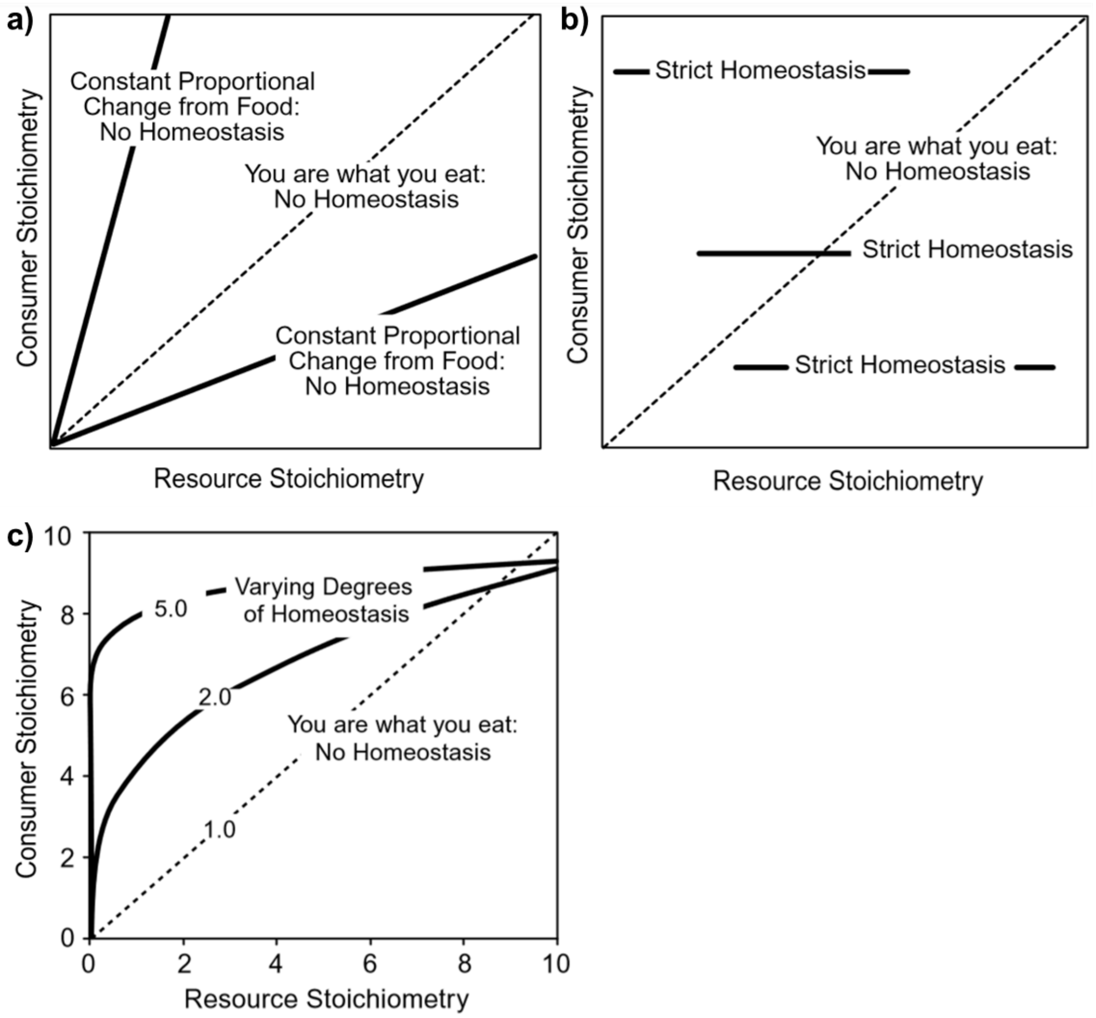

Figure 1.1: Generalised stoichiometric patterns of homeostasis and plasticity between consumer soichiometry and resource stoiciometry from Sterner and Elser (2017b). The dashed lines in a-c) represents the 1 : 1 line, where an organism's stoichiometry is identical to that of the resource. In this case, the organism exerts no control over its own stoichiometry and shows full plasticity. The solid lines in a) represents an organism whose stoichiometry alters with changing resource stoichiometry, albeit in a constant and proportional manner (i.e., non-homeostatic). In b) the solid lines represent an organism who exerts full control on its biomass stoichiometry, maintaining fixed stoichiometry despite changes in the resource. In c) the solid lines represent organisms that exhibit different degrees of homeostatic regulation, H, as per Sterner and Elser (2017b).

vascular plants predominantly found in aquatic systems (Sterner and Elser, 2017c). Foliar N:P ratio has been shown to be proportional to the N:P ratio of fertilisation input (Güsewell and Bollens, 2003) and foliar N:P ratio is negatively correlated with biomass (Güsewell, 2004). Therefore, increasing stoichiometric imbalance due to a widening of the N:P ratio of available N and P can negatively affect biomass. Plants may respond to imbalance due to deficiency by allocating resources to the acquisition of nutrients; namely, by promoting root growth (Hermans et al., 2006; Hodge, 2004, 2009), changes in root architecture (Drew and Saker, 1978; Kumar et al., 2020; Robinson, 1994), and promotion of symbiotic arbuscular mycorrhizal fungi (Govindarajulu et al., 2005; Kumar et al., 2019; Ryan et al., 2012; Veresoglou et al., 2012), all of which enhance nutrient uptake (see Chapter 2).

Microbial heterotrophs, on the other hand, have been shown to exhibit much more constrained biomass stoichiometry than plant biomass (Cleveland and Liptzin, 2007; Hartman and Richardson, 2013). Stoichiometric imbalance is common for soil microorganisms involved in decomposition due to the high C:nutrient ratios inherent to plant material (Cowling and Merrill, 1966) compared to the narrower C:nutrient ratios of their own biomass. Soil microorganisms alleviate this imbalance by production of extracellular enzymes (Sinsabaugh and Moorhead, 1994). Sinsabaugh et al. (2008) could even show that ratios of enzyme activity rates for the hydrolysis of C, N and P required for decomposition of plant matter were proportional to the respective stoichiometric imbalance on a global scale.

Changes in soil microbial stoichiometry and soil resource stoichiometry can feed back on terrestrial nutrient cycles. If soil C:N ratio is greater than that of the soil microbial biomass, there is net microbial immobilisation of N ($>$ 30:1). If the soil C:N is less than the microbial demand (12:5), there is net N mineralisation (Hodge et al., 2000). Manzoni et al. (2008) could show that soil microbes involved in decomposition shifted from net N immobilisation to net mineralisation as resource C:N ratio approached that of their own biomass. Therefore, lower stoichiometric imbalance can lead to greater nutrient availability for plants. Furthermore, changes in nutrient use efficiency (Mooshammer et al., 2014a,b; Sinsabaugh et al., 2013, 2016; Yuan et al., 2019) and nutrient turnover time (Spohn, 2016) may be an adaptation to mitigate the imbalance, which also impact on nutrient cycling.

Measures of soil stoichiometry are most often given in terms of total concentrations of C and nutrients in the soil. However, a significant proportion of C and nutrients are immobilised in the soil, and are, thus, not biologically available. Therefore, it is important to consider the stoichiometry of dissolved (biologically available) nutrients in the soil for a more accurate measure of stoichiometric imbalance, as well as the total concentrations, when comparing stoichiometries of the soil and soil microbial biomass (Griffiths et al., 2012).

Imbalance due to perturbations

Natural occurring phenomena such as freezing and thawing of the soil in winter (DeLuca et al., 1992; Wagner-Riddle et al., 2017), drought (Sun et al., 2020), and wildfires (Kong et al., 2021) cause great surges of nutrients to be displaced in terrestrial systems. These perturbations cause nutrient imbalances which may be temporary or have longer lasting impacts. In the case of freeze-thaw (FT) events, this leads to large-scale loss of N as nitrous oxide emissions (Ejack and Whalen, 2021; Wagner-Riddle et al., 2017), which has economical costs and environmental consequences.

In soils, changes in microbial community assembly can be both the cause and result of stoichiometric imbalance. It has been shown that changes in functional traits due to a shift in community assembly can impact overall nutrient turnover and stoichiometry (Aanderud et al., 2018; Wei et al., 2020). Changes in nutrient input can also impact the microbial community composition. Specifically, fungi have generally a higher capacity to store C (Adu and Oades, 1978), and thus have higher C:N ratio, than bacteria (Hodge et al., 2000). It has further been shown that organic matter with high C:N ratio promotes fungal growth over bacterial (Henriksen and Breland, 1999), leading to increases in fungal:bacterial biomass ratio, whereas increased N fertilisation leads to decrease in fungal:bacterial ratio (Bardgett et al., 1999). Shifts in fungal:bacterial ratio can thus change the overall microbial stoichiometry, which in turn can alter the nutrient demand and immobilisation/mineralisation of C and N in the system (Hodge et al., 2000). Principles of ecological stoichiometry can, therefore, be useful in understanding how changes in stoichiometry can feedback on ecosystem function and nutrient cycling.

Anthropogenic CO_2 emissions and agronomic use of N- and P-rich fertiliser are major drivers of stoichiometric imbalance in both terrestrial and aquatic systems (Guignard et al., 2017; Peñuelas et al., 2013, 2012; Sardans et al., 2012a). Agronomic N and P inputs through fertilisation often exceeds that which is recovered in biomass (Kanter, 2018; MacDonald et al., 2011; Tilman et al., 2002). This excess nutrient input has to go somewhere and usually ends up where it is not wanted. Nitrates are leached into groundwater causing pollution (Wang et al., 2019) and nitrous oxide emissions contribute to global heating (Tian et al., 2019). Whereas N undergoes various transformations in the soil and eventually leaves the soil system, P binds to soil particles and accumulates in the soil after fertilisation. These P enriched soil particles can later lead to eutrophication of water bodies when soil is eroded and the P-rich soil particles enter aquatic systems (Bennett et al., 2001; MacDonald et al., 2011).

It could be argued that nutrient limitations and stoichiometric imbalance due to nutrient deficiency in plants and soil fauna would be alleviated with such large-scale inputs of N and P, but the opposite has been shown to be true. High input of N and P can skew terrestrial community composition in favour of fast growing species that are well adapted to high nutrient concentrations (Leff et al., 2015; Tilman and Wedin, 1991), and in doing so out compete the less nutrient demanding species (Sistla and Schimel, 2012). High nutrient inputs, thus, lead to decreased biodiversity (Allison et al., 2007; Allison and Vitousek, 2004), which in turn diminishes the stoichiometric flexibility and functional diversity of a system (Dai et al., 2020; Sistla and Schimel, 2012). N deposition has been shown to increase foliar N:P ratios, which can lead to increased P limitation in herbivores (Sterner and Elser, 2017a). In agricultural systems, these effects are coupled with the removal of plant material. In natural systems, dead plant matter would normally remain and be decomposed, returning its respective nutrients to the soil. This natural cycling of nutrients is missing in agriculural systems and further exasperates nutrient limitation (Rhodes, 1995).

It may seem counter-intuitive, but high nutrient inputs often lead to increased C limitation in soils. Although atmospheric CO_2 levels are on the rise, soil C stocks are depleting (Minasny et al., 2011; Olson et al., 2016). Highly productive lands in terms of plant biomass production (e.g. arable land and forest plantations) are in fact the most C deficient and nutrient limited soils (Guo and Gifford, 2002). There is much potential for soil to sequester considerable quantities of C and N (Minasny et al., 2017), but they are more often sources of green-house gas emissions than sinks due to poor management.

Conversely, in recent years, it has been shown that atmospheric N deposition improves soil organic carbon (SOC) storage (Lu et al., 2021), although the stability of this additional sequestered C is contended (Forstner et al., 2019). At the same time, N addition has been shown to reduce soil microbial respiration and biomass (Treseder, 2008). Stoichiometric imbalance characterised by high biomass C:nutrient and N:P ratios is linked to high maintenance energy demand, such as metabolic quotient (Anderson and Domsch, 1985), and can indirectly be linked to low C-use efficiencies due to relatively high losses of C through respiration proportional to biomass (i.e., metabolic quotient) (Xu et al., 2017).

In light of these complex and often contradictory responses to changes in nutrient input, it is important to fully understand how stoichiometric imbalance between soil microbes and their resources (either soil or plant derived) can impact on ecosystems functioning and nutrient cycling. In agriculture, it is important to understand how the stoichiometry of the inputs effect the entire system in order to balance sustainable use of nutrients with maintenance of high yields. Considering that soil microbial biomass and necromass are the main drivers of stable soil organic matter (SOM) formation (Buckeridge et al., 2020; Kallenbach et al., 2016; Ma, 2018; Wang et al., 2021) this poses the following questions: How flexible is the microbial biomass for accrual of nutrients? How can soils be managed to promote nutrient sequestration and prevent nutrients losses due to perturbations such as freezing and drought? How do ambient nutrient conditions affect the potential of soil microbial stoichiometry to accrue and/or lose nutrients after perturbation events?

As mentioned before, ecological processes are dynamic, therefore stoichiometric imbalances are not in a constant state of being. Yet, most literature on stoichiometric imbalance considers and compares systems at one instance in time only. Little research has been done into the effect of variation in degree of stoichiometric imbalance/flexibility, either due to nutrient gradients or time evolution (Barrett and Burke, 2000; Chen et al., 2016; Sistla et al., 2015).

Stoichiometry, Nutrient Limitation, and Nutrient interactions

Stoichiometric imbalance goes hand-in-hand with nutrient limitation and, therefore, it is to be expected that the degree of nutrient limitation will change with changing nutrient availability along a nutrient gradient.

A commonly used definition of nutrient limitation was coined by Vitousek and Howarth (1991). They state that nutrient limitation occurs when the addition of a nutrient causes a positive response, for example, in growth rate, or changes the endpoint of the system. Classical nutrient limitation theory is based on Liebig's Law of the Minimum (Liebig, 1840, 1855), whereby an organism is limited by the most lacking nutrient in relation to its requirements. On addition of said limiting nutrient until requirement to that nutrient has been satisfied, the next most lacking nutrient (relation to its requirements) will become the next limiting nutrient. Ecological stoichiometry allows to assess when a system or organism is limited by a particular nutrient based on element ratios. In plants, foliar N:P ratio can determine whether a plant is N or P limited, e.g. $N:P > 16$ is indicative of P limitation and $N:P < 15$ is indicative of N limitation (Aerts, 1996; Koerselman and Meuleman, 1996). Nutrient limitation in heterotrophs has to be modelled differently to plants as heterotrophs are characteristically and foremostly limited by C. Thus, in this case, to determine when a nutrient was limiting, Threshold Element Ratio (TER) models were developed (Sterner and Elser, 2017a; Urabe and Watanabe, 1992, 1993), whereby the threshold ratio refers to the C:nutrient ratio where limitation to C and said nutrient is equal. Deviations from this ratio indicate a shift to increased limitation to one nutrient and decreased limitation to the other.

Yet, more recent understanding of nutrient limitation is that organisms do not always follow a sequential pattern of limitation to the scarcest available nutrient, rather they can be limited by a combination of two or more nutrients simultaneously (Ågren et al., 2012; Harpole et al., 2011; Saito et al., 2008). Furthermore, these nutrients are often required in a specific optimum ratio. This makes the matter much more complex as the co-limitation creates extra dimensions to the problem, such as whether the effects of each nutrient are independent or co-dependent, and whether the order in which the nutrients are applied has a bearing on the response

(Harpole et al., 2011). Furthermore, in contrast to the Liebig based approach of TER models where nutrient limitation status can be determined by relation to a critical stoichiometric threshold, shifts between limiting states in co-limiting models may even occur across a range of ratios (Ågren et al., 2012).

This current understanding of co-limitation is based on the widely recognised phenomenon of nutrient interactions, namely nutrient synergism, whereby the effect of adding N and P together is greater than the combined effect of adding N and P separately (Davidson and Howarth, 2007; Elser et al., 2007). This effect has been observed in fresh water, marine, and terrestrial systems alike (Elser et al., 2007).

Although co-limitation and nutrient interactions are now widely recognised, little is known about how nutrient interactions change with changing nutrient availability. Fertilisation experiments on plants mainly focus on the effect of changing one of either N or P. Fewer studies investigate the effect of changing both N and P (Duncan et al., 2018), and when they do, they often have two or more levels of N and only two P levels (high/low) in factorial design (Prystupa et al., 2004, 2003), or the number of P fertilisation rates is far less than the N fertilisation rate (Michaelson et al., 1982; Tigre, 2014). It is not well understood whether synergistic effects are present at all levels of N and P, or whether there is a specific fertiliser N:P ratio or minimum level of N and/or P fertilisation where the response may be observed.

As previously discussed, increases in agronomic N and P can lead to stoichiometric imbalance. Therefore, in order for a more efficient and responsible use of fertilisers, it would be valuable to determine the minimum amount of N and P fertilisation (or range of fertiliser N:P ratios) required to give synergistic response in crop plants (Sardans et al., 2012b).

1.2 Aims

The central aim of this thesis was to investigate the effect of stoichiometric imbalance due to a nutrient gradient on the accrual of carbon (C), nitrogen (N), and phosphorus (P) in plant biomass and in soil microbial biomass. In plants, we investigated how barley biomass changed with changing N and P fertilisation. Biomass C and -N were measured in shoot and root tissue of barley (*hordeum vulgare*) in a fertilisation pot experiment (Chapter 2). In soils, microbial parameters such as biomass C, -N and -P, as well as microbial respiration and soil C, -N and -P were measured along a nutrient gradient from a post-mining chronosequence (Chapters 3 and 4). We also investigated the effect of stoichiometric imbalance in soil microbial biomass due to a freeze-thaw (FT) event to shed light on post-harvest N losses in arable lands (Chapter 4).

Generalised hypotheses

While specific hypotheses of each investigation are detailed in each chapter, the overarching hypotheses of the thesis are:

1. There will be a threshold concentration of N and/or P fertilisation for observing synergistic interaction of N and P in barley biomass and for observing a shift from relative higher investment into root biomass to relative higher investment into shoots on crossing this threshold in varying degrees of N and P deficiency.

2. Accrual of nutrients in soil microbial biomass will increase and metabolic quotient will decrease with decreasing stoichiometric imbalance/increasing soil organic carbon (SOC) content.

3. There will be a shift in microbial stoichiometry due to a freeze-thaw event and there will be an effect of SOC on the size of the response to freezing.

1.3 Chapter summary

In this thesis, the theory of Ecological Stoichiometry is used to study stoichiometric imbalance due to changes in nutrient availability. The effect of a nutrient gradient, either by controlled fertilisation input (Chapter 2) or due to development of soil organic matter (SOM) over time in recultivated soils from a post-mining chronosequence (Chapters 3 and 4), is investigated. The contents of these chapters focus on agricultural issues as these pose the greatest challenges in stoichiometric imbalance in terrestrial ecology. These include the synergistic response of N and P fertilisation in barley (Chapter 2), SOM formation in recultivated arable lands (Chapter 3), and the response of microbial biomass to freeze-thaw event in post-harvest arable soils to investigate N losses (Chapter 4). In all three investigations, discrete responses to changes in nutrient availability were observed as opposed to gradual responses, and critical threshold values for P and C availability were observed in chapters 2 and 3, respectively.

Chapter 2: *A two-way N×P fertilisation gradient experiment on barley reveals shifts from additive to synergistic N-P interactions at critical P fertilisation level under nutrient deficiency.*

Barley was subject to varying degrees of nutrient deficiency in a controlled pot experiment by simultaneous reductions of N and P content of the Hoagland solution (a fertilisation solution containing the optimum concentrations of macro and microelements required for plant growth). This study aimed to investigate how plants respond to a two-way N and P fertilisation gradient in total biomass, biomass-C and -N accrual, and allocation of biomass and nutrients to shoots and roots. It aimed to find if there would be a threshold value of N and/or P where the addition of both N and P would have a synergistic effect and where allocation of resources would shift away from roots towards shoots as nutrient deficiency decreased.

Chapter 3: *Shifts in soil microbial stoichiometry and metabolic quotient provide evidence for a critical tipping point at 1% soil organic carbon in an agricultural post-mining chronosequence.*

This study aimed to investigate the development of soil organic matter (SOM) and changes in soil microbial biomass along a soil organic carbon (SOC) gradient. Soil microbial C, -N, -P, and measures of microbial activity, such as metabolic quotient, were measured along a chronosequence formed from the reclamation of land after open-cast brown-coal mining. Due to the unchanged protocol of reclamation over 50 years, this gave rise to a space-for-time chronosequence consisting of soils with exact starting conditions with ages spanning 1-52 years after time of restoration. It was expected that the youngest soils would have the most severe nutrient dilution due to the restoration process, and, thus, these soils' microorganisms would exhibit the largest degree of stoichiometric imbalance. It was expected that soil microbial stoichiometry would not be completely homeostatic, but would converge to an optimum microbial C:N:P ratio with increasing time after restoration. Likewise, it was expected that with increases in SOM over time the stoichiometric imbalance would also decrease.

Chapter 4: *Soil freezing-thawing induces immediate shifts in microbial and resource stoichiometry in Luvisol soils along a postmining agricultural chronosequence in Western Germany.*

This study aimed to show how soil microbial biomass responds to a freeze-thaw event in the first 18 hours after thawing in order to understand the mechanism of N losses observed after a freeze-thaw event. It was expected that soil microbial stoichiometry would change due to varying rates of transformation of nutrients after freezing. Using chronosequence soils selected based on carbon content (giving rise to a carbon gradient), the effect of carbon content on the magnitude of the freeze-thaw event could be investigated.

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2 Two-way N × P fertilisation experiment on barley reveals shift from additive to synergistic N-P interactions at critical P fertilisation level

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2.1 Abstract

In a pot experiment, we investigated the nitrogen-phosphorus (N-P) interaction on shoot and root biomass of barley (*Hordeum vulgare*) along a two-way nitrogen (N) and phosphorus (P) fertilisation gradient. We subjected the barley plants to varying degrees of N and P deprivation using a modified Hoagland solution. We chose 6 levels of N and 6 levels of P fertilisation, resulting in 36 N and P combinations, with N:P ratios ranging 2–120. We aimed to determine whether synergistic interactions of N and P on barley biomass (on both shoot and root level) would be the same for all levels of N and P fertilisation. We further aimed to determine if there was a critical level of N and P fertilisation rate where we would observe the plants investing relatively less resource into root biomass and more into shoots as nutrient availability increased, and expected to see a shift in shoot:root ratio. Lastly, we aimed to determine interactions between N and P fertiliser in nitrogen-use efficiency (NUE) and phosphorus-use efficiency (PUE). We observed N-P interactions in shoot and root biomass, and PUE. Most strikingly, a synergistic response of biomass to N and P fertilisation was observed only above a

critical level of P fertilisation ($P \geq 0.25\,\mathrm{mmol}$) when P was not limiting growth. We speculate that increasing stoichiometric imbalances due to widening in N:P ratio were driving the responses observed, and that the plants were less able to cope with greater imbalance when P was deficient. Furthermore, we did not find a significant N-P interaction on shoot-root allocation, but found that shoots became more responsive to N compared to roots above the critical P value. We provide, for the first time, stoichiometric evidence that critical levels for synergistic interactions between N-P may exist in crop plants, and further highlight the impact of P limitation on stoichiometric imbalance in plants.

2.2 Introduction

Nitrogen (N) and phosphorus (P) are the two most important macronutrients that limit plant growth (Elser et al., 2007; Sterner and Elser, 2008). Both nutrients can limit growth since both N and P are needed for core metabolic activities (Elser et al., 2000). Classic models of nutrient limitation are based on Liebig's Law of the Minimum (Liebig, 1840, 1855). The law states that the scarcest nutrient (in relation to the organisms' requirements) limits growth and once the requirements for this nutrient have been met, the next scarcest nutrient will become the new limiting nutrient (Ågren et al., 2012). Yet, in the last two decades, new models have emerged, in which nutrient limitation is a product of co-limitation or multiple-limitation to multiple nutrients simultaneously, whereby nutrients are required together in some optimum ratio (Ågren et al., 2012; Harpole et al., 2011; Saito et al., 2008).

Nutrients which are co-limiting may also interact (Elser et al., 2007). These so called nutrient interactions can be either synergistic (the combined effect on growth is greater than the sum of the individual effects of each nutrient) or negative (the combined effect is less than the sum of the individual effects) (Davidson and Howarth, 2007). Despite generally synergistic effects of N and P on cereal crop yield and nutrient-use efficiency, their potential interaction effects have been far less extensively studied in agroecosystems (Duncan et al., 2018a,b; Elser et al., 2007; Rietra et al., 2017).

Yet, to fully understand N-P interactions in crop yields and nutrient-use efficiencies, we must first look underground and see how plants invest in above- and belowground tissues across different N and P availabilities, thus considering scenarios where nutrient supply is neither optimum nor balanced. Plants are extremely adaptable to changing nutrient conditions and are able to optimally redistribute resources among the tissues where they are required (Hilbert, 1990; Sadras, 2006; Temperton et al., 2003; Werf and Nagel, 1996; Wilson, 1988). When nutrients are scarce, plants increase investment into root biomass, causing a decrease in shoot:root ratio (Hermans et al., 2006; Robinson, 1994; Scheible et al., 1997; Werf and Nagel, 1996). However, this optimisation can come with a cost to aboveground productivity leading to a shoot-root trade off (Kim and Li, 2016; Li et al., 2010).

Adaptations such as changes in roots architecture in response to variation in nutrient availability may allow for optimised nutrient uptake. In heterogeneous supply of N and P, localised increases in lateral roots have been observed in the areas of higher nutrient concentration (Drew, 1975; Drew and Saker, 1978; Drew et al., 1973) as well as overall increased nutrient uptake flux by roots to compensate for the non-uniform supply (Robinson, 1994). Moreover, Kumar et al. (2019) found that root diameter correlated with colonisation of arbuscular mycorrhizal fungi for optimised P uptake. There are also differing effects of N and P limitation on root architecture. Kumar et al. (2020) found that plant roots foraged in deeper layers when N was the limiting factor, but explored the topsoil when P was limiting. In a complementary experiment where N and P were added at different timepoints in the growing phase, delayed N addition had a stronger negative effect on biomass than delayed P (Duijnen et al., 2021).

Although there is plenty of evidence of the underground responses to changes in N and P availability independently, little is known about the effects of an N-P interaction on root growth, i.e., due to changes in both N and P. Duncan et al. (2018b) showed that there was a positive interaction between N and P on root growth as well as N-use efficiency and N retention in wheat. It is, however, not so well understood how nutrient interactions may shape the allocation of nutrients to shoots and roots. The aboveground studies of N-P interaction on cereals in most cases have a simple experimental design with few combinations of low vs high N and P fertilisation rates (Prystupa et al., 2004, 2003) or fewer P rates compared to N (Michaelson et al., 1982; Tigre, 2014). Fewer studies have investigated nutrient interactions across a high number of fertilisation rates. Duncan et al. (2018a) identified 11 fertilisation experiments on wheat in which the design had at least two N fertilisation rates and multiple P fertilisation rates. Through investigating N-P interaction on both the shoot and root level, it may be possible to gain insight into shoot-root trade-offs and give answers to the following questions: At what combined level of N and P do we see shifts to decreased root investment with increased N and P levels? What happens when we subject plants to gradients of N and P in magnitudes ranging from inadequate to adequate supply to the plant's requirements?

In a pot experiment, we investigated the N-P interaction on shoot and root biomass of barley (*Hordeum vulgare*) along a two-way N and P fertilisation gradient. We subjected the barley plants to varying degrees of N and P deprivation using a modified Hoagland solution (Hoagland and Arnon, 1950). We chose 6 levels of N and 6 levels of P fertilisation, resulting in 36 N and P combinations and N:P ratios ranging 2–120. This covers a wide range of different ratios and includes the narrow range of 4–6 (the range in which Sadras (2006) found attained the maximum yield in over 40% of the 1500+ crops he assessed).

We hypothesise that there will be positive interactions between N and P fertilisation treatments due to N-P colimitation. Due to trade-offs in plant's below vs. aboveground allocation, we expect to find a critical stoichiometric threshold as nutrient provision increases, where plant investments in root biomass would be reduced in favour of shoot biomass. Finally, we hypothesise that changes in barley biomass yield per unit of applied N or P fertilizer (i.e., N- and P-use efficiency) show similar patterns (i.e., would be statistically independent) along the fertilisation gradient.

To test these hypotheses, we set up a pot experiment with an N-P gradient using extremely low-nutrient soils from a mining site, where we expected microbial communities to be very limited.

2.3 Materials and Methods

2.3.1 Preparation of seeds for germination and pot preparation

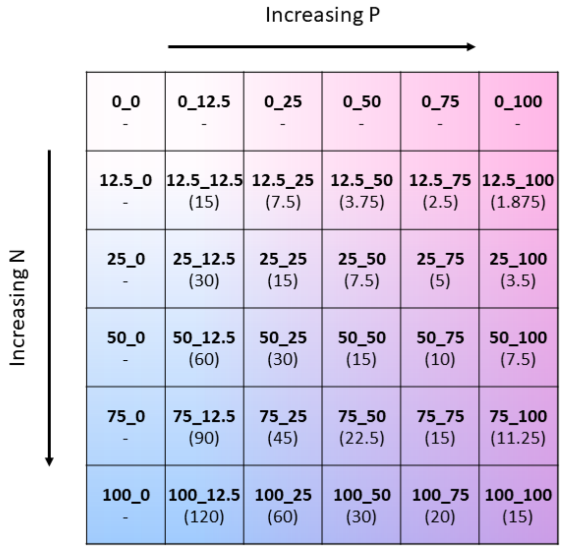
Barley (*Hordeum vulgare*) seeds were sterilized for 1 min with 70% ethanol under vacuum and for a further 2 min with chlorine bleach (5 % sodium hypochlorite) and finally washed with sterile water. Under sterile conditions, single seeds were planted into small plastic tubes (< 5 mL volume) with sterile sand and watered as required (Fig. S2.1A).

Pots with volume of ~ 2 L were prepared with a substrate of loess soil mixed with sand in ratio 1:4 (Fig. S2.1B). There were 36 fertilisation treatments each replicated 8 times, giving 288 pots in total. A nutrient-poor silt loess soil, devoid of soil organic matter, was collected from deep sandy layers at a lignite mining site in Jackerath (North Rhine-Westphalia Germany), at a depth of 5 to 12 m. The soil was oven dried overnight at 60°C to kill spores of arbuscular mycorrrhiza and any potential soil fauna (Endlweber and Scheu, 2006). Three seedlings per pot were planted and were placed in a temperature-controlled greenhouse. The plants were allowed to grow for 8 weeks in the greenhouse, were watered as necessary and supplied with fertiliser as per the experimental set up for the fertilisation gradient. After every fertilisation, plants were randomly repositioned to minimize edge effects.

2.3.2 Two-way N and P fertilisation gradient

The two-way fertilisation gradient was created by modifying both the N and P concentration of the Hoagland solution (Hoagland and Arnon, 1950), whilst keeping all other nutrient concentrations unchanged. The concentration of N and P containing substrates were altered to give separately 6 concentration levels: 0, 12.5, 25, 50, 75, and 100% of the original Hoagland solution. This gave 36 unique combinations of N and P concentrations (Fig. 2.1). In order to reduce N and

P in Hoagland solution, the nutrient salts KH_2PO_4, KNO_3, and $Ca(NO_3)_2$ were reduced accordingly. The missing potassium and calcium as a result of reduction of the nutrient salts were replaced by the addition of KCl and CaCl. The exact formulation of the modified Hoagland solution for each of the 36 treatments is found in the supplementary material (Table S2.1). Throughout the growing period the amount of solution applied to the plants gradually increased in accordance with the plant's requirements. Fertiliser solution was applied once weekly and summed to a total of 500 mL of solution applied per pot throughout the experiment. Absolute molar amounts of N and P applied over the full course of the experiment can be found in Table S2.2 and the N:P ratios of each treatment in Fig. 2.1.



0_0 -	**0_12.5** -	**0_25** -	**0_50** -	**0_75** -	**0_100** -
12.5_0 -	**12.5_12.5** (15)	**12.5_25** (7.5)	**12.5_50** (3.75)	**12.5_75** (2.5)	**12.5_100** (1.875)
25_0 -	**25_12.5** (30)	**25_25** (15)	**25_50** (7.5)	**25_75** (5)	**25_100** (3.5)
50_0 -	**50_12.5** (60)	**50_25** (30)	**50_50** (15)	**50_75** (10)	**50_100** (7.5)
75_0 -	**75_12.5** (90)	**75_25** (45)	**75_50** (22.5)	**75_75** (15)	**75_100** (11.25)
100_0 -	**100_12.5** (120)	**100_25** (60)	**100_50** (30)	**100_75** (20)	**100_100** (15)

Figure 2.1: Schematic of the two-way N_P fertilisation gradient depicted as a matrix. The values 0, 12.5, 25, 50, 75, and 100 correspond to % of the original N or P concentration in the Hoagland solution. Absolute molar concentrations of N and P for each treatment can be found in Table S2.2. N:P ratio of the fertiliser solution of each treatment in brackets.

2.3.3 Harvesting and analysis of plant material

A detailed record of plant height and number of plants per pot was taken every week. After sufficient growth, the barley plants were harvested at 48 days and roots, shoots, and ears (if present) were separated and dried at 60℃. Roots were washed before drying. The dry weight of the biomass was determined for both roots and shoots. Total carbon (C) and total nitrogen (N) contents were determined in both shoot and root tissue using a Flash 2000 Organic Elemental Analyzer (Thermo Scientific).

In order to determine whether there was a synergistic or negative effect of the combined N and P treatments, we calculated the expected yield (Y_{NP}) for biomass, total C and total N in both shoots and roots Eq. (2.1) as per Rietra et al. (2017). Where Y_N is the mean yield of the N only treatments (when $P = 0$ and $N > 0$), Y_P is the mean yield of P only treatments (when $N = 0$ and $P > 0$), Y_0 is the control (when $N = 0$ and $P = 0$), and Y_{NP} is the expected yield of N and P combined Eq. (2.1). We used the mean, as there was little within group variation of the

$\mathrm{N} = 0$ and $\mathrm{P} = 0$ treatments, i.e., there was no effect of P on $\mathrm{N} = 0$ treatments and no effect of N on $\mathrm{P} = 0$ treatments. The error on the expected yield (ΔY_{NP}) was estimated using the standard errors ΔY_{N}, ΔY_{P}, and ΔY_0 of their respective means Eq. (2.2). When the observed yield was greater than $\mathrm{Y_{NP}}$, there was a synergistic response. Observations below $\mathrm{Y_{NP}}$ indicated negative responses, and observations within $\mathrm{Y_{NP}} \pm \Delta\mathrm{Y_{NP}}$ indicated additive responses.

$$\text{Expected yield:} \quad \frac{\mathrm{Y_{NP}}}{\mathrm{Y_0}} = \frac{\mathrm{Y_N}}{\mathrm{Y_0}} \times \frac{\mathrm{Y_P}}{\mathrm{Y_0}} \tag{2.1}$$

$$\text{Estimated error:} \quad \Delta\mathrm{Y_{NP}} = \mathrm{Y_{NP}} \times \sqrt{\left(\frac{\Delta\mathrm{Y_N}}{\mathrm{Y_N}}\right)^2 + \left(\frac{\Delta\mathrm{Y_P}}{\mathrm{Y_P}}\right)^2 + \left(\frac{\Delta\mathrm{Y_0}}{\mathrm{Y_0}}\right)^2} \tag{2.2}$$

Shoot:root (SR) ratios were calculated for biomass, total C and total N. For example, SR-Biomass = shoot biomass (mg)/root biomass (mg), SR-Total C = Shoot total C/Root total C, SR-Total N = Shoot total N/Root total N. Nitrogen-use efficiency (NUE) and phosphorus use efficiency (PUE) were calculated as the biomass per applied N and P fertilisation for roots and shoots respectively. For example, Shoot NUE = shoot biomass (mg)/applied N fertilisation (mmol) and Shoot PUE = shoot biomass (mg)/applied P fertilisation (mmol).

2.4 Data handling and Statistics

To avoid bias in the data, only pots which contained all 3 barley plants at the end of the growing period were included in the analysis. There were 66 pots which did not meet this criterion due to failure to grow or predation by rodents in the greenhouse.

To get an overview of the whole dataset (total 222 pots), we applied Manova models using the predictor variables N and P fertiliser treatments and $\log_{10}$(N:P ratio). From each significant Manova model, Anova statistics were extracted to see the effect of the predictor variables (e.g., N and P fertilisation) on each individual response variable (i.e., protected Anova, (Scheiner and Gurevitch, 2001)). The results of the Manova and Anova models can be seen in detail in the Supplementary Material (Tables S2.4 to S2.7).

All analyses and data handling were conducted in R (version 3.5.0) with the use of packages: *dplyr* (Wickham et al., 2018b), *tidyr* (Wickham et al., 2018c), *broom* (Robinson et al., 2021), and *purrr* (Henry and Wickham, 2019). Figures were produced using the R packages *ggplot2* (Wickham et al., 2018a), *ggpubr* (Kassambara, 2020), and *RColorBrewer* (Neuwirth, 2014). Statistical model validation was carried out as per Zuur et al. (2010). Significance levels reported throughout the manuscript are as follows: $p > 0.05$ (ns), $p \leq 0.05$ (*), $p \leq 0.01$

(**), and $p \leq 0.001$ (***).

2.5 Results

2.5.1 Biomass, total C, total N, and shoot:root ratios

The gradient in N and P fertiliser caused significant change across nearly all response variables (Fig. 2.2), and significant interactions between N and P fertiliser treatments and plant tissue (shoot/root) were found (Suppl. Manova model Tables S2.4, S2.6, S2.9 and S2.11).

As expected, total barley biomass, total C, and total N in shoots and roots significantly increased both with increasing N and increasing P fertilisation, and there was a significant N × P interaction (Suppl. Table S2.5). The interaction could be clearly seen in the difference in response of biomass, total C, and total N to N fertilisation at different levels of P fertilisation (Fig. 2.2). When P was low ($P \leq 0.25$ mmol), biomass and total C in shoots and roots (Fig. 2.2a and 2.2b) did not increase significantly with increasing N fertilisation, and there was generally no response to N addition. Only in treatments where $P > 0.25$ mmol were there strong effects of N fertiliser, where increases in N fertilisation led to large increases in biomass and total C. There was also little or no difference due to the effect of increasing P in treatments where $P > 0.25$ mmol.

Barley total biomass, total C and total N in shoots and roots showed synergistic responses to combined N and P fertilisation compared to yields expected if N and P were applied separately (Fig. 2.2 black lines), but generally when $P > 0.25$ mmol and $N > 0$. For shoot total N (Fig. 2.2c-i), the synergistic response occurred at a lower P threshold, in treatments where $P > 0.062$ mmol. Generally, additive responses were observed in treatmeants where $P \leq 0.25$ mmol, except in total N, where only $P = 0.062$ mmol treatments showed an additive response. Exceptions also occured in biomass and total C where $P = 0.25$ mmol in the particular treatments where the N and P Hoagland proportions were balanced (e.g., N_P 25_25, corresponding to 0.25 mmol N and 0.125 mmol P), where a synergistic response was seen.

In the treatments where $P \leq 0.25$ mmol, peaks in total biomass and total C were observed at each P level (Fig. 2.2a and 2.2b). The peaks corresponded to the treatments where the Hoagland proportions of N and P were equal (e.g., 25_25, 50_50, etc). However, in treatments where $P > 0.25$ mmol it was not possible to determine if biomass had peaked. In roots (Fig. 2.2b), the peaks occurred only in N_P treatments 12.5_12.5 and 25_25.

The shift in the relationship of the response variables to N at a critical P value was more clearly seen when plotted against log transformed N:P ratio of the applied fertiliser treatment (Fig. 2.3). When looking at the P levels independently, increases in biomass, total C and total N with increasing N:P ratio were caused by increases in N fertilisation (as P was constant) and the resulting relationships were linear (Suppl. Table S2.6). Biomass, total C and total N in shoots and roots increased with increasing N:P ratio when $\mathrm{P} > 0.25\,\mathrm{mmol}$ (Suppl. Table S2.6). However, when $\mathrm{P} \leq 0.25\,\mathrm{mmol}$, there was either no effect of increased N:P ratio or a negative effect. The linear dependency made it suitable to compare slopes (α) of the relationships for each P level (Fig. 2.3a-iii, b-iii and c-iii). The slopes (α) of the regressions of biomass, total C and total N to $\log_{10}(N : P)$, in both roots and shoots, increased with increasing P fertilisation. Here we could show very clearly that as P level increased, the effect of N:P ratio (or simply N) on the response variables increased. In particular, we see a great shift in the slopes (α) between $\mathrm{P} = 0.125\,\mathrm{mmol}$ and $\mathrm{P} = 0.25\,\mathrm{mmol}$ in both shoots and roots. The effect of increasing P was strongest in shoots compared to roots (see full Anova and regression statistics, Tables S2.7 and S2.8).

Shoot:root ratio of biomass and total C (SR-Biomass and SR-Total C, Fig. 2.4a and 2.4b respectively) increased with increasing N and P fertilisation, but there were no significant N × P interactions (Suppl. Table S2.9). SR-Total N ratio (Fig. 2.4c) increased with increasing N fertiliser but there was no effect of P, nor a significant N × P interaction (Suppl. Table S2.10).

2.5.2 Nutrient-use efficiencies

Generally, NUE decreased with increasing N fertilisation and increased with increasing P fertilisation in shoots and roots (Figs. 2.5a-i and 5a-ii). Similarly, PUE decreased with increasing P fertilisation and increased with increasing N fertilisation in shoots and roots (Figs. 2.5b-i and 2.5b-ii). No N × P interaction was observed in NUE in either shoots or roots, i.e., the rate of decrease in NUE due to increased N did not change with increasing P, but the magnitude of the NUE increased with increasing P (Suppl. Tables S2.11 and S2.12). Whereas with PUE, there was an interaction in both roots and shoot. PUE in shoots generally increased with increasing N, but, unlike NUE, the relationship was not the same for all P levels. When P was high ($\mathrm{P} \geq 0.25\,\mathrm{mmol}$) PUE increased with increasing N but only up to $\mathrm{N} = 3.75\,\mathrm{mmol}$. Above this level there was no further change in PUE with increasing N. In the low P treatments, initially PUE increased strongly with increasing N but peaked and then decreased again with increasing N. There was no overall statistical effect of N on PUE in roots Suppl. Table S2.12).

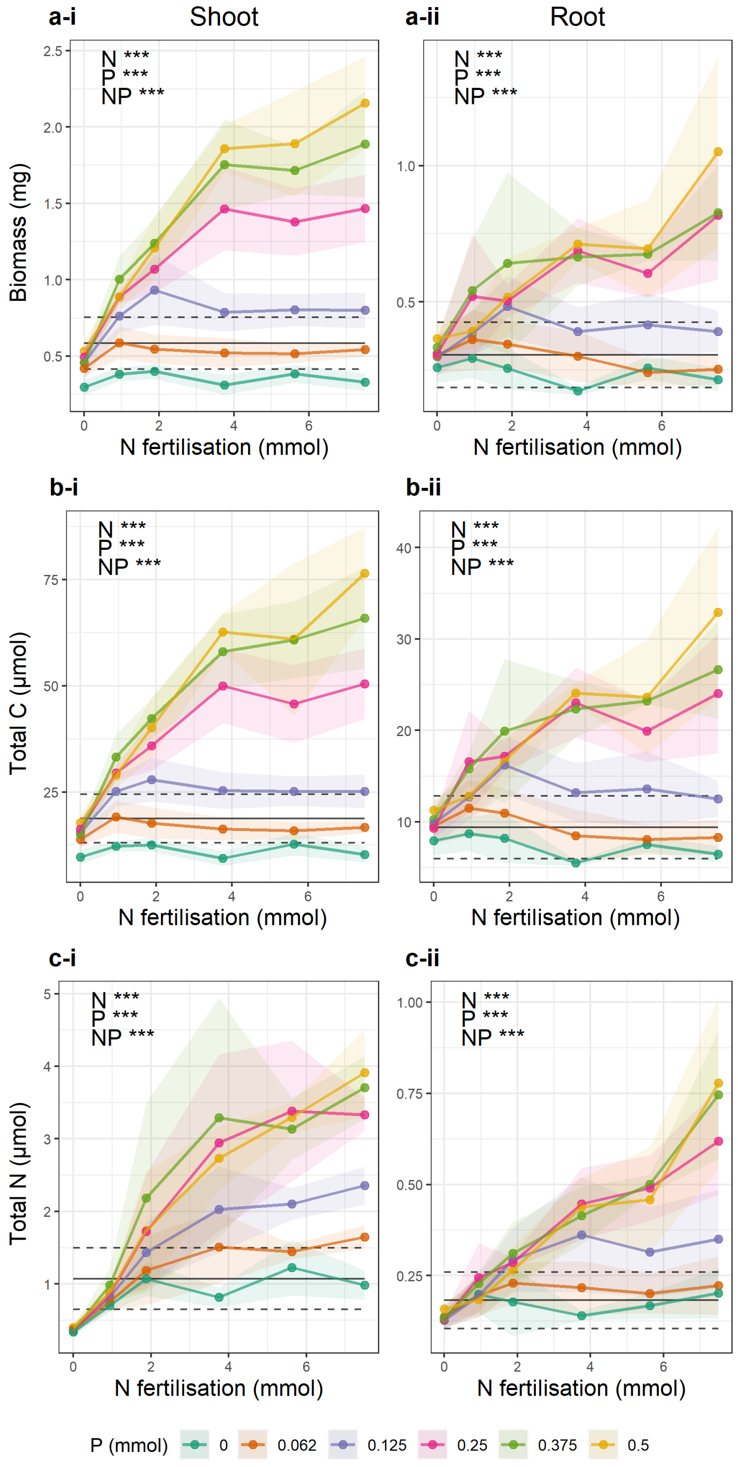


Figure 2.2: Change in a) biomass, b) total C and c) total N in respect to N fertilisation (x-axis) and P fertilisation (coloured lines) in i) shoots and ii) roots. Points show mean value for treatment and shaded area around the lines drawn by connecting the points ±1 standard deviation from the mean. Black lines indicate the expected yield Y_{NP} if N and P were applied separately. The dashed black lines give $\pm\Delta Y_{NP}$ (the estimated error on Y_{NP}, see methods). When the observed yield was greater than Y_{NP}, there was a synergistic response. Observations below Y_{NP} indicated negative responses, and observations within $Y_{NP} \pm \Delta Y_{NP}$ indicated additive responses.

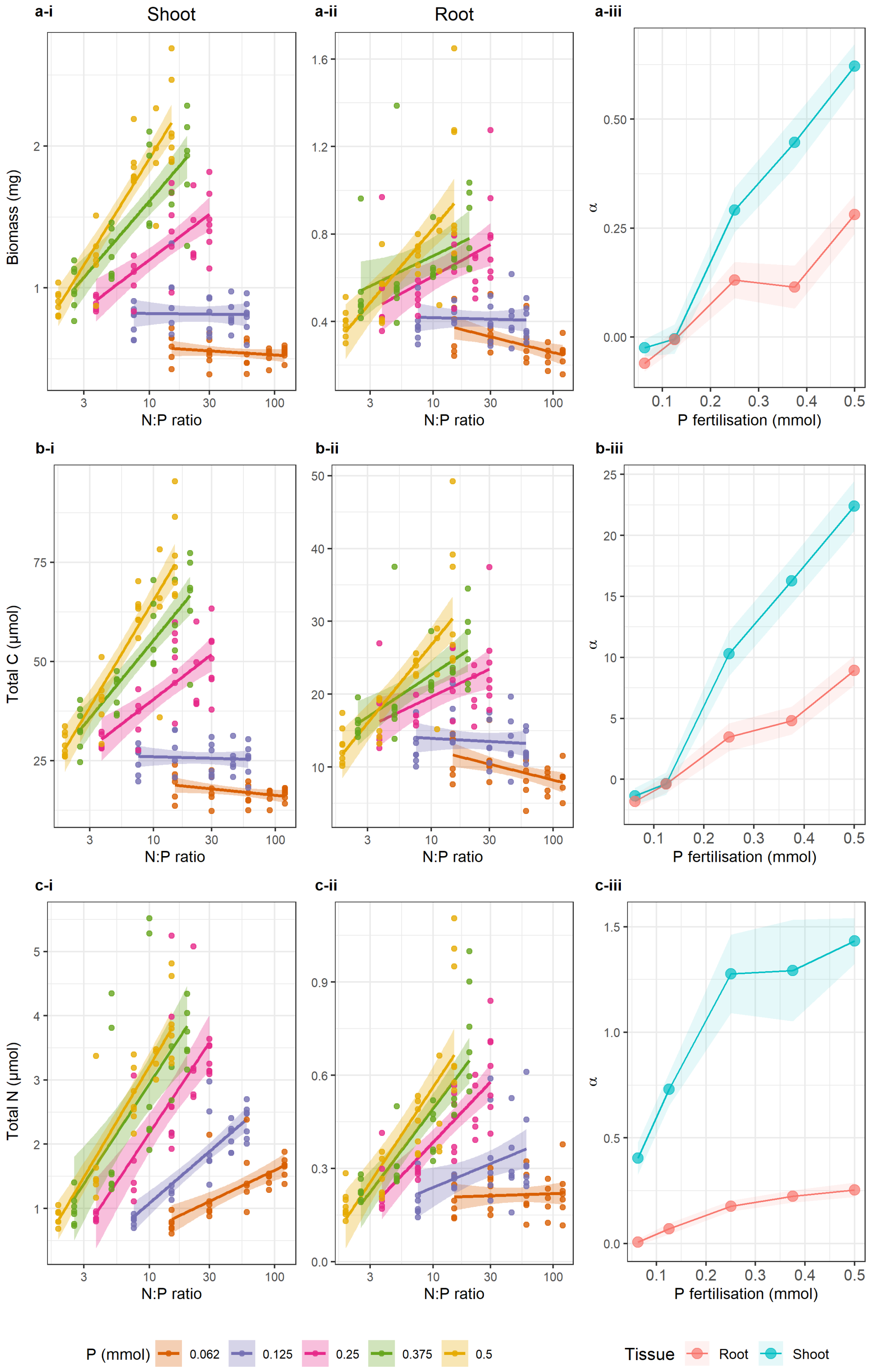


Figure 2.3: Biomass (a), Total C (b) and Total N (c) with changing N:P ratio of the applied fertiliser (log transformed x axis) and P fertilisation (lines) for i) shoots and ii) roots. Panel iii) show the slopes (α) of the linear regressions of each response variable with N:P ratio, for both roots and shoots, with respect to P fertilisation.

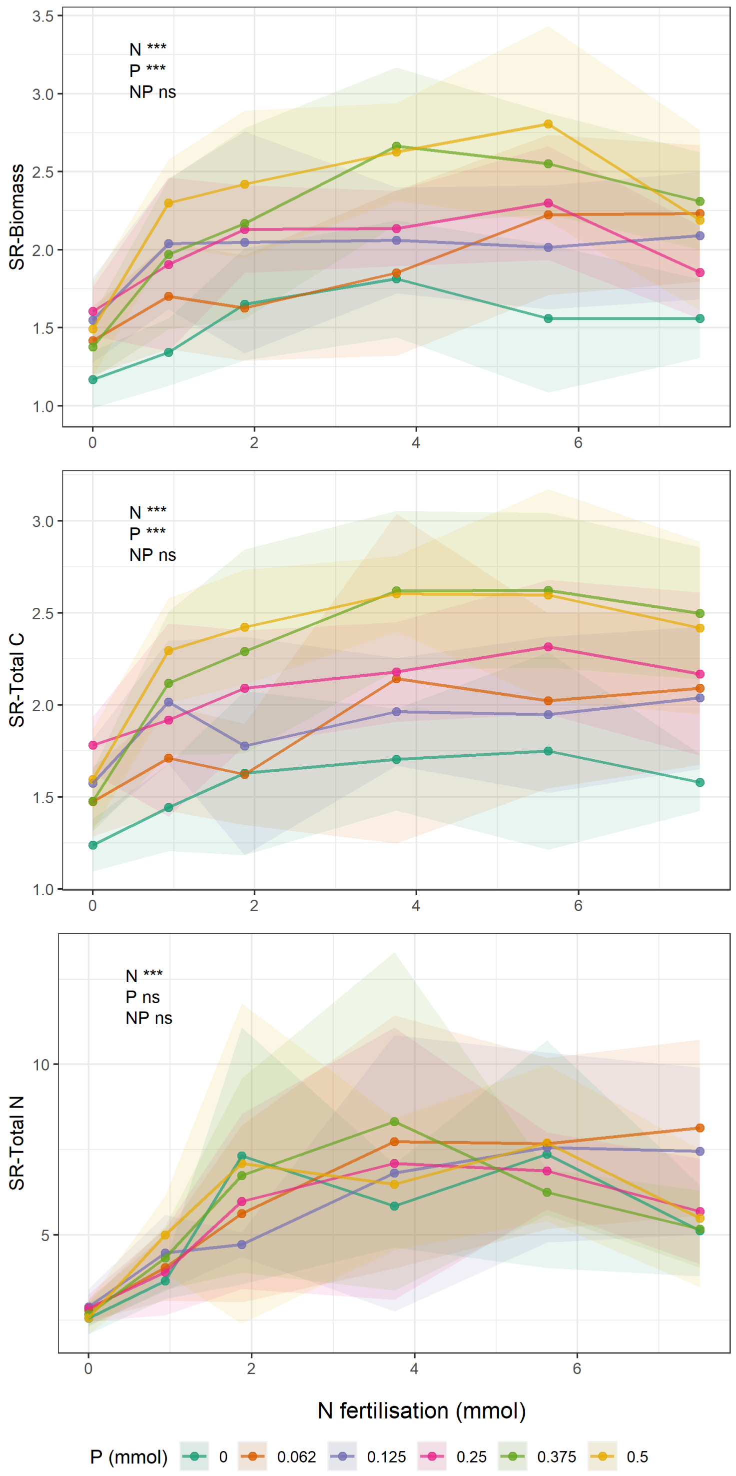


Figure 2.4: Change in shoot:root ratios in respect to N fertilisation (x-axis) and P fertilisation (lines) for the different response variables a) biomass, b) total C, and c) total N. Points show mean value for treatment and shaded area around the lines drawn by connecting the points ±1 standard deviation from the mean.

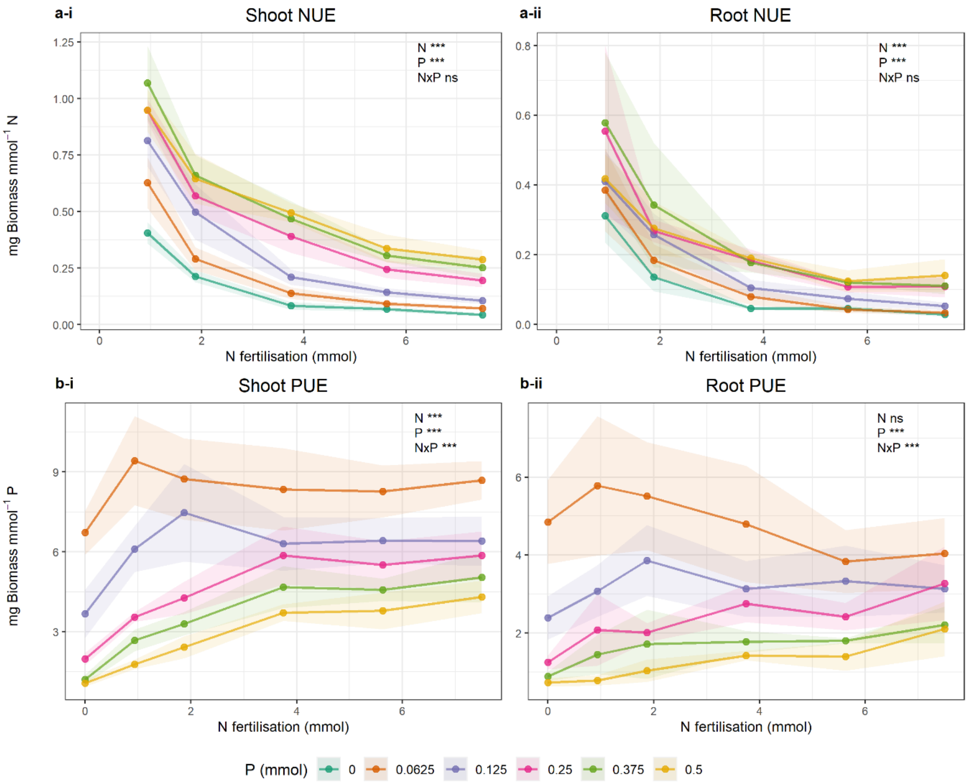


Figure 2.5: Nutrient-use efficiency, a) NUE and b) PUE in in i) shoot- and ii) root biomass with changing N fertilisation (x-axis) and P fertilisation (coloured lines). Nutrient-use efficiency was calculated as the biomass (mg) per mmol nutrient applied. Dots represent the mean and shaded areas correspond to ±1 standard deviation.

2.6 Discussion

2.6.1 Synergistic N-P interactions and critical level of P

As expected, the treatments with low N and low P fertilisation resulted in low yields of biomass, total C and total N, and it was to be expected that there would be increases in yields with increasing N and P. Yet, what is interesting in our data is that we saw that the rate of increase in biomass, total C and total N due to increasing N fertilisation was not the same for all levels of P fertilisation. In fact, we saw synergistic interactions between N and P only above a critical threshold of P fertilisation, in both shoots and roots.

The critical level of P observed for biomass and total C in this study could be interpreted as the threshold between when P was deficient (growth limiting) and sufficient (not growth limiting). When $P > 0.25$ mmol, increases in P had no or only minimal effect on yield (i.e., not growth limiting), whereas N had a strong positive effect on yield above the threshold P level (Fig. 2.2). The synergistic effect of N and P occurred in biomass, total C and total N in both shoots and roots (Fig.2.2), whereby the yield when N and P were applied together was greater than the expected yield if N and P were applied separately. This result further confirms the synergistic effect as seen in various studies (Duncan et al., 2018a; Rietra et al., 2017; Tigre, 2014; Zubillaga et al., 2002). Yet, crucially, in our results, the synergistic effect was only observed when $P > 0.25$ mmol. When P was less than or equal to the critical level, there was an additive effect; at this level of P, the effect of N and P applied together was the same as the expected yield. Interestingly, the critical value of P which determined a shift between synergistic and negative N-P interaction was lower in shoot total N ($P = 0.062$ mmol) compared to the other variables (Fig. 2.2c-i). This shows that only the smallest amount of P aided the acquisition of shoot N immensely, which corroborates with findings from a key study investigating the effect of an N-P gradient in wheat (Duncan et al., 2018b).

2.6.2 Effect of widening N:P ratio

The effect of the critical P value could be explained due to the ever increasing stoichiometric imbalance caused by the widening of the N:P ratios as P decreased and N increased (Ågren et al., 2012; Güsewell, 2005; Yan et al., 2015). When the ratio of the nutrients supplied deviates from the optimum, an imbalance is created causing a relative excess of one nutrient to a relative deficiency in others (Elser et al., 2007; Reich, 2017). This can also lead to feedbacks, i.e., as the demand of one nutrient is satisfied, this can lead to a higher demand of other nutrients (Reich, 2017). The range of N:P ratios of the highest P level ($P = 0.5$ mmol) in our study was 1.9 – 15, whereas the range of N:P ratios of the lowest ($P = 0.062$ mmol) was 15 – 120 (Suppl. Table S2.3). This fits well with the findings of Sadras (2006), whereby crop species had an N:P ratio of between 4 and 6 when managing optimal yield. Our plants only saw such an N:P range when supplied with enough P ($P \leq 0.25$ mmol), but went higher than this range when supplied with lower than 0.25 mmol P.

Breakpoints in foliar N:P ratio have been shown to exist which determine nutrient limitation, whereby foliar $N : P > 16$ is indicative of P limitation and $N : P < 14$ is indicative of N limitation (Aerts, 1996; Koerselman and Meuleman, 1996). However, we did not see a breakpoint as such in our results, as ranges of fertiliser N:P ratios for each P level were not discrete but overlapped (Fig. 2.3). Yet, despite this, we still clearly saw a threshold level of P due to the large shifts in slopes between P treatments $P > 0.25$ mmol and $P \leq 0.25$ mmol (Fig. 2.3, Suppl. Table S2.8), most notably in biomass and total C in both shoots and roots. The differences between slopes were less extreme in total N but slopes still increased with increasing P level (Fig. 2.3c). Ågren et al. (2012) discussed the possibilities that shifts in nutrient limitation can occur over a range of N : P ratios rather than a discrete deviation from a fixed optimum ratio. This, too, could explain our result of synergistic interactions occurring across a range of N : P ratios, albeit when N : P ratio was low.

We can speculate further that the plants in P deficiency were less equipped to cope with widening N : P imbalance compared to when N was limiting, and therefore a positive effect of N was not observed when P was limiting. This was also observed in an N × P fertilisation experiment on *Arabidopsis thaliana* with 3×3 levels of N and P fertilisation (Yan et al., 2015), whereby growth rate of green leaves increased with fertiliser N : P ratio in N limited conditions, but decreased when P limited (at the lowest P fertilisation level).

2.6.3 Effect of balanced N:P ratios

When looking at the response curves for biomass, total C and total N for the individual P levels (Fig. 2.2) we generally saw peaks in the curves where N and P levels were balanced (e.g., 25% N and 25% P, etc, of original Hoagland solution), with N:P ratio of 15. Yet, interestingly, we saw this effect only in the treatments equal to or below the critical P level, where generally additive interactions were observed. The curves of the higher P levels did not appear to saturate unlike in the lower P levels. Therefore, it was not possible to ascertain whether the peak responses had been reached. It is well accepted that balanced application of nutrients results in the most positive response (Ericsson, 1995; Güsewell, 2004; Knecht and Goransson, 2004), yet our data show that this was only the case when P was limiting. This could mean that above the critical level, where P was not limiting, balanced application was less important and increases in N generally had positive effects. Whereas when P was limiting, the best-case scenario for the plant was to receive balanced nutrient supply, as increased N supply led to large stoichiometric imbalance (Ågren et al., 2012; Güsewell, 2004). Furthermore, in the treatments where P was equal to the critical value (0.25 mmol), there was generally an additive effect of N and P, except for when both N and P levels were balanced (25_25). Here we saw, in this instance only, that the effect of N and P was synergistic. As soon as N increased again, the effect was lost and returned to an additive response. Again, this is probably due to the inability of the plant to react well to changes in N:P ratios when P was limiting.

A further effect of balanced supply of nutrients was demonstrated in the phosphorus use efficiency (PUE). When P was limiting ($P \leq 0.25\,\text{mmol}$), PUE peaked when N and P supply were balanced, but levelled off as N:P ratio widened (Fig. 2.5b-i and b-ii). This further illustrates the importance of a balanced supply of nutrients when P was low.

Wide N:P ratio of plant tissue has been shown to result in reduced growth rate (Cernusak et al., 2010; Yan et al., 2015), but there are fewer studies on the direct effect of applied nutrients with varying N:P ratio on plant growth. Some of the results here differ from barley root responses in N-P timing addition (Duijnen et al., 2021) or less varied N:P stoichiometry fertilisation experiments (Kumar et al., 2020). Kumar et al. (2020) found an expected linear increase in shoot biomass when moving from low N/low P to high N/high P with the other factors in between (low N/high P; high N/low P). In contrast, having N applied late was far more detrimental than adding P late (Duijnen et al., 2021), which is in contrast to our findings here.

2.6.4 Shoot-to-root allocation

As in our previous results (Fig. 2.2), we expected to see an interaction between N and P in the shoot:root ratios and that there would be a critical value for N and/or P where there would be a clear shift from investment in roots (low shoot:ratio) to investment in shoots (high shoot:ratio). Yet, we did not observe this in our results.

Shoot:root ratios of biomass (SR-biomass), total C (SR-total C) and total N (SR-total N) increased with increasing N and P (Fig. 2.4). In other words, decreases in N and P caused decreases in shoot:ratio, meaning that there was relative increase in investment into root mass when nutrient supply was low , as reported by (Ågren and Franklin, 2003; Hilbert, 1990; Werf and Nagel, 1996; Wilson, 1988). SR-biomass and SR-total C were significantly affected by both N and P, but interestingly there was no N-P interaction. The effect of N on SR-biomass and SR-total C did not change with changing P. This is contrary to the previous results and to our hypothesis. Moreover, SR-total N was only affected by N and not P. Therefore, P did not have an effect on allocation of N in plant tissue. The reason we did not see an interaction between N and P may be because the magnitudes of the N-P interactions were the same for both shoot and roots (i.e., the effect of N on biomass changed in a similar magnitude with respect to changing P in both roots and shoots), and so in calculating the shoot:root ratio the interaction simply cancelled out.

However, when comparing the responses of biomass, total C and total N to N fertilisation (Fig. 2.2) and N:P ratio (Fig. 2.3) between shoots and roots, differences were observed. The synergistic effect of N and P was higher in shoots compared to roots, and shoots appeared to respond more strongly to N than roots (Fig. 2.2). The responses to N:P ratio (Fig. 2.3) showed that when P was limiting, the magnitude of the responses to increased N was the same for shoots and roots (same magnitude slopes). Whereas when P was not limiting, the magnitude of the responses to N were suddenly much greater in shoots compared to roots (increased slopes). This could mean that when P was not limiting, relatively more resource was directed to shoots than roots in comparison to when P was limiting. Results to this effect have been reported by Hilbert (1990) and Scheible et al. (1997). But to our knowledge, this is the first study to investigate N-P interactions of this scale on root biomass and nutrient content.

2.6.5 Nitrogen- and phosphorus-use efficiencies

Our calculations of NUE and PUE, were, in essence, a standardisation of the biomass to the amount of fertiliser applied. As a result, we compare how much biomass was produced per mmol N or P applied with increasing N and P. As expected, NUE decreased with increasing N fertilisation and PUE decreased with increasing P fertilisation, in both shoots and roots. Yet, NUE and PUE increased with increasing P and N, respectively. Duncan et al. (2018b) showed that NUE in wheat increased with addition of P, and also potassium (K), compared to just N alone and attributed this to increases in root mass and architecture. Similarly, Mehrparvar et al. (2021) showed that when sunflower plant's P and K requirements were met, the required N fertilisation rate decreased whilst NUE increased. Our NUE data fit with these findings but there is little literature on the effect of N on PUE for comparison with our results. Similar results of N having positive effect on plant P dynamics, and vice versa, were observed in Yan et al. (2015). They showed that P increased N resorption efficiency and likewise, N increased P resorption efficiency.

A decrease in NUE due to decreased P availability could be explained by investments of N for P mining (i.e., production of phosphatase enzymes) which could trade-off against direct investment of N for growth, as was shown for microorganisms (Ramin and Allison, 2019). Similarly, Marklein and Houlton (2012) demonstrated that P-mineralising phosphatase enzyme activity in roots increased with N fertilisation under P limitation, and Fujita et al. (2010) showed that increased N fertilisation has been shown to promote phosphatase activity for improved P uptake.

We expected, as with previous results, that there would be an interaction between

N and P for NUE and PUE. This would mean that, for example, the rate of decrease in NUE due to increased N would change with increasing P and result in different slopes for the different P levels. Yet, we did not see this, as the relationship of NUE to N remained unchanged for all P levels in both shoots and roots. However, with PUE there was an N-P interaction in both roots and shoots, as the relationship of PUE to N was not the same for each P level. As explained above, PUE peaked with balanced N and P supply when P was low. But for the higher P levels, a different pattern emerged. We saw for the first time in this investigation a potential critical value of N for PUE in shoots where $P > 0.25\,\text{mmol}$ (Fig. 2.5b-i). When P was not limiting, shoot PUE increased with increasing N fertilisation and peaked at $N = 3.75\,\text{mmol}$. Above this level, PUE in shoots remained constant despite further increases in N. The saturation of PUE occurred at the N level containing 50% of the original Hoagland solution. Hoagland solution should contain the perfect amount of nutrients to enable a plant to grow, and yet 50% N was all that was required to reach the maximum PUE.

2.7 Conclusions and outlook

Our data show two main findings: Firstly, there were positive synergistic N-P interactions in biomass, total C, total N in both roots and shoots. Secondly, the synergistic effects of N and P were only observed above a critical level of P fertilisation ($P \geq 0.25\,\text{mmol}$). Below this critical level, N-P interactions were additive. We provide, for the first time, stoichiometric evidence that critical levels for synergistic interactions between N-P may exist in crop plants, which could determine whether there is under- or overyielding of crop biomass or nutrient content. The evidence of a critical value of P in our study further demonstrates the vital role of P in plant growth (Hermans et al., 2006). We showed that the plants were able to grow in very poor nutrient conditions, but they did suffer. In low P treatments (below the critical level), the plants did not show increases in biomass or nutrient content, even with ample N addition.

We speculate that increasing stoichiometric imbalances due to widening in N:P ratios were driving the responses observed, and that the plants were less able to cope with greater imbalance when P was deficient. We could show that changes in N:P ratio can have differing effects depending on the absolute P availability. This could mean that if P is deficient, the plant may not cope well with small changes in N:P ratio, whereas the plant may cope better with same change in N:P ratio when P is less limiting. This highlights that, when nutrient availability is low, it is much more important to have balanced supply of N and P compared to when nutrient availability is high. This has wider implications for nutrient cycling, as nutrient imbalance in plants has been shown to have negative feedback on soil organic matter formation (Ding et al., 2021). One solution to reduce fertiliser waste is to reduce fertiliser application, but we demonstrate here that it should be done carefully, in balance with other nutrients, so as not to create a dangerous imbalance at very low nutrient levels.

We did not see any shifts in the shoot:root ratios; therefore, we cannot say if there was a shift of resource allocation from roots to shoots (or vice versa) at a distinct level of N or P. However, we did see that the response of shoots and roots to N was the same below the critical P value, but above the critical value, shoots became more responsive to N. This could then be interpreted as the critical level of P required to enable plants to increase resource investment into shoots. There was no combined critical value of N and P, around which resource allocation shifted between shoots and roots, but solely a critical P value. Importantly, absolute amount of available P determined how the plant reacted to changes in N. This outcome could possibly change the way we interpret stoichiometry results, since stoichiometry usually deals with ratios as a way to understand the ecological effects of relative changes in nutrients. Our study strongly suggests that we may need to identify key thresholds beyond which the system and the stoichiometric interaction functions quite differently. These results contribute further to the understanding of terrestrial nutrient limitation and are especially important since it is predicted that terrestrial systems are to become increasingly P limited in the future due to anthropogenic N input (Peng et al., 2019; Yuan and Chen, 2015).

Conflict of Interest

None declared.

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2.9 Supplementary Material

Figure S2.1: Experimental setup: (A) Seedlings for germination. (B) Final pot arrangement.

Table S2.1: Table showing the recipes for fertilisation solutions of the 36 different treatments. N and P level are given in terms of percentage of the full Hoagland solution. Concentration of each compound of the Hoagland solution are given in $\mathrm{mmol\,L^{-1}}$.

Treatment		**Concentration of compounds** ($\mathrm{mmol\,L^{-1}}$)					
N level (%)	P level (%)	KH_2PO_4	KNO_3	$Ca(NO_3)_2$	$MgSO_4$	KCl	CaCl
0	0	0	0	0	2	6	5
12.5	0	0	0.625	0.625	2	5.375	4.375
25	0	0	1.25	1.25	2	4.75	3.75
50	0	0	2.5	2.5	2	3.5	2.5
75	0	0	3.75	3.75	2	2.25	1.25
100	0	0	5	5	2	1	0
0	12.5	0.125	0	0	2	5.875	5
12.5	12.5	0.125	0.625	0.625	2	5.25	4.375
25	12.5	0.125	1.25	1.25	2	4.625	3.75
50	12.5	0.125	2.5	2.5	2	3.375	2.5
75	12.5	0.125	3.75	3.75	2	2.125	1.25
100	12.5	0.125	5	5	2	0.875	0
0	25	0.25	0	0	2	5.75	5
12.5	25	0.25	0.625	0.625	2	5.125	4.375
25	25	0.25	1.25	1.25	2	4.5	3.75
50	25	0.25	2.5	2.5	2	3.25	2.5
75	25	0.25	3.75	3.75	2	2	1.25
100	25	0.25	5	5	2	0.75	0
0	50	0.5	0	0	2	5.5	5
12.5	50	0.5	0.625	0.625	2	4.875	4.375
25	50	0.5	1.25	1.25	2	4.25	3.75
50	50	0.5	2.5	2.5	2	3	2.5
75	50	0.5	3.75	3.75	2	1.75	1.25
100	50	0.5	5	5	2	0.5	0
0	75	0.75	0	0	2	5.25	5
12.5	75	0.75	0.625	0.625	2	4.625	4.375
25	75	0.75	1.25	1.25	2	4	3.75
50	75	0.75	2.5	2.5	2	2.75	2.5
75	75	0.75	3.75	3.75	2	1.5	1.25
100	75	0.75	5	5	2	0.25	0
0	100	1	0	0	2	5	5
12.5	100	1	0.625	0.625	2	4.375	4.375
25	100	1	1.25	1.25	2	3.75	3.75
50	100	1	2.5	2.5	2	2.5	2.5
75	100	1	3.75	3.75	2	1.25	1.25
100	100	1	5	5	2	0	0

Table S2.2: Absolute values in mmol of N and P added for the respective N and P treatment levels.

Proportion of Hoagland solution (%)	**N (mmol)**	**P (mmol)**
0	0	0
12.5	0.9375	0.0625
25	1.875	0.125
50	3.75	0.25
75	5.625	0.375
100	7.5	0.5

Table S2.3: Min, max and range of N:P ratio values for each P fertilisation level ($P > 0$).

P fertilisation (mmol)	**N:P min**	**N:P max**	**Range**
0.0625	15	120	105
0.125	7.5	60	52.5
0.25	3.75	30	26.25
0.375	2.5	20	17.5
0.5	1.875	15	13.125

2.9.1 Statistical models

Table S2.4: Manova model 1 summary ($n = 219$). (Response variables ~ N.fertilisation*P.fertilisation).

Model term	**Wilks λ**	**F**	**df1**	**df2**	p
N fertilisation	0.22	127.16	6	209	< 0.001
P fertilisation	0.26	99.01	6	209	< 0.001
N × P interaction	0.42	48.63	6	209	< 0.001

Table S2.5: Summary of the individual Anova analyses of each response variable from Manova model 1 (Response variable ~ N.fertilisation*P.fertilisation).

		Model term					
		N fertilisation		**P fertilisation**		**N × P interact.**	
Response var.	Plant tissue	F	p	F	p	F	p
Total biomass	root	63.75	< 0.001	188.04	< 0.001	94.73	< 0.001
	shoot	227.79	< 0.001	568.59	< 0.001	209.1	< 0.001
Total C (mmol)	root	83.66	< 0.001	253.63	< 0.001	129.14	< 0.001
	shoot	226.41	< 0.001	580.35	< 0.001	231.11	< 0.001
Total N (mmol)	root	280.54	< 0.001	128.2	< 0.001	142.75	< 0.001
	shoot	335.2	< 0.001	105.9	< 0.001	72.66	< 0.001

Table S2.6: Manova summary from model 2 (n = 219). (Response variables ~ $\log_{10}$(N:P ratio)*P.fertilisation)

Model term	**Wilks λ**	**F**	**df1**	**df2**	p
$\log_{10}$(N:P)	0.41	33.62	6	143	< 0.001
P fertilisation	0.15	140.04	6	143	< 0.001
$\log_{10}$(N:P)*P interaction	0.29	57.29	6	143	< 0.001

Table S2.7: Summary of Anova analyses for each response variable of Manova model 2 (Response variable ~ $\log_{10}$(N:P)*P.fertilisation)

		Model term					
		N fertilisation		**P fertilisation**		**N × P interact.**	
Response var.	Plant tissue	F	*p*	F	*p*	F	*p*
Total biomass	Root	16.72	< 0.001	110.09	< 0.001	83.85	< 0.001
	Shoot	78.07	< 0.001	684.31	< 0.001	268.45	< 0.001
Total C (µmol)	Root	25.53	< 0.001	169.45	< 0.001	130.98	< 0.001
	Shoot	74.38	< 0.001	639.18	< 0.001	269.35	< 0.001
Total N (µmol)	Root	5.62	0.02	168.6	< 0.001	112.89	< 0.001
	Shoot	18.81	< 0.001	213.53	< 0.001	81.8	< 0.001

Table S2.8: Statistics of linear models giving slope (α) shown in Fig. 2.3 for biomass, total C, total N in roots and shoots for each P fertilisation level. (Linear model: Response variable ~ $\log_{10}$(N:P))

Resonse variable	**Tissue**	**P fertil. (mmol)**	**Slope (α)**	**Std. err.**	**T stat.**	p
Biomass	Shoot	0.0625	-0.025	0.019	-1.283	0.21
		0.125	-0.004	0.034	-0.129	0.898
		0.25	0.291	0.051	5.749	< 0.001
		0.375	0.447	0.058	7.716	< 0.001
		0.5	0.622	0.05	12.332	< 0.001
Biomass	Root	0.0625	-0.06	0.019	-3.159	< 0.01
		0.125	-0.006	0.022	-0.277	0.784
		0.25	0.131	0.042	3.118	< 0.01
		0.375	0.115	0.049	2.326	0.028
		0.5	0.282	0.045	6.282	< 0.001
Total C	Shoot	0.0625	-1.352	0.663	-2.039	0.051
		0.125	-0.35	0.91	-0.384	0.703
		0.25	10.301	1.836	5.61	< 0.001
		0.375	16.27	1.87	8.699	< 0.001
		0.5	22.409	2.046	10.952	< 0.001
Total C	Root	0.0625	-1.813	0.575	-3.151	< 0.01
		0.125	-0.391	0.716	-0.546	0.589
		0.25	3.456	1.137	3.039	< 0.01
		0.375	4.819	1.131	4.26	< 0.001
		0.5	8.917	1.203	7.412	< 0.001
Total N	Shoot	0.0625	0.404	0.076	5.296	< 0.001
		0.125	0.73	0.078	9.355	< 0.001
		0.25	1.277	0.185	6.899	< 0.001
		0.375	1.293	0.24	5.38	< 0.001
		0.5	1.432	0.109	13.175	< 0.001
Total N	Root	0.0625	0.006	0.015	0.379	0.707
		0.125	0.069	0.026	2.65	0.013
		0.25	0.177	0.025	7.097	< 0.001
		0.375	0.223	0.028	7.878	< 0.001
		0.5	0.254	0.034	7.572	< 0.001

Table S2.9: Manova summary from model 3 ($n = 219$). (Shoot:root ratio (SR) of response variables ~ N.fertilisation*P.fertilisation)

Model term	**Wilks λ**	**F**	**df1**	**df2**	p
N fertilisation	0.77	20.84	3	212	< 0.001
P fertilisation	0.68	34.01	3	212	< 0.001
N × P interaction	0.95	3.58	3	212	0.015

Table S2.10: Summary of Anova analyses for each response variable of Manova model 3. (shoot:root ratio (SR) of response variables ~ N.fertilisation*P.fertilisation)

	Model term					
	N fertilisation		**P fertilisation**		**N × P interaction**	
Response variable	F	p	F	p	F	p
SR-total biomass	36.82	< 0.001	52.02	< 0.001	0.41	ns
SR-total C (mmol)	42.55	< 0.001	73.71	< 0.001	2.17	ns
SR-total N (mmol)	53.58	< 0.001	0.01	ns	2.7	ns

Table S2.11: Manova summary from model 4 ($n = 219$). (Response variables ~ N.fertilisation*P.fertilisation). Response variables were nitrogen-use efficiency (NUE) and phosphorus-use efficiency (PUE) in shoots and roots, i.e., Shoot-NUE, Shoot-PUE, Root-NUE and Root-PUE.

Model term	**df**	**Wilks λ**	**F statistic**	**df1**	**df2**	p
N fertilisation	1	0.23	121.2	4	147	< 0.001
P fertilisation	1	0.21	136.15	4	147	< 0.001
N × P interaction	1	0.82	8.11	4	147	< 0.001

Table S2.12: Summary of Anova analyses for each response variable of Manova model 4. Response variables were nitrogen-use efficiency (NUE) and phosphorus-use efficiency (PUE) in shoots and roots.

		Model term					
		N fertilisation		**P fertilisation**		**N × P interact.**	
Tissue	Nutrient-use efficiency	F	p	F	p	F	p
Root	NUE	191.61	< 0.001	15.17	< 0.001	0.01	ns
	PUE	2.12	ns	248.55	< 0.001	23.13	< 0.001
Shoot	NUE	428.62	< 0.001	94.28	< 0.001	2.22	ns
	PUE	31.93	< 0.001	412.16	< 0.001	23.86	< 0.001

3 Shifts in soil microbial stoichiometry and metabolic quotient provide evidence for a critical tipping point at 1% soil organic carbon in an agricultural post-mining chronosequence

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3.1 Abstract

Soil microbial stoichiometry (MB-C:MB-N:MB-P) and microbial maintenance respiration (i.e. metabolic quotient, qCO_2) were monitored along a nutrient gradient in soils from a 52-year space-for-time chronosequence of reclaimed agricultural land after brown-coal mining. Land reclamation produced loess soils of initially low (0.2%) SOC. Consecutive agricultural land management led to a gradual recovery of SOC contents. Our data revealed sudden shifts in microbial stoichiometry and metabolic quotient with increasing SOC at a critical value of 1% SOC. As SOC increased, accrual rate of C into microbial biomass decreased, whereas microbial N increased. Simultaneously, metabolic quotient strongly decreased with increasing SOC until the same critical value of 1% SOC and remained at a constant low thereafter. The microbial fractions of the soil in samples containing $< 1\%$ SOC were out of stoichiometric equilibrium and were inefficient at immobilising C due to high maintenance respiration. Increasing SOC above the threshold value shifted

the soil microbes towards a new equilibrium where N became growth limiting, leading to a more efficient acquisition of C. The shift in microbial N accrual was preluded by high variation in microbial biomass N in soils containing 0.5–0.9% SOC indicative of a regime shift between microbial stoichiometric equilibria. Our data may help establishing a quantitative framework for SOC targets that, along with agricultural intensification, may better support feedback mechanisms for a sustainable accrual of C in soils.

3.2 Introduction

Chronosequences of post-mining areas are ideal model systems to study soil processes related to the formation of soil organic matter (SOM) over time, because these soils, typically being reclaimed over decades from the same carbon-poor overburden substrates, form a succession of soil development with almost identical starting conditions and well-defined dates of origin (Bartuska and Frouz, 2015; Dworschak and Rose, 2014). As SOM content is linked to crop yield through its nutrient content and water retention in soil (Oldfield et al., 2019), it is important to gain a deeper understanding of the mechanisms promoting carbon (C) sequestration in agricultural soils (Singh et al., 2018). Agricultural management however, too often promotes C-depletion of SOM (Liu and Greaver, 2010; Panettieri et al., 2014; Paustian et al., 2004), while these soils instead could represent a globally important C sink (Chabbi et al., 2017; Minasny et al., 2017; Poulton et al., 2018). Traditionally, the build-up and persistence of soil organic carbon (SOC) was thought to depend on the chemical ”recalcitrance” of plant inputs to decomposition, yet recent research provides compelling evidence for microbial necromass as the main contributor to the stable SOM fraction in soils (Kallenbach et al., 2015; Kallenbach et al., 2016; Miltner et al., 2012, 2009). It is now thought that after microbial death, with each iterative turnover of the microbial community, a fraction of its necromass is stabilised by the mineral soil matrix, leading to a gradual accrual of SOC (Chenu and Stotzky, 2002; Kögel-Knabner et al., 2008; Liang et al., 2017). This theory implies a reconsideration of the build-up and maintenance of soil carbon stocks, which requires a better understanding and management of soil microbial biomass Fang et al., 2020; Kallenbach et al., 2015; Kallenbach et al., 2016; Khan and Joergensen, 2019; Kirkby et al., 2013.

Ecological stoichiometry sets physiological constraints on the incorporation of C and other nutrients into microbial biomass (Marklein and Houlton, 2012; Mooshammer et al., 2014; Sinsabaugh and Moorhead, 1994; Sinsabaugh et al., 2009), and provides a compelling framework for the management of SOC (Buchkowski et al., 2019; Hessen et al., 2004). However, the regulation of the build-up and maintenance of microbial biomass in soils is still not well understood. Generally, microbial biomass C (MB-C) grows linear with increasing SOC content (Anderson and

Domsch, 1989), but microbial biomass N (MB-N) and P (MB-P) may not grow at the same rate, leading to stoichiometric mismatch between microbial consumer and resource (Khan and Joergensen, 2019; Mulder et al., 2013). Soil microorganisms can respond to such a stoichiometric imbalance by either adjusting their stoichiometry to match the resource (plasticity) or by maintaining a fixed stoichiometry (homeostasis) (Spohn, 2016). Both require adaptations on the individual level, e.g. by expelling or storing nutrients (Manzoni and Porporato, 2009) and adjusting exoenzyme production (Allison and Vitousek, 2005; Sinsabaugh et al., 2009) and/or on the community level through shifts in community composition to species that are better adapted to the constraints of the resource (Ma et al., 2019; Zechmeister-Boltenstern et al., 2015). However, a consensus on the stoichiometric plasticity of soil microbial biomass and a good mechanistic understanding of the factors driving the relationship between soil and microbial stoichiometry has not yet been reached (Cleveland and Liptzin, 2007; Ehlers et al., 2010; Fanin et al., 2017, 2013; Hartman and Richardson, 2013; Li et al., 2012; Xue et al., 2019).

In contrast to aquatic systems, nutrients in soil are mostly bound to organic matter and only a fraction is freely accessible to microorganisms (Griffiths et al., 2012). This transient availability of readily accessible C substrates is the main limiting factor for microbial activity in (Demoling et al., 2007; Kaiser, 1994; Kamble and Bååth, 2014; Kuzyakov and Blagodatskaya, 2015) soils. As a result of this, the vast majority of soil microbial biomass remains in a dormant state most of the time while maintaining only basic metabolic processes (Blagodatskaya and Kuzyakov, 2013; Joergensen and Wichern, 2018).

The specific metabolic quotient (qCO_2), typically given as the ratio of basal C mineralisation rate (respired CO_2) to unit biomass C (MB-C), measures the energy required for the long-term maintenance of the microbial biomass in soils ("maintenance respiration", Anderson and Domsch (1985a) and Anderson and Domsch (1985b)). Stoichiometric imbalance requires higher maintenance respiration (Griffiths et al., 2012) and can lead to a gradual decline of soil microbial biomass if C is not sufficiently supplied (Anderson and Domsch, 1985a; Joergensen and Wichern, 2018). Still, the relationship between soil stoichiometry and maintenance respiration is not well explored. The metabolic quotient (qCO_2) is a measure of the fundamental physiological state of the microbial community. It is influenced by the fungal to bacterial biomass ratio in soils (Nannipieri et al., 2003; Sakamoto and Oba, 1994) and has been widely used as an indicator of change in the eco-physical status of soil microorganisms (Anderson and Domsch, 2010) for example during succession (Insam and Domsch, 1988; Insam and Haselwandter, 1989), land use change (Hartman and Richardson, 2013; Xu et al., 2017), or due to soil management (Zhou et al., 2017) and disturbance (Insam et al., 1996; Odum, 1985).

In this study we investigated changes in microbial stoichiometry and metabolic quotient along a soil nutrient gradient of a post-mining agricultural chronosequence, formed from the reclaimed land of the open-cast brown coal mine Inden, North Rhein-Westphalia, Germany. Homogeneous agricultural loess soils have been restored in this region according to a standardized protocol for over 50 years (Dworschak and Rose, 2014; Lucas et al., 2019; Pihlap et al., 2019), forming an ideal space-for-time substitution (Pickett, 1989; Walker et al., 2010) for the study of stoichiometric changes during soil development after restoration. We studied both the cultivated arable fields and their arable margins (grass strips directly bordering the arable fields), because we expected the unmanaged margins would have higher SOM contents due to the lack of ploughing nor regular removal of plant biomass. Half a century of soil development since restoration has given rise to a SOM gradient along the chronosequence, ranging from SOC poor freshly deposited young soils to the more developed arable soils after 52 years of cultivation with maximum SOC content in arable margins.

We hypothesised that 1) Soil microbial biomass in the chronosequence soils would not follow strict homeostasis; 2) Soil microbes would be in a state of stoichiometric imbalance in young, nutrient poor soils; 3) Soil microbial stoichiometry will gradually converge to an optimum ratio with increasing age/SOM content; and 4) The metabolic quotient (qCO_2) will be high in soils with stoichiometric imbalance and would decrease as optimum ratio is reached.

We found that microbial stoichiometry was flexible and converged towards the extractable C:N with increasing age after restoration. Further, changes in stoichiometry were not gradual as we expected but instead gave evidence of a critical threshold of SOC due to shifts in microbial stoichiometry and energy requirements for maintenance respiration once a critical value of 1% SOC was passed. These results shed new light on the processes governing the conversion of microbial biomass for the build-up of soil organic matter in C poor agricultural soils.

3.3 Materials and Methods

3.3.1 Formation of chronosequence soils

The study was conducted in a 52-year post-mining agricultural chronosequence near to Inden, Germany (50°52′44.6″N 6°19′4.4″E; Fig. S3.1). Mean annual temperature is 9.8℃ and the mean annual precipitation is 829 mm. The mining company RWE Power AG (Essen, Germany) extract lignite from the earth via opencast mining. This involves completely removing the topsoil and subjacent layers of tertiary sands to a depth of > 100 m in order to expose the lignite seam beneath. After lignite has been extracted, the cavity is filled again with the material which was previously removed. The process of extraction and restoration is continuous as the mining company follow the lignite seam through the landscape. Large conveyor belts continuously transport material from the front of the mine (where excavation proceeds) to the back of the mine (where exaction has ended) to refill the cavity and restore the land to its previous usage. This has given rise to a $>$ 50 years chronosequence of restored agricultural soils, whereby the restored soils closest to the mine are the youngest and those further away increase in age. RWE have kept detailed records of the mine's trajectory through the landscape and the dates of when the land was restored. The reclamation process has hardly changed in decades (Dumbeck, 1992; Dworschak and Rose, 2014). At the front of the mine diggers first remove the top soil loess layer, occasionally reaching down to 12 m depth, and carefully separate it from deeper substrate layers of tertiary sands. Then the loess and sandy substrates are each separately mixed and moved by conveyor belts to fill up the backside of the mine, where on top of the tertiary sands a 2 m layer of homogenized loess (containing 1% former topsoil, 0.2% SOC) is evenly spread (Dworschak and Rose, 2014). The ceaseless process creates a continuum of very homogeneous but successively older agricultural loess soils, while the open-cast mine continues to advance through the landscape.

3.3.2 Management of reclaimed land after mining

Reclaimed soils typically received in the first year an initial fertilization of 60, 120 and 180 kg ha^{-1} of, N and P and K fertilizer respectively, and are cultivated for three subsequent years with alfalfa (Medicago sativa) without any further fertilizer or biocide applications. Four to seven years after reclamation, fields typically undergo a wheat and barley crop rotation. Cereals are fertilized by NPK and CAN (Calcium ammonium nitrate) mineral fertilizers, and receive in addition in the fourth and seventh year of reclamation 30 t ha^{-1} of organic fertilizer (compost), resulting in a total annual fertilisation rate of 200, 80, 60 and 40 kg ha^{-1} of N, P, K and Mg fertilizer, respectively. After seven years, fields are returned to the previous owners and are usually managed with a sugar beet-winter wheat crop rotation. The perimeters of the arable fields were left unmanaged and are only between 20–100 cm wide. These strips we here name ”arable margins”. They differ only in that they were not intensively cultivated, i.e. had a continuous grass cover and were seldomly ploughed. Due to their narrow width we assume that the arable margins receive the same mineral fertiliser input as the arable fields. Minimum sampling distance between arable field and arable margin soils was 10 m (Fig. S3.2).

3.3.3 Soil sampling

Soil sampling took place in March 2016 in ten reclaimed sites of ages 1, 2, 3, 4, 5, 10, 26, 37, 45 and 52 years after reclamation at the time of sampling. The location of sampling sites was determined by the exact recordings of the former locations of the mine (Fig. S3.1). Most sampling sites were less than 3000 m apart and the maximum distance between sampling sites was 6000 m. At each site, soil was sampled from the reclaimed arable field and from the arable margins directly bordering the arable fields (factor "Soil Origin"). Information on the crop growing at time of sampling can be found in the supplementary material (Table S3.1). Per field, five technical replicates, each consisting of five pooled soil cores (6 cm ⊘, with a depth of 10 cm), were collected (for more details see Roy et al. (2017)). At the adjacent arable margins three technical replicates consisting each of two soil cores were sampled. All samples were sieved (2 mm), roots and stones removed and stored at 4°C prior to analysis.

3.3.4 Measurement of soil nutrients, microbial stoichiometry and microbial respiration

Soil organic C (SOC) was measured after the removal of inorganic C by addition of 10% HCl (Nelson and Sommers, 1996) and determined by combustion together with total soil N (N_{tot}) using a C/N element analyser (Flash 2000 Thermo Fisher Scientific GmbH, Germany). Soil extractable P was measured using the Calcium-Lactate extraction.

Soil microbial biomass C (MB-C)and N (MB-N), and extractable C (C_{ext}) and N (N_{ext}) were determined via the chloroform fumigation-extraction (CFE) method (Vance et al., 1987) using the respective extraction constants for C and N, $k_{EC} = 0.45$ and $k_{EC} = 0.54$ (Joergensen, 1996; Wu et al., 1990). Samples were shaken with 0.5 M K_2SO_4 on a platform shaker for 30 min (1:4 soil : extractant ratio) to extract available C and N. Extracts were frozen until measurement with a Multi N/C 2100S (Analytik Jena, Germany). Soil microbial P (MB-P) was determined using a modified anion exchange membrane method (Kouno et al., 1995; Myers et al., 1999) using hexanol instead of chloroform to induce cell lysis. P concentration in the extracts was measured using the Murphy and Riley colorimetric test (Murphy and Riley, 1962) with a microplate reader (photometric wavelength 710, VarioskanTM, Thermo Electron Corporation, Germany). Some extracts for MB-C /MB-N analysis were lost during failure of the Multi N/C analyser, resulting in unequal numbers of replicates between sites.

The metabolic quotient (qCO_2; CO_2-C mmol MB-C $mol^{-1}\,h^{-1}$) was calculated as the microbial basal respiration to unit microbial biomass C (MB-C). Microbial basal respiration was measured over 24 h with a micro-compensation apparatus (Scheu, 1992). Prior to measurement, fresh soil with 3 g equivalent dry soil were adjusted to 50% water holding capacity and incubated overnight at 20°C. Respiration rates were converted from O_2 consumption to CO_2-C respired using the ideal gas constant (e.g. 1 µL O_2 ~12/22.4 = 0.53 µg CO_2-C).

3.3.5 Statistical analyses

Non-linear shifts in microbial nutrient accrual and metabolic quotient (qCO_2) with increasing SOC were revealed by breakpoint analysis. Data were subsequently $\ln(x+1)$ transformed for linearity and subset into two groups by a soil nutrient content grouping variable with two factors (soil nutrient content either above or below a critical value). Segmented regression was performed on the subset data to give individual slopes for the two groups. To determine whether the two slopes of the segmented regressions were significantly different from the slopes where no grouping criteria was applied, an ANCOVA analysis was performed (linear model with grouping variable interaction). All statistical analyses were conducted in R (version 3.5.0) using the *base* and *stats* packages (Team, 2018). The segmented function in the package *segmented* was used to calculate breakpoints in the data (Muggeo, 2008). Other R packages used for graphing and data handling include, *ggplot2* (Wickham et al., 2018a), *dplyr* (Wickham et al., 2018b) and *tidyr* (Wickham et al., 2018c), *lattice* (Sarkar, 2008) and *latticeExtra* (Sarkar and Andrews, 2013).

3.4 Results

3.4.1 Development of soil nutrients and microbial stoichiometry over time after restoration

Changes in soil nutrient levels (SOC, total N, extractable P) and microbial biomass (MB-C, MB-N, MB-P) followed a similar pattern with increasing age after restoration (Fig. S3.3a-f). Initially, soil nutrients and microbial biomass increased at the same rate in both arable and arable margins in soils aged 1–5 years. After 10 years the accumulation of SOC and microbial biomass ceased in the arable soils, but continued to increase in the margin soils (Fig. S3.3a-e). SOC ranged between 0.21–0.93% in arable soils and 0.22–4.47% in the margin soils.

Plotting the ratios of soil C:N versus microbial biomass C:N (MB-C:MB-N), with the soil C:N calculated either from SOC and total N ($SOC{:}N_{tot}$) or from the extractable C and N ($C_{ext};N_{ext}$) (Fig. 3.1 1a, b) showed slopes strongly deviating from the 1:1 line (where microbial C:N is exactly balanced by the C:N of the soil resource). Generally, variation in MB-C:MB-N relative to soil stoichiometry was high in young soils and decreased with soil age, indicating a progressively constrained stoichiometry within the soil microbial community with increasing soil age after recultivation. In particular the changes in microbial C:N relative to extractable soil C:N appeared vertically directed towards the 1:1 line as indicated by significant linear relationships in soils aged 4, 10 and 26 years after recultivation (Fig. 3.1 b). In soils ages > 26 years, the slopes decreased and the values converged close to the 1:1 line within a narrow range of extractable soil C:N ($C_{ext}{:}N_{ext}$). This was true for both arable and margin soils, despite significant differences in total amounts of C and N (Fig. S3.3a-b). We calculated the final microbial C:N ratio as the MB-C:MB-N values where the slope crossed the 1:1 line in soils aged 37, 42 and 52 years, these were 4.4, 4.6 and 5.6 respectively.

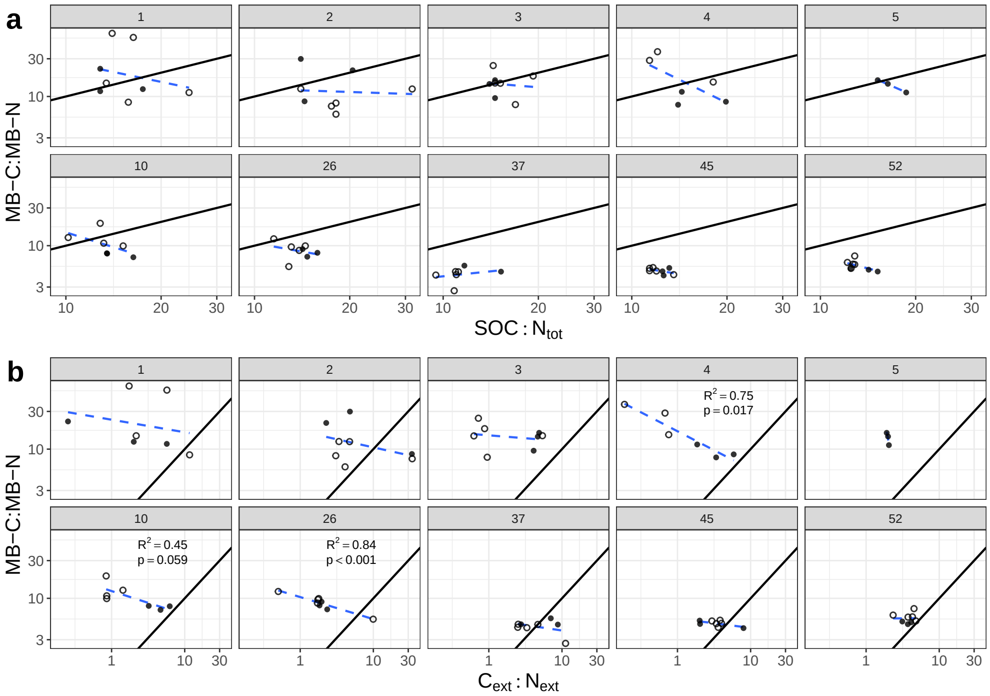

Figure 3.1: Scatter plots and slopes (dashed line) of (a) molar soil total C:N ratio (SOC:N_{tot}) versus molar microbial biomass C:N ratio (MB-C:MB-N) and (b) molar available C:N ratio from extractable C and N (C_{ext}:N_{ext}) versus molar microbial biomass C:N ratio (MB-C:MB-N) in soils of different ages (1–52 years) after recultivation for arable field (filled circle) and arable margin (open circle). Values are shown in comparison to the 1:1 (solid) line. Adjusted R^2 and p-values are given in case of significant linear relationships.

3.4.2 Microbial biomass accumulation as a function of SOM

Nutrient contents of soil microbial biomass generally increased with increasing SOC content. However, the rate of increase in MB-C and MB-N was not constant with increasing SOC (Fig. 3.2) due to shifts in microbial stoichiometry around a threshold value of $810\,\text{mmol}\,\text{kg}^{-1}$ SOC ($\sim$ 1% SOC). In soils containing $< 810\,\text{mmol}\,\text{kg}^{-1}$ SOC, MB-C increased strongly with increasing SOC (slope $\mu 1 = 1.2$, Table 3.1). Above this threshold, accrual rate of MB-C decreased by 50% MB-C compared to the soils below the threshold (slope $\mu 2 = 0.58$, Table 3.1). Conversely, increasing SOC content had the opposite effect on microbial N accumulation (Fig. 3.2). There was a weak accumulation of MB-N with increasing SOC in soils containing

$< 450\,\mathrm{mmol\,kg^{-1}}$ SOC (slope $\mu 3 = 0.48$, 3.1), whereas above the critical value of $810\,\mathrm{mmol\,kg^{-1}}$ SOC, microbial N accumulation rate doubled (slope $\mu 4 = 0.81$, Table 3.1). The shift in microbial N accrual was preluded by a region of high variance within MB-N in soils containing 450–700 mmol $\mathrm{kg^{-1}}$ SOC (0.5–0.9% SOC). Microbial P (MB-P) increased gradually with SOC (slope $\mu 5 = 0.31$, Table 3.1) and no shift in P accrual rate was observed. Soil total N (N_{tot}) was strongly correlated to SOC (Fig. S3.4), therefore when MB-N was plotted as a function of N_{tot} (Fig. 3.3) a similar shift in MB-N was observed. Here, the shift to increased accrual rate of MB-N (slope $\mu 7 = 1.03$, Table 3.1) occurred at critical value of $32\,\mathrm{mmol\,kg^{-1}}$ N_{tot} ($\sim 0.045\%$ N_{tot}).

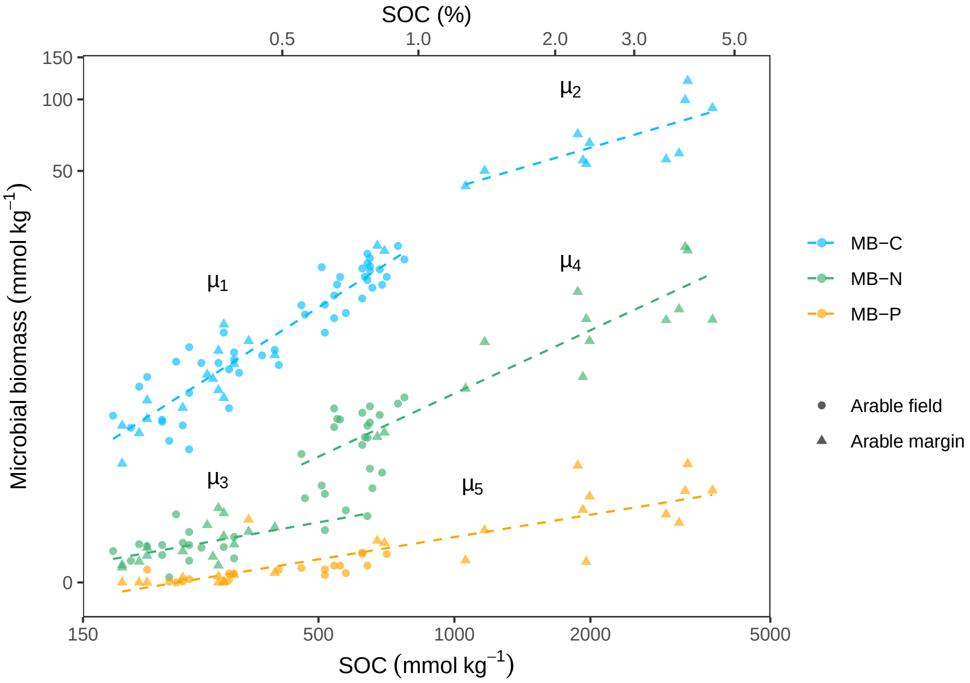

Figure 3.2: Accrual of C (MB-C), N (MB-N) and P (MB-P) in microbial biomass ($\mathrm{mmol\,kg^{-1}}$) with increasing soil organic C (SOC, $\mathrm{mmol\,kg^{-1}}$) in arable field soils (circles) and bordering arable margin soils (triangles). Data were $\ln(x + 1)$ transformed and segmented linear regression was applied. See Table 3.1 for regression statistics. Upper x-axis SOC unit given in %.

Parallel to the shifts in accrual rate of MB-C and MB-N, there was also a shift in the relationship of metabolic quotient (qCO_2) to SOC at the same critical value of $810\,\mathrm{mmol\,kg^{-1}}$ ($\sim 1\%$) SOC (Fig. 3.4). qCO_2 decreased log-linearly with increasing SOC until the critical value (slope $\mu 8 = -0.72$, Table 3.1); above the critical value qCO_2 remained at a low level of 4.46 ± 1.26 (CO_2-C $\mathrm{mmol\,h^{-1}}$ MB-C $\mathrm{mol^{-1}}$) independent of increasing SOC.

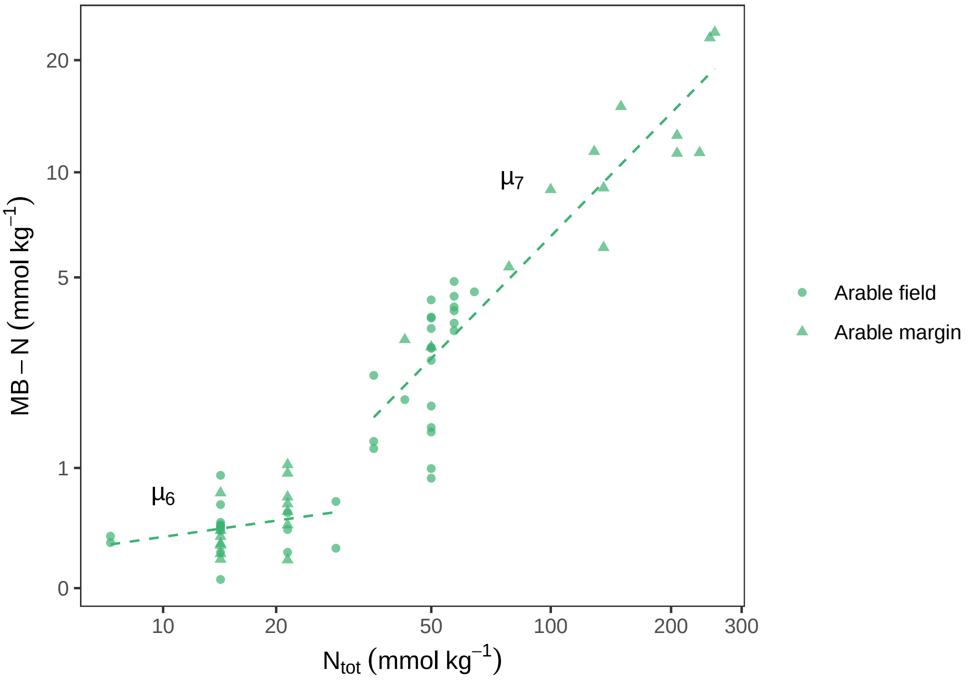

Figure 3.3: Accrual of microbial biomass N (MB-N) as a function of soil total N (N_{tot}) in arable field soils (circles) and bordering arable margin soils (triangles). Data were $\ln(x+1)$ transformed for normality and segmented linear regression was applied. See Table 3.1 for regression statistics.

Table 3.1: Table of segmented regression statistics and ANCOVA model results for individual slopes $\mu 1$- $\mu 9$ of figs. 3.2 to 3.4. All variables were $\ln(x+1)$ transformed for linearity. Microbial biomass carbon (MB-C), -nitrogen (MB-N) and -phosphorus (MB-P), and metabolic quotient (qCO_2) (y variables in figs. 3.2-3.4) were modelled as a function of soil organic carbon (SOC) or soil total nitrogen (N_{tot}) (x variables in figs. 3.2-3.4). Wherever a critical threshold in x or y (x_c or qCO_2) was identified via breakpoint analysis, the data were subset into two groups: e.g. $x \leq x_c$ and $x > x_c$. Segmented linear regression was carried out on the subset data, resulting in two slopes per xy variable pair. To determine whether the two slopes of the segmented regressions were significantly different from the slopes where no grouping criteria was applied, an ANCOVA analysis was performed (linear model with grouping variable interaction). All ANCOVA models showed a significant difference to the model without grouping variable interaction (confidence interval, $p < 0.01$). ANCOVA results shown in the table are from the regression model including grouping variable. Significance levels: $p < 0.1$ (*); $p < 0.05$ (*), $p < 0.001$ (***).

		Linear regression of $\ln(x+1)$ transformed data subset according to the breakpoint								ANCOVA			
Slope ref.	**Fig. ref.**	x	y	**n**	**R^2**	**Slope**	**Intercept**	**Significance**	**Critical value (variable,** mmol kg^{-1}**)**	**R^2**	**F**	**df**	**Significance**
$\mu 1$	3.2	SOC	MB-C	67	0.88	**1.2**	-4.85	***	SOC <= 810	0.93	537.4	[3,75]	***
$\mu 2$	3.2	SOC	MB-C	12	0.61	**0.58**	-0.27	**	SOC > 810				
$\mu 3$	3.2	SOC	MB-N	39	0.59	**0.48**	-2.31	***	MB-N <= 1.0	0.95	390.2	[3,67]	**
$\mu 4$	3.2	SOC	MB-N	32	0.83	**0.81**	-3.74	***	MB-N > 1.0				
$\mu 5$	3.2	SOC	MB-P	46	0.74	**0.31**	-1.69	*	N/A	N/A	N/A	N/A	N/A
$\mu 6$	3.3	N_{tot}	MB-N	33	0.09	**0.17**	-0.13	ns	N_{tot} <= 32	0.91	261.6	[3,75]	***
$\mu 7$	3.3	N_{tot}	MB-N	39	0.85	**1.03**	-2.73	***	N_{tot} > 32				
$\mu 8$	3.4	SOC	qCO_2	62	0.53	**-0.72**	6.7	***	SOC <= 810	0.63	42.57	[3, 69]	***
$\mu 9$	3.4	SOC	qCO_2	11	0.03	**0.09**	0.95	ns	SOC > 810				

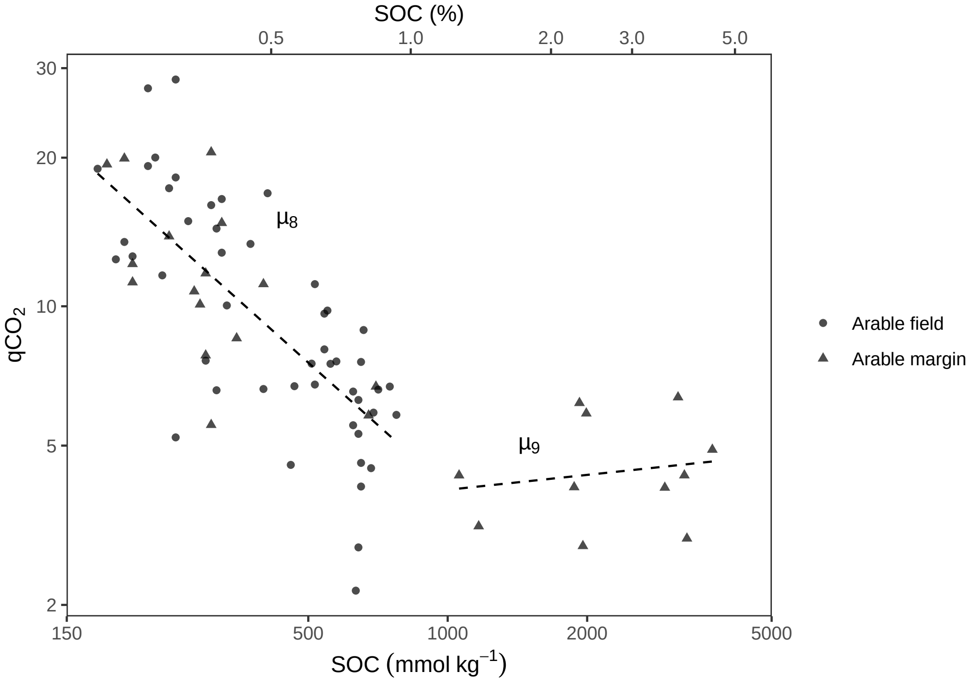

Figure 3.4: The metabolic quotient (qCO$_2$) as a function of soil organic C (SOC) content in arable field soils (circles) and bordering arable margin soils (triangles). Data were $ln(x+1)$ transformed for normality and segmented linear regression was applied. See Table 3.1. for regression statistics. Upper x-axis SOC unit given in %.

3.5 Discussion

In the recultivation chronosequence the relationships of microbial C:N ratio to soil C:N ratio were driven by soil age after recultivation (Fig. 3.1a, b). Values above the 1:1 line indicate good N availability due to a narrow soil C:N relationship. Yet the microbial biomass was characterized by surprisingly high C:N ratios, especially in soils aged $< 10\,\mathrm{y}$ (Fig. 3.1a, b). These young soils showed the largest differences between microbial and resource (soil) C:N ratio, demonstrating a substantial stoichiometric imbalance. According to high MB-C:MB-N, Pihlap et al. (2019) assumed N-limitation of microorganisms by investigating young reclaimed soils of the Garzweiler chronosequence with roughly comparable loess substrates in 50 km distance of our sites. With increasing soil age variation in microbial stoichiometry decreased as microbial C:N converged towards the 1:1 relationship with soil C:N, pointing to a weak homeostatic regulation of microbial C:N in these soils (Scott et al., 2012). Since total SOC and soil N are not immediately accessible to microorganisms, the extractable C and N give a much better approximation of the potentially available resources in soil (Griffiths et al., 2012). The (linear) convergence of microbial C:N towards to the 1:1 line (Fig. 3.1b) clearly shows that the microbes better adjust to the available resource (C_{ext}:N_{ext}) rather than to the total resource (SOC:N_{tot}) stoichiometry, irrespective of soil origin being arable or arable margin.

Our estimation of MB-C:MB-N in soils aged 37-52 years was approximately 5:1, which is lower than the global average as reported by Cleveland and Liptzin (2007) but well within range of previously reported microbial biomass C:N values for agricultural fields (Kallenbach and Grandy, 2011). It shows that stoichiometric imbalance was a strong driver to balance soil microbial communities towards equilibrium with soil stoichiometry.

There is intensive discussion about the extent of variation in soil microbial stoichiometry and the factors driving it (Cleveland and Liptzin, 2007; Fanin et al., 2017, 2013; Hartman and Richardson, 2013; Li et al., 2012; Xue et al., 2019). Changes of the homogeneous soil substrate along the chronosequence revealed regular stoichiometric patterns of alterations in soil and micro-organisms, which enable a better mechanistic understanding of the processes underlying variation in microbial stoichiometry and the associated formation of SOC.

3.5.1 Critical thresholds in microbial stoichiometry

Variation in microbial stoichiometry was not random. The much higher accrual rate (i.e. slope) of MB-C in soils with low SOC content in comparison to the accrual of MB-N and MB-P (Fig. 3.2) reflect a higher microbial demand for C than N and P (Mulder et al., 2013). The most limiting element C, showed a strong increase in microbial biomass with increasing SOC relative to the other elements until a critical threshold (1% SOC), where microbial stoichiometry was characterized by decreasing C and increasing microbial N demand (Fig. 3.2). Well-developed soils function as a slow-release fertilizer, where nutrients bound to SOM are gradually released upon mineralization by extracellular enzymes (Sanderman et al., 2017). This buffering pool of C and nutrients was however largely lacking in the SOM-poor, less developed agricultural soils. Farrell et al. Farrell et al. (2014) demonstrated that under such conditions microbial uptake of dissolved N-containing low-molecular weight molecules, such as peptides and amino acids, is primarily driven by microbial C-demand rather than N-demand. As soil organic C and N content increased above the critical value, the stoichiometric demands of the microbes could be better satisfied, as shown by the increased assimilation rate of N into microbial biomass with increasing SOM (slope $\mu 4$, Fig. 3.2). This demonstrates a shift from principal C limitation below the critical value, to microbial co-limitation of C and N above, where N became the next growth limiting nutrient after C (Ma et al., 2019; Traoré et al., 2016). The accrual of C, N and P into microbial biomass with increasing SOM follows exactly the same pattern in both arable and margin soils until 1% SOC, but only margin soils that surpass 1% SOC show a sudden decrease in the accrual rate of MB-C and an increase in the accrual rate of MB-N.

3.5.2 High microbial maintenance respiration due to stoichiometric imbalance

A parallel shift in qCO_2 occurred at the same critical 1% SOC value and revealed high C losses below the threshold, indicating stoichiometric imbalance of the microbial community (Schimel and Weintraub, 2003; Sinsabaugh et al., 2013, 2016). In soils containing less than 1% SOC, MB-C was low while the microbes showed a high demand for C (Fig. 3.2) but simultaneously high C losses per unit biomass (Fig. 3.4), indicating that a large proportion of the ingested C was lost through respiration and could not be converted to microbial biomass. In addition, microbial C:N ratio was surprisingly high relative to available soil N in the young, SOC-poor reclaimed soils (Fig. 3.1b) further corroborating high C demand. The apparent inability of the microbes to assimilate more C into biomass likely results from high microbial investments for resource acquisition in the resource-poor soils that trade-off against microbial growth (Malik et al., 2019; Ramin and Allison, 2019). These processes likely constrain the rates of C accrual in soil.

Generally, rates of C accrual from microbial biomass via necromass into SOC are not constant, but depend on the C saturation status of the soil matrix, which is a function of clay content (Frouz, 2017; Stewart et al., 2007). Rates of C accrual decrease as soils approach C saturation (Kimetu et al., 2009; Stewart et al., 2009), but this does not necessarily equate to an increase of C accrual in soils far from C saturation. Instead, empirical measurements of C sequestration in post mining chronosequences show a hyperbolic function with reduced rates of C and N accrual in both, soils close to and far from C saturation (Bartuska and Frouz, 2015; Frouz, 2017). Assuming that the proper functioning of a soil is maintained by mineralisation of C and nutrients from SOM, a minimum amount of SOM would be required for the necessary feedback processes to work. In agreement, Frouz (2017) concluded from empirical measurements of changes in SOC of chronosequence soils over time that a ”soil that has already accumulated some C will have a greater ability to accumulate additional C”. Assuming that the conversion rates of C into microbial biomass via microbial necromass directly feeds back on the formation of SOC (Buchkowski et al., 2019), reduced rates of C accrual in soils far from C saturation (Bartuska and Frouz, 2015; Frouz, 2017) could be explained by increased microbial metabolic expenditures due to stoichiometric mismatch.

We infer that increasing SOC above the critical 1% SOC value transitioned the microbes into a new state where incorporation of C into biomass was more efficient (Fig. 3.4), as demonstrated by a the parallel shifts in microbial stoichiometry and qCO_2. This result is in line with previous studies which showed that decreases in the ratio of soil C to the next the growth limiting nutrient (C:X) lead to decreases in qCO_2 (Hartman and Richardson, 2013; Keiblinger et al., 2010; Spohn, 2015; Spohn and Chodak, 2015), demonstrating the coupling of nutrient limitation to qCO_2 and nutrient use efficiency. The rapid decrease in qCO_2 in the chronosequence soils on approach to the critical value again indicates that microbes were out of stoichiometric equilibrium but moved quickly into a more nutrient efficient stoichiometric state once soil nutrient content exceeded the critical value of 1% SOC. Correspondingly, Insam and Domsch Insam and Domsch (1988) on the study of forest restoration in post open cast mining land reported a dramatic decrease of qCO_2 once soils nutrient content exceeded 1% SOC, demonstrating that this observation is not only limited to agricultural soils.

3.5.3 Microbial stoichiometric transitions occur at different critical values for different nutrients

Our data do not only indicate a shift in stoichiometric state due to the crossing of a critical threshold level of SOM, but also indicate that these microbial stoichiometric transitions occur at different critical values for different nutrients. N_{tot} was highly correlated to SOC (Fig. S3.4), therefore a similar pattern towards increasing microbial N limitation was observed when MB-N was described as a function of soil N_{tot} (Fig. 3.3). The shift in MB-N occurred at $32\,\mathrm{mmol\,kg^{-1}}$ soil N_{tot}. Soils with this concentration of total N contained approximately $400\,\mathrm{mmol\,kg^{-1}}$ SOC ($\sim 0.5\%$ SOC, Fig. S3.4). Whereas the shift in C accrual rate occurred at 1% SOC (Fig.1), meaning that the shift towards microbial N limitation (at $\sim 0.5\%$ SOC) commenced at an earlier critical value of SOC.

Furthermore, shifts in microbial N accrual show that stoichiometric transitions were not smooth, but preceded a phase of high variability between 30–60 mmol kg^{-1} N_{tot} (0.04–0.08% N_{tot}). This high variation has been observed on the approach to a tipping point, which can be indicative of a system entering a critical state (Clements and Ozgul, 2018). According to Scheffer et al. (Scheffer et al., 2012) a decisive prerequisite for a tipping point is a positive feedback which drives the change to an alternative state after exceeding a threshold. Thus, once a critical level of N_{tot} (and SOC) was reached (Fig. 3.2), the more efficient incorporation of C into the microbial community (as confirmed by qCO_2) generates a positive feedback which would be the prerequisite of a more efficient accrual of SOM from necromass (Ma, 2018).

3.5.4 Towards a quantitative framework for SOC targets

Our results show that by increasing SOM the microbial community shifted from an unstable, metabolic inefficient state (away from stoichiometric equilibrium) to a stable, more nutrient-efficient state on the crossing of a critical soil organic C content of 1%. The concept that soil functions are dependent on critical values of SOM has been previously explored, in particular the effect of a 1% SOC critical value on crop yield and SOM decomposition rates in agricultural soils (Aune and Lal, 1997; Loveland and Webb, 2003). Considering the persistence of SOM as an ecosystem property (Schmidt et al., 2011), where the proper functioning of a soil is maintained by mineralisation of C and nutrients from SOM, a minimum amount of SOM appears essential for the necessary feedback processes to operate. Still, threshold ratios and nutrient limitations are most likely site-specific, depending on parent soil material and climatic constraints. It is important to note that the arable soils of the chronosequence did not acquire SOC in excess of 1% even 52 years after soil reclamation. Thus, the consequence of a threshold model for SOM accumulation in soil is that soils will remain in the inefficient state unless the critical tipping point is reached. This may well explain the repeated failure of arable field soils in becoming a stable C sink (Minasny et al., 2017; Schulp et al., 2008). Current meta analyses suffer from the underrepresentation of agricultural soils, which are known for having the lowest C contents (Guo and Gifford, 2002). Our data further indicate that it is crucial to compare soils of similar origin in order to detect clear patterns that help to explain stoichioimetric variability. Our data are a first step towards establishing a quantitative framework for SOC targets that, along with agricultural intensification, support sustainable feedback mechanisms for the accrual of C in soils.

3.6 Conclusion

Overall, our combined results show that by increasing SOM the microbial community shifted from an unstable, metabolic inefficient state (away from stoichiometric equilibrium) to a stable, more nutrient-efficient state on the crossing of a critical soil organic C content of 1%. Microorganisms in the young chronosequence soils, where stochiometric imbalance was greatest, persisted in a nutrient inefficient state with high C demand but low acquisition of biomass C. Our data show that when SOC increased above a critical value of 1% SOC, soils entered into an alternative stoichiometrically stable state where microbial C acquisition was more efficient. Similar mechanisms may underly the functioning of all natural and managed soil systems, but threshold ratios and nutrient limitations will be likely site-specific, depending on parent soil material and climatic constraints. Agricultural soils have the lowest C stocks, therefore efforts to improve this may be fruitless if soils are still below a critical threshold value, even if C inputs are increased. A better understanding of critical elemental threshold ratios of soil microbial biomass may ultimately lead to an improved management of the processes governing the build-up of organic matter in soil.

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3.8 Supplementary Material

Table S3.1: Table detailing the soil age and crop plant growing on the arable field at time of sampling

Year of restoration	**Age of soil at time of sampling**	**Crop plant growing at time of sampling**
2015	1	Alfalfa (Medicago sativa)
2014	2	Alfalfa (Medicago sativa)
2013	3	Winter wheat (Triticum sp.)
2012	4	Winter barley (Hordeum vulgare)
2011	5	Winter barley (Hordeum vulgare)
2006	10	Winter wheat (Triticum sp.)
1990	26	Winter wheat (Triticum sp.)
1979	37	Winter wheat (Triticum sp.)
1971	45	Winter wheat (Triticum sp.)
1964	52	Winter wheat (Triticum sp.)

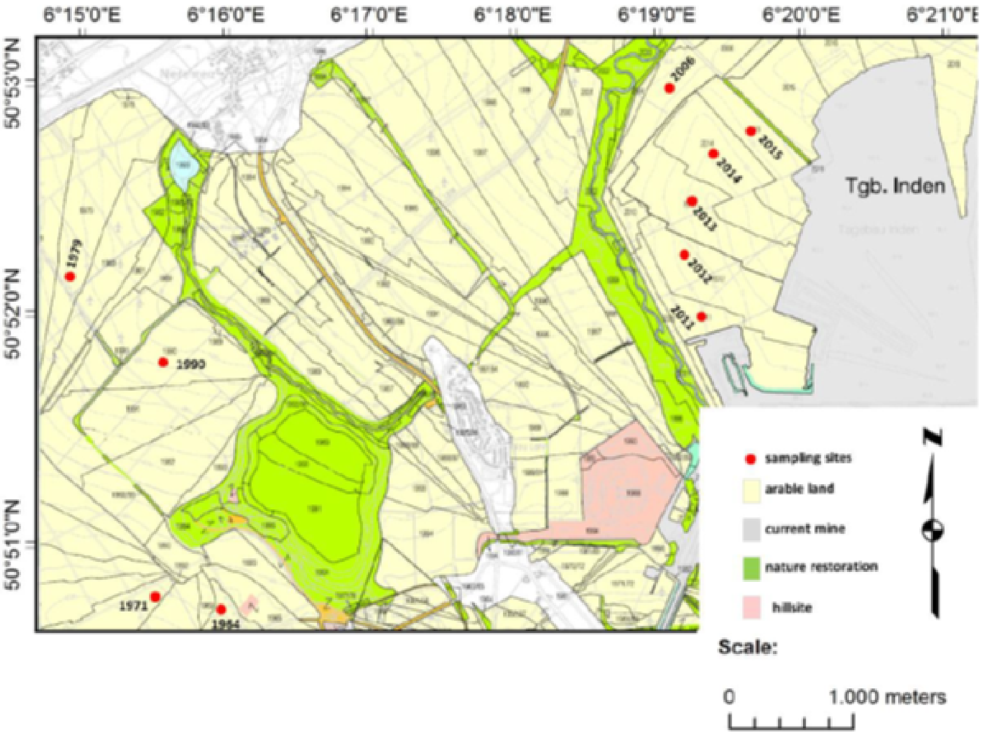

Figure S3.1: Map of the Inden mine and restored land in 2016. The red points mark the locations of the study sites sampled for the chronosequence with their year of restoration.

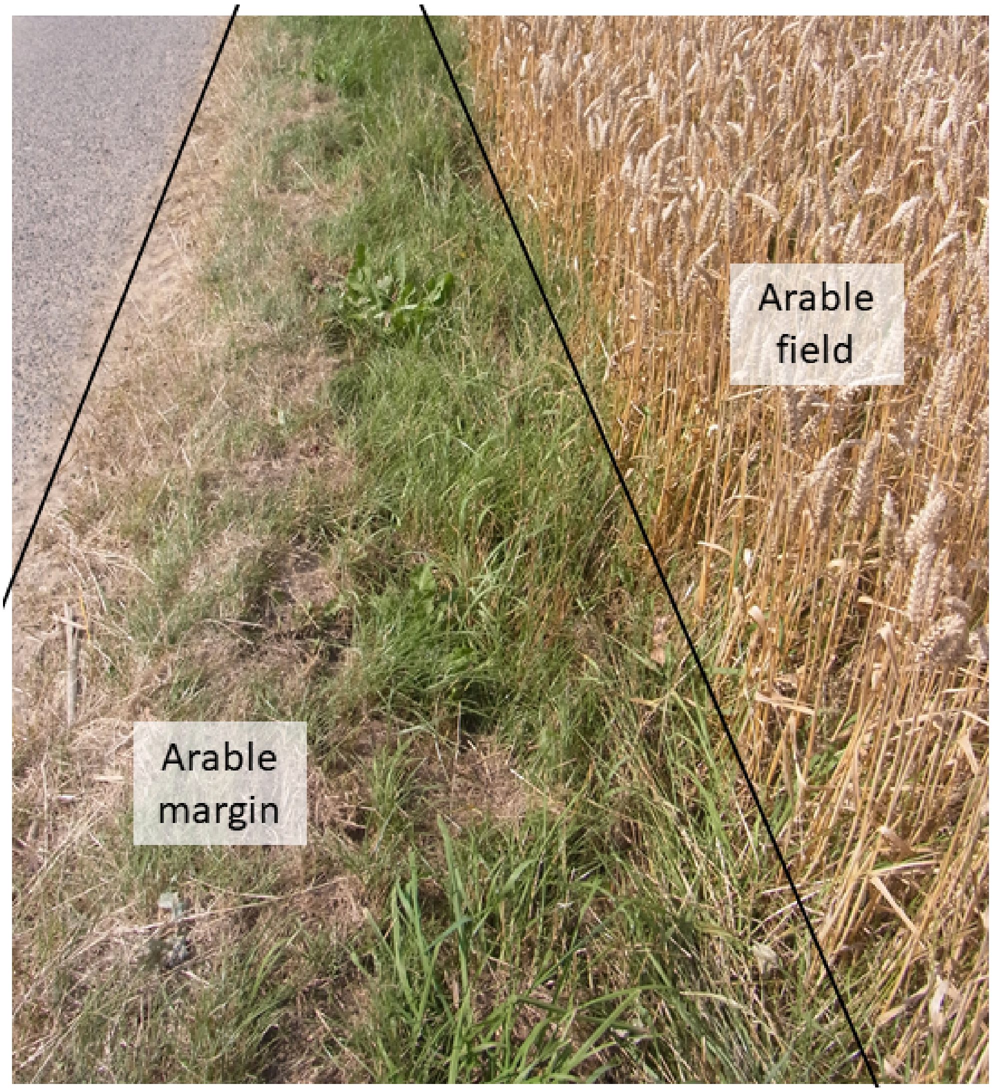

Figure S3.2: Photo of 37 year old restored land showing the proximity of the arable margin with cover of mixed grasses (left) to the arable field growing wheat (right).

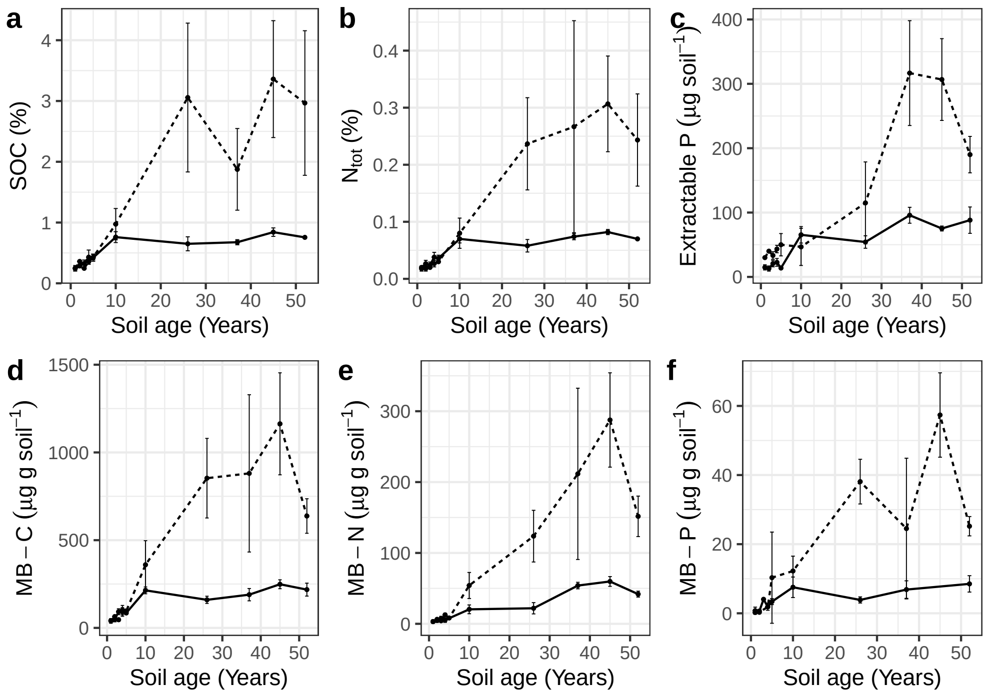

Figure S3.3: Development of soil nutrients and microbial stoichiometry over time after restoration (soil age) for arable fields (solid line) and arable margins (dashed line). Mean and standard deviation plotted over soil age for a) soil organic carbon (SOC), b) total N (N_{tot}), c) extractable P, d) microbial biomass C (MB-C), e) microbial biomass N (MB-N), and f) microbial biomass P (MB-P).

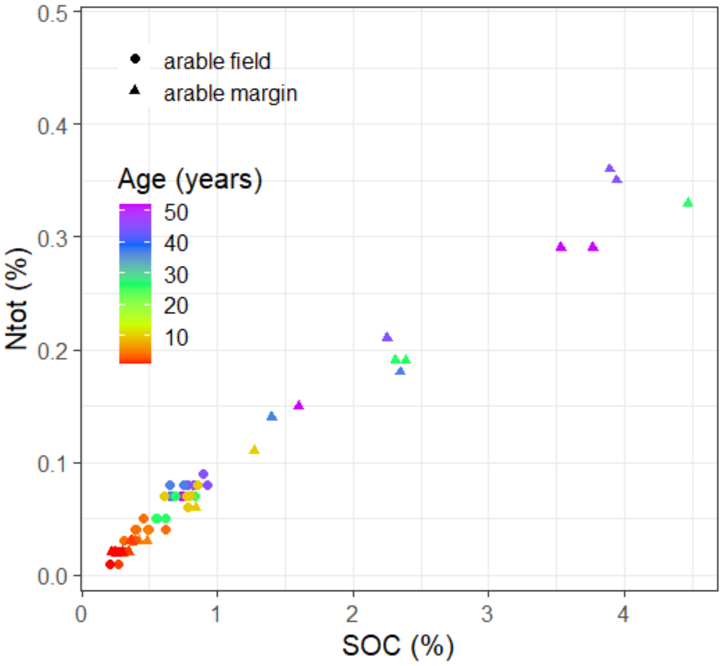

Figure S3.4: Correlation of soil organic carbon (SOC) and soil total nitrogen (N_{tot}) in arable fields (circles) and arable margins (triangles) of increasing age since soil reclamation (Pearson's $r = 0.96$, $n = 79$, df = 77, t = 59.88, $p < 0.001$).

4 Soil freezing-thawing induces immediate shifts in microbial and resource stoichiometry in Luvisol soils along a postmining agricultural chronosequence in Western Germany

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4.1 Abstract

Freeze-thaw (FT) events exert a great physiological stress on soil microorganisms and hence impact biogeochemical processes in soils. As numerous environmental factors affect microbial and chemical responses to FT, a better understanding of the leverage factors that regulate the responses to FT events is required. To date, FT-induced shifts and transformations in microbial and resource stoichiometry have received particularly little attention. We exposed fifteen Luvisol soils with different time after restoration and corresponding differences in soil organic C contents from a postmining agricultural chronosequence to a single FT event and analysed changes in soil chemistry and microbial stoichiometry one hour and eighteen hours after thawing. FT considerably altered soil biochemical attributes within the first hours of thawing. Dissolved organic carbon (C) and total dissolved nitrogen (N) concentrations immediately increased. Subsequent disproportional losses of dissolved organic C led to shifts in available resource stoichiometry. Microbial biomass C declined substantially after FT, and its relative losses were positively correlated with enhanced dissolved organic C contents. Thus, microbial cell lysis likely led to the significant increase of dissolved organic C. Moreover, microbial biomass C losses were disproportionally higher in C-rich soils, suggesting that soil microorganisms in high-C soils might be particularly prone to FT stress. Microbial biomass N marginally decreased one hour after thawing, yet returned to initial levels eighteen hours after thawing. The alternating responses of microbial biomass C and N caused a strong stoichiometric reduction of the microbial C:N ratio. The resulting microbial oversaturation with N relative to C is likely the first step in the chain of processes that generally lead to the high N losses commonly recorded in agricultural soils in the aftermath of FT events. Metabolic activity of the soil microbial community increased with the relative decline of the microbial biomass C:N ratio eighteen hours after thawing, suggesting increased levels of microbial metabolic expenditure due to stoichiometric shifts. The strength of the FT-driven biochemical responses was strongly dependent on soil organic C content, indicating that high-C soils might be especially vulnerable to initial C and N losses due to shifts in microbial stoichiometry.

4.2 Introduction

Soil freeze-thaw (FT) events have a profound impact on the ecophysiology of soil microorganisms (Miura et al., 2019; Mooshammer et al., 2017; Schimel et al., 2007; Schimel and Mikan, 2005; Sharma et al., 2006; Williams et al., 2015) and hence the biogeochemistry of terrestrial ecosystems (Bardgett et al., 2008; Congreves et al., 2018). Soil microorganisms can adapt to freezing (or rather near-freezing) temperatures by (i) upregulating protein synthesis, (ii) increasing membrane fluidity (through changes in the fatty acid composition), (iii) taking up/producing polysaccharides or compatible solutes for cryo-protection or (iv) regulating specific metabolic pathways (reviewed e.g., in Margesin and Miteva (2011) and Shivaji and Prakash (2010)). Yet, despite these adaptations, a substantial portion of the soil microbial biomass is usually killed during FT events (Feng et al., 2007; Han et al., 2018; Schimel and Clein, 1996; Song et al., 2017; Yanai et al., 2004). This die-off could either be a consequence of the freezing itself, or due to the physiological stress induced with the subsequent thawing of soils when soil microorganisms have to rapidly reverse their physiological adaptations to prevent cell rupture (Schimel et al., 2007). As a result, readily available organic compounds enter the soil where they are lost either through leaching or gaseous emissions, or metabolised by the remaining viable microorganisms, which explains the frequently observed increase of CO_2 and N_2O emissions from soils after FT events (Congreves et al., 2018; Joseph and Henry, 2008; Matzner and Borken, 2008; Song et al., 2017).

Lossses of soil carbon (C) and nitrogen (N) after FT can be substantial (Congreves et al., 2018; Matzner and Borken, 2008) and have been linked to inherent soil microbial dynamics (Christensen and Christensen, 1991; Herrmann and Witter, 2002; Matzner and Borken, 2008; Risk et al., 2013). Sharma et al. (2006) investigated CO_2 and N_2O along with microbial gene expression and community responses to FT in a microcosm study and found increased activity and expression of denitrifying genes of the soil microbial community after FT. Herrmann and Witter (2002) could further show that microbial necromass C contributed ca. 65% to the C mineralisation flush upon FT. Moreover, recent evidence suggests that C and N liberated from microbial cell lysis rather than aggregate disruption fueled N_2O emissions after FT (King et al., 2021). This suggests that soil microorganisms are strong drivers of ecosystem responses to FT events.

Although the significance of microbial stoichiometry in predicting terrestrial C and N fluxes has been acknowledged recently (see e.g., Buchkowski et al. (2015, 2019)), stoichiometric shifts and transformations during and after disturbances received little attention so far. Empirical evidence suggests that FT events can strongly alter the nutrient availability in soils. For example, Schimel and Mikan (2005) showed that soil microorganisms shifted their N usage from N-poor detritus at sub-zero temperatures to N-rich microbial products when soils were near 0℃. The observed N release from microbial products could either be a consequence of an intracellular adaptation (e.g., cytoplasmic release due to osmoregulatory processes) of the surviving microorganisms to above 0℃ temperatures, or be related to the microbial die-off after FT (Schimel et al., 2007). Herrmann and Witter (2002) and Schimel and Mikan (2005) showed that the die-off and turnover of microbial biomass strongly contributed to dissimilatory C losses (i.e., respiration) after FT. However, Clein and Schimel (Clein and Schimel, 1995) further demonstrated a decoupling of microbial transformations of labile soil C and N pools during these FT events. This shift in resource availability after FT events will most likely affect the stoichiometry of microbial consumers. For example, relatively high dissimilatory C losses of the microbial community after FT could lead to an oversaturation of N relative to C in the microbial biomass and the soil environment, while relatively high N losses through leaching and denitrification could have the opposite effect. As stoichiometric imbalances between soil microorganisms and their resources can have major implications for ecosystem-level C and N fluxes (Sterner and Elser, 2002; Zechmeister-Boltenstern et al., 2015), a better understanding of FT effects on microbial stoichiometry is urgently required. This is particularly important in the light of current climatic projections which predict increases in the frequency and magnitude of annual soil FT cycles (Henry, 2008).

Several factors such as soil physicochemical parameters (Schmitt et al., 2008; Song et al., 2017; Yanai et al., 2004) or the intensity, duration and frequency of FT events shape the chemical and microbial responses (Feng et al., 2007; Sorensen et al., 2018; Stres et al., 2010). In their review, Griffiths and Philippot (Griffiths and Philippot, 2013) discussed the role of soil carbon content and microbial nutrient contents on the resistance and resilience of microbial population to stress, citing studies where carbon limited microbes showed increased resistance to heat (Allison et al., 2010) and chemical stressors (Overbeek et al., 1995). A very recent study of repeated FT cycles on similar chronosequence soils found that soils with higher SOC content showed the largest relative losses of microbial biomass carbon (Rosinger and Bonkowski, 2021).

Due to this multifactorial complexity of FT effects on soil (Henry, 2007), experimental setups are required that allow to disentangle potential driving factors of microbial responses to FT events and the underlying mechanisms. In this study, we set out with a mechanistic approach to investigate microbial and chemical responses to a single FT event within the first hours after thawing, as this time period appears critical for microbial C and N turnover processes. We chose a severe and rapid freeze thaw event, freezing soils at −21°C for 48 hours and thawing at 10°C, to amplify the response of the microbes. We used soils from a 56-year postmining agricultural chronosequence in Western Germany, which provided a unique opportunity to study microbial and chemical responses across a soil organic C (SOC) and age gradient (Walker et al., 2010). The SOC contents varied between 0.29–4.65%, and time since recultivation ranged from 2–33 years (from here on referred to as ”soil age”). Samples were taken from agricultural fields and their directly adjacent arable margins. Dissolved organic C (DOC), total dissolved N (TDN), microbial biomass C (MB-C) and N (MB-N) were measured before freezing, 1 hour and 18 hours after thawing. We also investigated the microbial respiratory responses, i.e. basal respiration and metabolic quotient (qCO_2), before freezing and 18 hours after thawing as measures of eco-physiological responses to FT (Anderson and Domsch, 2010; Hartman and Richardson, 2013; Xu et al., 2017).

We hypothesize firstly that in comparison to before freezing, MB-C and MB-N would decrease within the first hours after thawing, while soil available nutrient concentrations would increase due to microbial death. Secondly, we hypothesize a subsequent change in soil microbial (MB-C:N ratio) and resource (DOC:TDN ratio) stoichiometry at 1 hour and 18 hours after thawing due to non-equivalent transformations of C and N by the remaining microbial population. Thirdly, we predict that factors such as SOC content and soil age shape soil microbial and chemical responses to FT.

4.3 Material and Methods

4.3.1 Study site and formation of chronosequence

The sampling site is located in the reclaimed agricultural lands after open-cast mining adjacent to the Garzweiler mines, ca. 10 km south of Mönchengladbach (6°15′0″E to 6°21′0″E; 50°50′5″N to 50°53′0″N). Mean annual temperature and precipitation is 9.8℃ and 829 mm, respectively. The primary land use in this region is for agriculture due to the fertile loess soils. Beneath the arable fields runs an enormous lignite seam, which sits relatively close to the surface, making it an ideal location for open-cast mining. Open-cast lignite mining, here conducted by the energy company RWE Power AG (Essen, German), involves completely removing the topsoil and subjacent tertiary materials (up to 100 m deep) to expose the lignite seam beneath. The excavation and subsequent restoration of the land is a continuous process, whereby material excavated at the front of the mine is transported (via conveyor belts) to the back of the mine to fill the cavity where exaction has been completed. The final top layer of the restored land typically consists of a mixture (1:100 ratio) of loess material of the former topsoil (characterized as Luvisol) and the parent material (unweathered loess from the Weichselian glaciation period; Lucas et al., (Lucas et al., 2019)) which is applied in a 2 m homogenous layer (Dworschak and Rose, 2014). Given that this process has mostly been unchanged for decades and due to excellent records of the restoration sites, this has given rise to a unique space-for-time substitution (Pickett, 1989) of soils with near exact starting conditions spanning over 50 years.

4.3.2 Management of restored lands

Once the cavity has been restored, the land is managed by RWE AG Power for 7 years before being handed back to the previous owner. The soil type is Luvisol, and soil texture of the restored soils is a silty clay loam with around 65% silt and 31% clay (Lucas et al., 2019). The bulk density of recultivated loess not affected by soil tillage soils is around 1.6 g cm^{-3} (Pihlap et al., 2019). The reclaimed lands for agriculture typically receive an initial fertilization of 60, 120 and 180 kg ha^{-1} of each, N and P and K fertilizer in the first year, and are cultivated for three subsequent years with alfalfa (*Medicago sativa*) without any further fertiliser or biocide applications. Four to seven years after reclamation, fields typically undergo a wheat and barley crop rotation. Cereals are fertilised with mineral NPK and CAN (Calcium Ammonium Nitrate) fertilisers, and receive 30 t ha^{-1} of compost in the fourth and seventh year of reclamation, resulting in a total annual fertilisation rate of 200, 80, 60 and 40 kg ha^{-1} of N, P, K and Mg fertiliser, respectively. After seven years, fields are returned to the previous owners and are usually managed with a continuation of the sugar beet-winter wheat crop rotation (Lucas et al., 2019; Pihlap et al., 2019). More details can be found in (Rosinger and Bonkowski, 2021).

The perimeters of the arable fields are left unmanaged and have initial widths of ca. 100 cm. We refer to these strips as ”arable margins” from here on. The arable margins, consisting of the same homogenous soil as the arable fields, differ only in that they were not intensively cultivated, i.e., had a continuous grass cover and were seldomly ploughed. Due to their narrow width, we assume that the arable margins received similar mineral fertiliser input as the arable fields. Arable margins studied in a neighboring chronosequence (adjacent to the Inden mine) had elevated SOC contents compared to the arable fields (Clayton et al., 2021), and for this reason were included in the study to broaden the range of SOC contents.

4.3.3 Soil sampling

Soil samples were taken in July/August 2018 on 8 different sampling sites along the chronosequence, with 2, 3, 4, 10, 12, 16, 23, 24 and 33 years after time of restoration, from both the arable field and arable margins. Five soil samples were taken randomly at each sampling site with a minimum distance of 20 m between replicates. The soil samples themselves consisted of 5 pooled subsamples taken within a 1×1 m square. Samples were taken from the upper A_p horizon to a depth of 10 cm using a soil auger (5.5 cm diameter). The minimum distance between the sampling sites was ca. 500 m, whereas the minimum distance between arable field and margin samples at each site was ca. 10 m (see Clayton et al. (2021) for detailed sampling procedure). Samples were put in plastic bags and brought to the laboratory the same day, where they were sieved at 2 mm and stored at 4℃. Thus, a total of 80 soil samples (8 sites $\times 2$ management types $\times 5$ soil samples) were taken.

4.3.4 Freeze-thaw experimental design

For the freeze-thaw experiment we took a subsample consisting of 15 soils chosen at random from the chronosequence soils, which covered a wide range of SOC contents (0.29-4.65%) and time after restoration (4–33 years) (see Table 4.1). We chose a severe freeze and rapid thawing method to induce a large response from the microbial biomass, whereby small volumes of soil (5 g) were frozen at -21℃

for 48 hours and thawed at 10°C. We decided on 3 timepoints to analyse the soils: before freezing (control), 1 hour after thawing, and 18 hours after thawing.

Table 4.1: Age of soil at time of sampling (years after restoration), sampling location, soil pH, SOC and total N contents (%) of the 15 soils used in the experiment

Soil	Soil age (years)	Management type	pH	SOC (%)	Total N (%)
1	4	arable field	7.45	0.39	0.04
2	16	arable margin	7.23	0.52	0.07
3	4	arable margin	7.32	0.8	0.04
4	4	arable field	7.41	0.81	0.03
5	24	arable field	7.24	1.05	0.04
6	33	arable field	6.62	1.16	0.09
7	12	arable margin	7.15	1.37	0.07
8	16	arable field	7.08	1.4	0.08
9	10	arable margin	7.16	1.92	0.17
10	33	arable margin	6.89	2.09	0.14
11	33	arable margin	6.95	2.13	0.18
12	10	arable margin	7.22	2.42	0.22
13	23	arable margin	6.62	2.52	0.2
14	10	arable margin	7	2.68	0.22
15	33	arable margin	7.05	4.65	0.41

The severe temperature difference and small volume of soil was intended to isolate the effect of one cycle of freezing. In doing so we hope to reveal the underlying mechanisms of stoichiometric changes in microbial biomass and soil biochemistry by amplifying the soil microbial responses to a freeze event. However, according to the German weather service (DWD), soils in the study area typically undergo up to ten FT cycles in the winter period, when soil temperatures typically cycle around the freezing point. Differences between daily minimum and maximum air temperatures of more than 21°C within 72 hours have been recorded, showing that soils in this area are exposed to severe but short temperature extremes around the freezing point.

4.3.5 Soil preparation

Soil microbial biomass and soil dissolved nutrients were measured using a chloroform-fumigation extraction method and microbial respiration via a micro-compensation apparatus (see Section 4.3.6 Soil analysis). In preparation for analysis, approximately 50 g of fresh soil per sample were incubated at 10°C for 7 days in 50 mL plastic tubes in threefold replication and water content was adjusted to 50% water holding capacity with deionised water. The threefold replication was required for each of the three freezing treatments, as chloroform extraction renders the sample unusable afterwards. For the chloroform extraction, 4× ca. 5 g of fresh soil from each sample were measured out into small plastic snap vials with lids, placed into a plastic bag and loaded into the freezer and frozen at −21°C for 48 hours. Once removed from the freezer the soils were placed in a temperature-controlled room at 10°C. At the respective timepoints, one sample per treatment was placed in the chamber for chloroform fumigation and the other (non-fumigated) sample directly prepared for extraction (see Section 4.3.6 Soil analysis). Likewise, two further subsamples of 5 g fresh soil per sample were taken for the control treatment and analysed accordingly. For the respiration analysis, again, two subsamples of 3 g fresh soil were weighed as before into plastic vials. One half of the subsamples were frozen as described and the control subsamples were directly measured on the respiration apparatus (see Section 4.3.6 Soil analysis). The frozen samples were removed from the freezer, and placed directly in the respiration apparatus for thawing, where respiration rate was measured every hour for up to 18 hours.

4.3.6 Soil analysis

Soil pH was measured in H_2O (5:1 w/w) using a pH electrode. Total soil C and N contents were determined by combustion using a C/N element analyser (Flash 2000 Thermo Fisher Scientific GmbH, Germany). For the SOC content, soils were treated with 10% HCl in order to remove inorganic C (Nelson and Sommers, 1996) and dried overnight at 60°C. Thereafter, the organic C content was determined by combustion as described.

MB-C and MB-N were analysed using the chloroform-fumigation extraction method (Vance et al., 1987; Witt et al., 2000). Briefly, two subsamples of 5 g fresh soil were taken from each soil treatment. One subsample was shaken in 25 mL of 0.5 M K_2SO_4 for 30 minutes and centrifuged at 4500 rpm for 10 minutes. Thereafter, 3 mL of the supernatant were transferred into new 15 ml plastic tubes and frozen until further determination of DOC and TDN. The other subsample was fumigated under vacuum (using a desiccator) with 50 mL of ethanol-free chloroform for 24 hours at room temperature prior to extraction (as above). Extracts were analysed with a Multi N/C analyser (2100S, Analytik Jena, Jena, Germany). MB-C and MB-N were calculated as the difference between fumigated and non-fumigated DOC and TDN, respectively. MB-C and MB-N were not corrected by any extraction efficiency coefficient. All results are expressed in µg g dry soil^{-1}.

Basal respiration was measured by an automated electrolytic micro-respiratory apparatus (Scheu, 1992). Briefly, ca. 3 g of fresh soil were weighed into glass vessels and connected to the respirometer. The O_2 consumption rates were measured at 21°C every 60 minutes and subsequently converted to CO_2 production using the molar gas constant. Basal respiration is expressed in µg CO_2-Cg dry soil^{-1}h^{-1}. The metabolic quotient (qCO_2) was calculated as basal respiration per unit MB-C and is expressed in mg CO_2-C g MB-$C^{-1}h^{-1}$.

4.3.7 Statistical analysis

We conducted a multivariate analysis of covariance (MANCOVA) to test for significant differences in DOC, TDN, the DOC:TDN ratio, MB-C, MB-N, the MB-C:N ratio, basal respiration and qCO_2 between the time points before freezing and 1 hour as well as 18 hours after thawing. FT treatment was used as a fixed factor (from now on referred to as "FT" or "FT treatment"), and SOC content and soil age were treated as covariates. We used a SS type III model to test for significant treatment effects as well as significant interactions with SOC content and soil age, respectively. To evaluate the statistical significance of the overall model, the Wilks' lambda distributions (λ) and derived F-values, *p*-values and the partial eta squared (η^2) for main and interaction effects are stated. Equality of variances (Levene's test) was given for all measured parameters ($p > 0.05$) except for the DOC:TDN ratio ($p = 0.026$). As the freshly recultivated loess material contains carbonates which disaggregate over time in the upper soil horizon of the chronosequence, soil age and soil pH were negatively correlated ($R = -0.724$, $p = 0.002$). The use of both factors in a single one-way MANCOVA showed that soil age (Wilks' $\lambda = 0.595$, $F = 0.987$, $p = 0.482$, partial $\eta^2 = 0.159$) appeared to be the stronger explanatory variable as compared to soil pH (Wilks' $\lambda = 0.634$, $F = 0.858$, $p = 0.629$, partial $\eta^2 = 0.141$) for biochemical parameters (i.e., MB-C, MB-N, MB-C:N ratio, DOC, TDN, DOC:TDN ratio). The same was true for the respiratory responses (i.e., basal respiration and qCO_2; statistics for soil age: Wilks' $\lambda = 0.815$, $F = 1.241$, $p = 0.307$, partial $\eta^2 = 0.097$; statistics for pH: Wilks' $\lambda = 0.948$, $F = 0.311$, $p = 0.869$, partial $\eta^2 = 0.026$). Accordingly, we omitted the colinear soil pH as a covariate. Moreover, SOC and total N were positively correlated ($R = 0.973$, $p < 0.001$). Using the same approach as above, the SOC content (for biochemical responses: Wilks' $\lambda = 0.885$, $F = 0.724$, $p = 0.58$, partial $\eta^2 = 0.059$; for respiratory responses: Wilks' $\lambda = 0.583$, $F = 1.03$, $p = 0.436$, partial $\eta^2 = 0.165$) was kept in the model as it had the slightly higher explanatory power compared to the soil N content (for biochemical responses: Wilks' $\lambda = 0.984$, $F = 0.094$, $p = 0.984$, partial $\eta^2 = 0.008$; for respiratory responses: Wilks' $\lambda = 0.593$, $F = 0.995$, $p = 0.473$, partial $\eta^2 = 0.16$). Because SOC content and soil age were only weakly correlated ($R = 0.472$, $p = 0.076$), both parameters were kept in the main analysis. Moreover, we tested whether the sampled management type (i.e., arable field or arable margin) had an effect on the analysis outcome. Because management type showed no significant effect in the final MANCOVA models with all other covariates, this factor was not further considered.

Paired sample t-tests using a Bonferroni correction for multiple comparisons were conducted to evaluate significant differences between the three time points before freezing, 1 hour after thawing and 18 hours after thawing. We used linear regression analyses and Pearson correlation coefficients to evaluate the relationship between SOC content and relative MB-C losses 18 hours after thawing (as compared to pre-freezing values) and to evaluate the relationships between ΔMB-C and ΔDOC as well as ΔMB-N and ΔTDN 18 hours after thawing. Δ refers to the percent differences between values before freezing and 18 hours after thawing. We refer to significant differences at $p < 0.05$ (with *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$). All statistical analyses and figures were conducted in SPSS 26.

4.4 Results

SOC content significantly shaped the outcome of the FT treatment in the overall model for both the biochemical and respiratory responses, while soil age had no predictive power (Table 4.2, 4.3). Test of between subject effects on individual parameters revealed that FT treatment effects for MB-C, MB-N, DOC and basal respiration were stronger in interaction with SOC content (Table 4.2, 4.3). Soil age only affected the response of basal respiration to FT (Table 4.3).

Table 4.2: Test statistics of a MANCOVA analysis to evaluate the effect of the FT treatment (non-frozen control, 1 hour after FT and 18 hours after FT) as well as interactions between FT and SOC content (FT × SOC) and time since restoration (FT × Soil age) on soil biochemical parameters (MB-C, microbial biomass C; MB-N, microbial biomass N; DOC, dissolved organic C; TDN, total dissolved N). Test statistics of the full model are given as well as of individual parameters separately

Full model statistics

	df	**F**	p	**Wilks' λ**	**partial η^2**
FT treatment	1,18	12.44	<0.001	0.028	0.696
FT × SOC	3,18	8.13	<0.001	0.063	0.602
FT × Soil age	3,18	0.59	0.895	0.724	0.102

Statistics on individual parameters

	FT treatment		**FT × SOC**		**FT × Soil age**	
	F	p	F	p	F	p
MB-C	0.725	0.544	33.25	<0.001	1.061	0.378
MB-N	0.042	0.988	25.341	<0.001	0.255	0.858
MB-C:N ratio	32.898	<0.001	0.694	0.562	0.186	0.905
DOC	7.992	<0.001	21.455	<0.001	0.413	0.745
TDN	7.27	0.001	0.468	0.706	0.284	0.837
DOC:TDN ratio	12.005	<0.001	1.996	0.132	0.23	0.875

MB-C contents declined progressively with time after the FT event (Fig. 4.1a); averaged over all samples, declines were ca. 15% 1 hour after thawing and ca.

Table 4.3: Test statistics of a MANCOVA analysis to evaluate the effect of the FT treatment (non-frozen control and 18 hours after FT) as well as interactions between FT and SOC content (FT × SOC) and time since restoration (FT × Soil age) on basal respiration and the metabolic quotient (qCO_2). Test statistics of the full model are given as well as of individual parameters separately

Full model statistics

	df	**F**	p	**Wilks' λ**	**partial η^2**
FT treatment	1,4	7.306	<0.001	0.374	0.389
FT × SOC	3,4	5.258	0.001	0.471	0.314
FT × Soil age	3,4	1.889	0.128	0.738	0.141

Statistics on individual parameters

	FT treatment		**FT × SOC**		**FT × Soil age**	
	F	p	F	p	F	p
Basal respiration	7.388	0.003	12.278	<0.001	4.213	0.027
qCO_2	9.186	0.001	0.715	0.499	0.043	0.958

49% 18 hours after thawing as compared to before freezing. Across the sample set, the MB-C declines 18 hours after thawing were between 11-69%, while MB-N contents remained rather unchanged (Fig. 4.1b). We recorded a marginal MB-N decrease 1 hour after thawing followed by a slight increase back to pre-freezing values (Fig. 4.1b). This caused significant shifts in the MB-C:N ratio 18 hours after thawing from 8.1 (±0.5) before freezing to a MB-C:N ratio of 7.8 (±0.7) and of 4.6 (±0.5), 1 hour and 18 hours after thawing, respectively (Fig. 4.1c). Linear regression analysis showed a significant negative relationship between SOC content and relative changes in ΔMB-C from before freezing to 18 hours after thawing, with relatively higher MB-C losses after thawing with increased SOC contents (Fig. 4.3a).

DOC contents increased progressively after the FT treatment by 16.5 and 39.9% 1 hour and 18 hours after thawing, respectively (Fig. 4.1d). TDN contents 1 hour after thawing were still similar to pre-freezing values, but significantly increased 18 hours after thawing (by 2.4 and 14.2% as compared to before freezing and 1 hour after thawing, respectively) (Fig. 4.1e). Because DOC contents increased disproportionately to TDN contents, the DOC:TDN ratio increased in time after thawing (Fig. 4.1f). Furthermore, DOC increased as MB-C was lost as demonstrated by a negative correlation between ΔMB-C and ΔDOC (Fig. 4.4a). Such a relationship was not found for ΔMB-N and ΔTDN (Fig. 4.4b). Basal respiration decreased on average by 16.1% 18 hours after thawing (Fig. 4.2a). The metabolic quotient in contrast increased 18 hours after thawing by ca. 40% (Fig. 4.2b). Importantly, high-SOC soils showed relatively stronger increases in qCO_2 after thawing than low

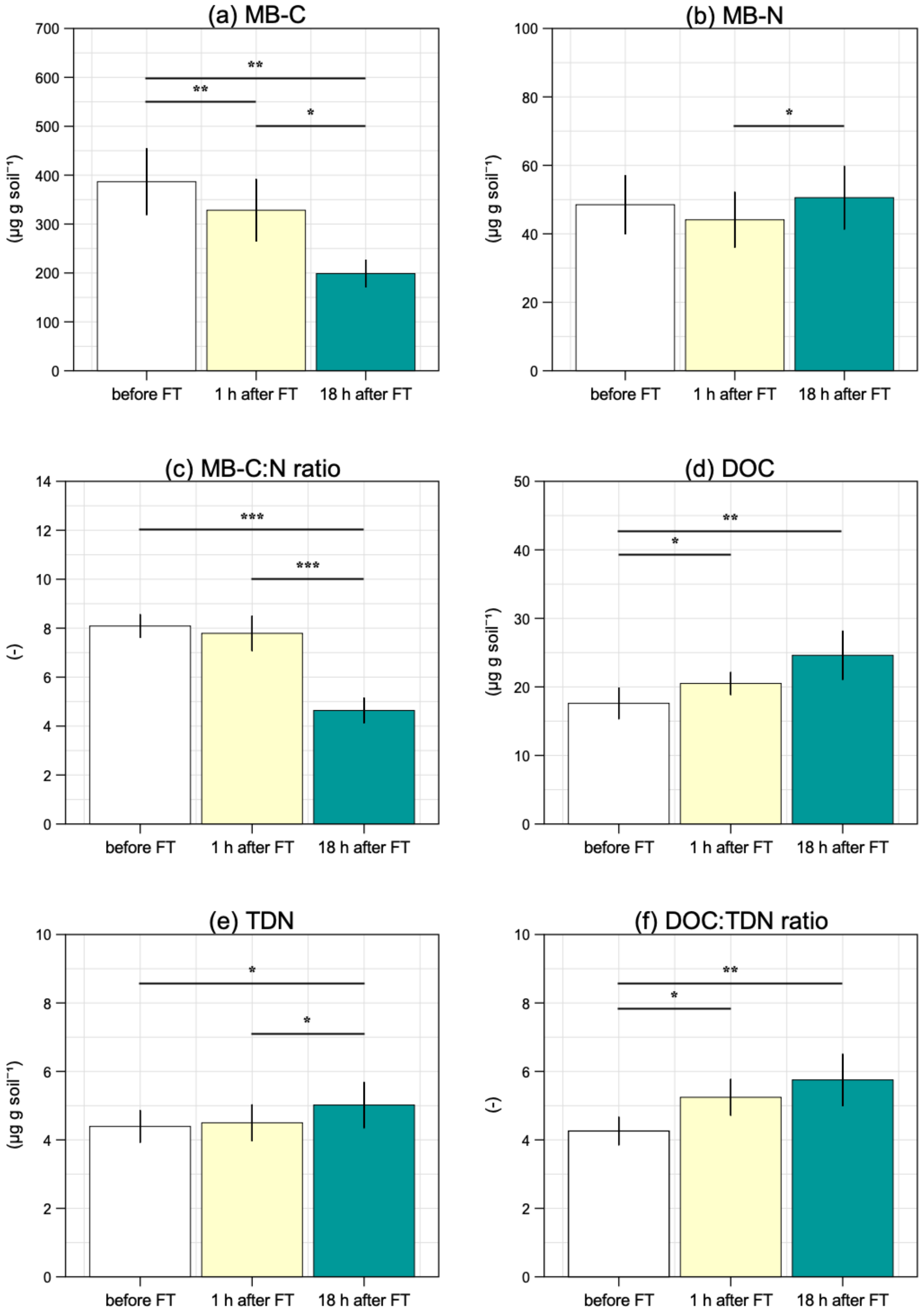

Figure 4.1: (a) Microbial biomass C (MB-C), (b) microbial biomass N (MB-N), (c) MB-C:N ratio, (d) dissolved organic C (DOC), (e) total dissolved N (TDN) and (f) the DOC:TDN ratio of field and marginal field soils 1 h after thawing (light-yellow bars) and 18 h after thawing (dark-green bars). White bars represent non-frozen control samples. Bars display the mean ± SE ($n = 15$), and asterisks indicate significant differences between FT treatments (with *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) as revealed by paired sample t-tests

SOC soils (Fig. 4.3b). Moreover, the ΔqCO_2 increased with decreasing ΔMB-C:N ratios (Fig. 4.5).

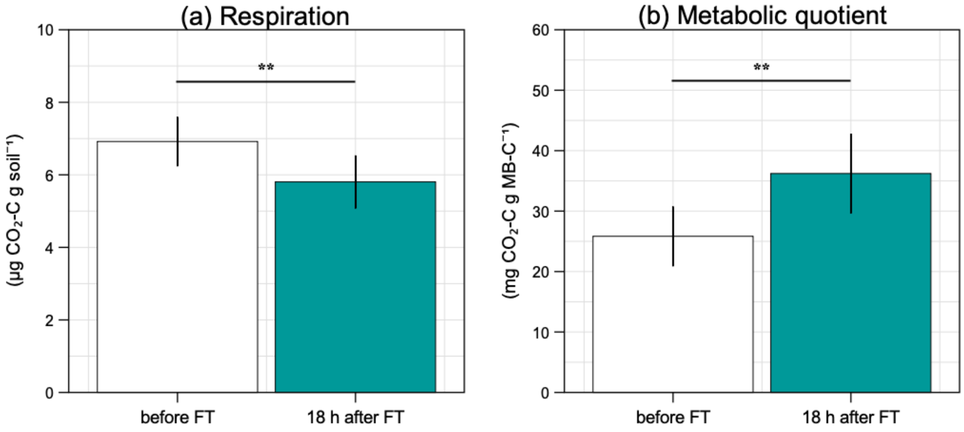

Figure 4.2: (a) Soil microbial respiration and (b) the metabolic quotient of field and marginal field soils of non-frozen control samples (white bars) and 18 h after thawing (dark-green bars). Bars display the mean ± SE ($n = 15$), and asterisks indicate significant differences between FT treatments (with *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) as revealed by paired sample t-tests

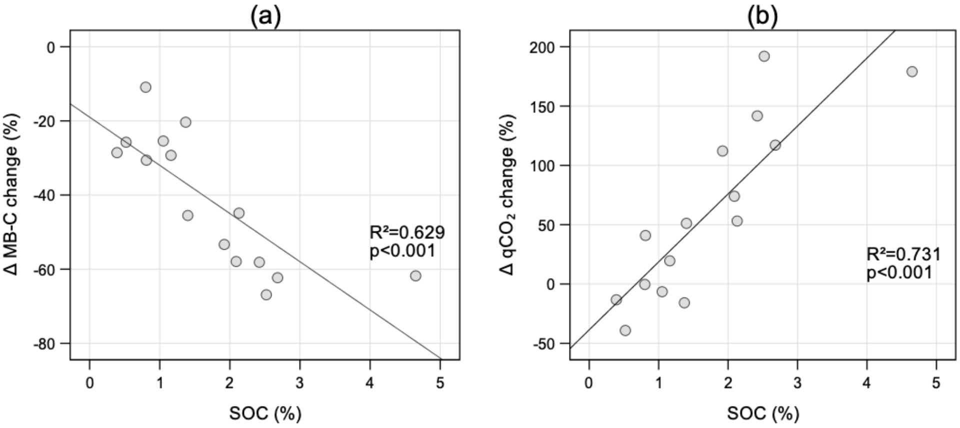

Figure 4.3: The relationship between the SOC content and (a) relative MB-C and (b) relative qCO_2 changes (%) between non-frozen controls and 18 h after thawing of field and marginal field soils (n = 15). Given are R^2 and p-values as revealed by linear regression analyses and Pearson correlation coefficients

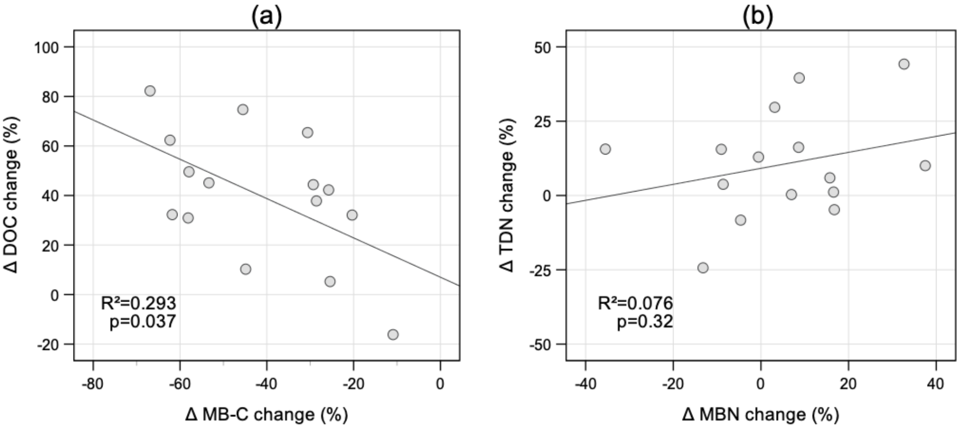

Figure 4.4: The relationship between (a) relative MB-C and DOC changes (in %) and (b) relative MB-N and TDN changes (in %) between non-frozen controls and 18 h after thawing of field and marginal field soils (n = 15). Given are R^2 and p-values as revealed by linear regression analyses and Pearson correlation coefficients

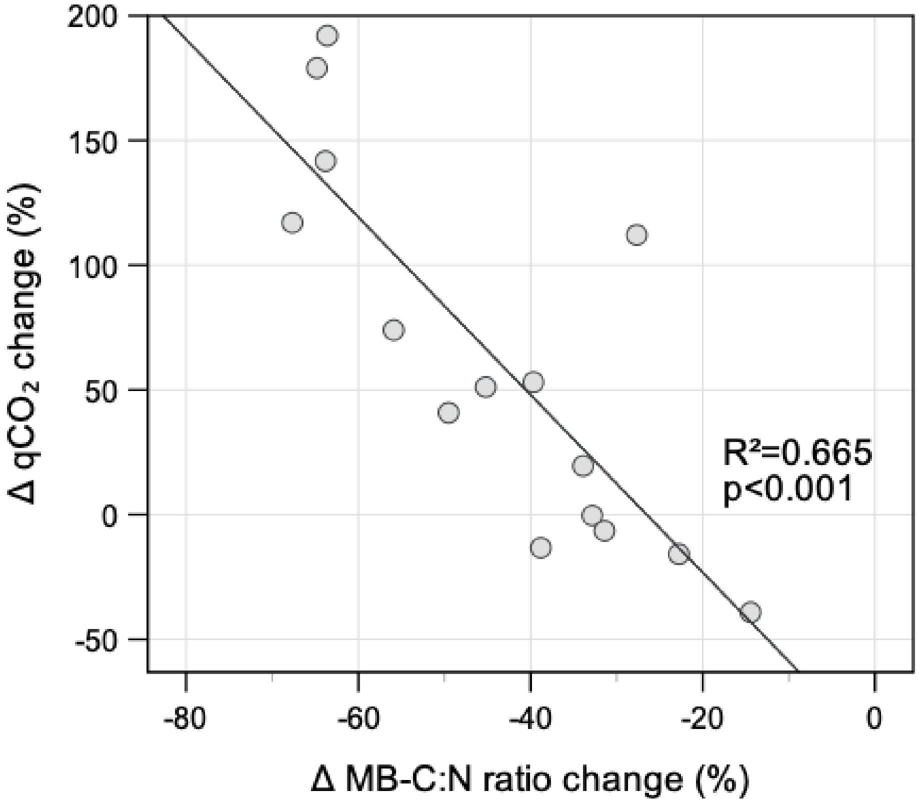

Figure 4.5: The relationship between relative MB-C:N ratio and qCO_2 changes (in %) between non-frozen controls and 18 h after thawing of field and marginal field soils (n = 15). Given is the R^2 and *p*-value as revealed by linear regression analysis and Pearson correlation coefficient

4.5 Discussion

As expected, FT led to substantial losses of MB-C within the first 18 hours of thawing (Fig. 4.1a) (Feng et al., 2007; Han et al., 2018; Schimel and Clein, 1996), confirming our first hypothesis. Averaged over all samples, nearly 50% of MB-C was lost within the first 18 hours of soil thawing; this magnitude is in correspondence with a recent study conducted along the same chronosequence (Rosinger and Bonkowski, 2021) and other studies (Schimel et al., 2007; Song et al., 2017; Yanai et al., 2004). MB-C losses within 18 hours of thawing varied a lot between soil samples and ranged between 11 and 69%.

Contrary to our first hypothesis, MB-N contents briefly dropped 1 hour after soil thawing, yet increased thereafter and appeared to return to control levels at 18 hours after thawing (Fig. 4.1b)(Mooshammer et al., 2017). This suggests that N released from disrupted microbial cells during freezing was quickly incorporated in the remaining and regrowing microbial biomass. Recent studies confirm that soil microorganisms can indeed store considerable amounts of N and P in their biomass (Bingham and Cotrufo, 2016; Kamp et al., 2015).

The observed contrasting MB-C and -N responses to FT led to great microbial stoichiometric shifts. The resulting MB-C:N ratios 18 hours after thawing were significantly lower as compared to ratios prior to freezing and 1 hour after thawing (Fig. 4.1c); reducing from 8.1 before freezing, to 7.8 and 4.6 one hour and 18 hours after thawing, respectively. This finding is in line with our second hypothesis and a recently published global meta-study on FT effects (Gao et al., 2021). Shifts in MB-C:N ratio due to FT may indicate rapidly changing environmental conditions, higher micro-faunal predation or microbial community shifts (Dörsch et al., 2004; Perez-Mon et al., 2020; Song et al., 2017). We cannot comment on whether the above mechanisms apply here, but our data rather indicate that N derived from microbial necromass was immediately assimilated, while C was lost by respiration by the re-growing microbial biomass, which lead to a short-term oversaturation of MB-N relative to MB-C.

This result was supported by the significant increase in qCO_2 in most soils 18 hours after thawing (Fig. 4.2b). The metabolic quotient reflects the efficiency by which C is assimilated per unit microbial biomass and has been commonly used as a stress indicator (Anderson and Domsch, 1990, 1993; Wardle et al., 1995) in response to changes in environmental factors such as soil temperature (Hagerty et al., 2014), soil texture (Jiang et al., 2013) or substrate availability (Blagodatskaya et al., 2011). We show linear proportional increases in ΔqCO_2 with increasing SOC contents and with a stronger stoichiometric mismatch (Fig. 4.3b, 4.5), demonstrating a clear link between microbial metabolic responses and stoichiometry. The microbial stoichiometric mismatch was a strong determinant of the efficiency by which C was assimilated per unit microbial biomass, which could be caused for example by intracellular osmoregulatory processes after thawing (Schimel et al.,

2007) or enhanced expression of denitrifying genes by the microbial community (Sharma et al., 2006). In their global-scale meta-analysis, Xu et al. (2017) found a negative correlation between qCO_2 and the microbial N:P ratio, indicating that stoichiometric shifts or mismatches can generally affect the metabolic quotient of soil microorganisms. As a result, FT disturbances can lead to a decoupling of MB-C and -N dynamics in soil, and may cause a stoichiometric mismatch of the soil microbial biomass (Sterner and Elser, 2002), which seems to represent a great physiological stress for the soil microbial community. Because we know that soil microorganisms along the agricultural chronosequence are mainly C limited (Clayton et al., 2021), FT might further exacerbate the inherent state of C limitation. Although our data suggest that soil microorganisms have the capacity of intracellular N storage within the first hours of thawing, soil microorganisms might adjust to this inherent stoichiometric mismatch (i.e., high MB-N relative to -C within the first hours after thawing) by releasing excess N to the soil environment in the aftermath of FT events, where it is prone to being lost via leaching or gaseous emissions (King et al., 2021; Matzner and Borken, 2008). This might particularly be the case when C availability is low. Further studies are required to investigate how microbial stoichiometric dynamics continue to evolve, and how this is related to subsequent soil C and N losses within short time periods after a FT event.

Within the time frame of our study, our data suggest an increased risk of C loss through leaching, as indicated by increased DOC concentrations after thawing (Fig. 4.1d) (Matzner and Borken, 2008). The ability of the soil microbial community to re-assimilate the available C as well as edaphic conditions at a later stage might dictate the magnitude of C losses from the soil. The increased DOC:TDN ratio after thawing further indicated a shift in available nutrients towards increased availability of C relative to N (Fig. 4.1f). Shifts in C:N:P resource stoichiometry were found to profoundly affect microbial community composition and community network structures (Aanderud et al., 2018; Wei et al., 2020), microbial nutrient limitation for growth (Aanderud et al., 2018; Griffiths et al., 2012) and resource partitioning towards different metabolic pathways (Chen et al., 2019) , with direct effects on soil ecosystem properties such as C mineralisation rates (Wei et al., 2020). Further studies should aim to investigate whether FT events and subsequent stoichiometric shifts also trigger such changes.

Furthermore, our data clearly showed that high-SOC soils suffered relatively higher MB-C losses as compared to low-SOC soils (Fig. 4.3a), confirming our third hypothesis. High-SOC soils tend to harbour a more active microbial biomass (Allison et al., 2007; Barrett and Burke, 2000). Since FT damage is greater when soil microorganisms are more active and growing (Schimel and Clein, 1996), the microbial communities in high-SOC soils were likely more vulnerable to FT effects. Another explanation could be that soils with increased organic matter have increased water holding capacity (Clein and Schimel, 1995). In this case, an increased number of expanding water molecules could lead to more microbial cells being damaged and ruptured. Although microorganisms in high-SOC soils showed a lower resistance to FT, it remains to be determined whether they are more resilient and recover faster after such stresses (Griffiths and Philippot, 2013). Increases in DOC contents 18 hours after thawing ranged between 10 and 45% (Fig. 4.1d), which is substantial but within the range of previous reports (Song et al., 2017). Our data do show a significant increase in DOC along with reduced CO2 emissions 18 hours after thawing (Han et al., 2018). The reduced MB-C after FT was accompanied by proportional increases of DOC (Fig. 4.4a), suggesting that significant amounts of DOC originated from microbial cells (Larsen et al., 2002).

Interestingly, MB-C losses in low-SOC soils emerged within the first hour after thawing, while the major MB-C losses in the high-SOC soils mainly occurred 18 hours after thawing. This temporal pattern suggests that it is not just cell disruption due to soil freezing, but physiological processes related to the subsequent thawing that are also responsible for the decrease in MB-C (Schimel et al., 2007). Thus, while soil freezing might be the main diminishing factor for MB-C in the low-SOC soils, the process of thawing and the inherent physiological stress (Schimel et al., 2007) might be the more significant disturbance factor in high-SOC soils.

Comparing the response of soils of defined origin along a chronosequence allows insights in fundamental mechanisms and factors of soil microbial and biochemical responses to FT events (Walker et al., 2010). Regarding our third hypothesis, SOC content indeed shaped the microbial response to FT, while soil age was of minor importance (Table 4.2, 4.3). These factors represent potentially different mechanisms that drive biochemical responses to FT. For example, high SOC contents have been associated with higher metabolic activities (Allison et al., 2007; Barrett and Burke, 2000), suggesting a higher susceptibility of more active microbial communities to FT (Schimel and Clein, 1996). On the other hand, soil age does not solely imply an increase in SOC content but also changes in edaphic characteristics such as organic matter quality (Chaudhuri et al., 2015; Clark et al., 2012; Egli et al., 2010; Kumar et al., 2018; Shi et al., 2006). Similarly, Delgado-Baquerizo et al. (Delgado-Baquerizo et al., 2020) could show that soil age drives local-scale ecosystem properties such as soil N:P and C:P ratios, microbial biomass, or shifts in fungal:bacterial dominance. Further studies are needed to dissect the specific factors associated with SOC content and soil age and its influence on soil biochemical responses to soil FT.

4.6 Conclusion

To conclude, we show that a single severe FT event resulted in considerable biochemical alterations across all our analysed soils within the first hours of thawing. We observed both DOC and TDN increases. Disproportional increases in DOC contents however led to available resource stoichiometric shifts. MB-C declined significantly after FT; because losses were positively correlated with relative DOC changes, significant amounts of DOC must have originated from microbial cell lysis. Moreover, MB-C losses were disproportionally higher in high-SOC soils, suggesting that soil microbes in high-SOC soils might be particularly prone to FT stress. MB-N marginally decreased 1 hour after thawing, yet returned to initial levels 18 hours after thawing. Hence, the observed disproportionate MB-C losses as well as increased metabolic activity immediately after FT led to an oversaturation of MB-N relative to -C as identified in the strong shift of MB-C:N stoichiometry within the first hours after thawing. The resulting microbial stoichiometric mismatch provides a mechanisitc explanation for the subsequently high N losses commonly recorded in agricultural soils in the aftermath of FT events. Increased MB-C:N ratio shifts 18 hours after thawing were found in line with increased metabolic activity (i.e., qCO_2), which advocates increased levels of microbial metabolic expenditures with disturbance-related stoichiometric shifts. Our study further allowed the partial dissection of SOC content and soil age as leverage factors of FT-driven changes in soil biochemistry. SOC content was the strongest shaping factor, while soil age showed less predictive power. Future studies on larger sample sets are required to further dissect the underlying mechanisms associated with SOC content.

Declarations

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Data availability

The presented data will be submitted to the BonaRes data repository (https://datenzentrum.bonares.de/data-portal.php).

Code availability

Not applicable.

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5 General conclusions

In this thesis, the response of terrestrial systems to stoichiometric imbalance was investigated. Chapter 2 investigated barley growth in varied degrees of N and P fertilisation. Chapter 3 investigated changes in soil microbial stoichiometry over a soil nutrient gradient starting from extremely low nutrient contents. In both these chapters, it could be shown that responses to stoichiometric imbalance were not gradual and for the first-time reported threshold critical values that determined the response of a system to stoichiometric change. Lastly, in Chapter 4, a potential stoichiometric mechanism for N losses in soil after a freeze thaw event caused by stoichiometric imbalance in the remaining microbial biomass was provided.

In systems where a nutrient is limiting, it is to be expected that when said nutrient is added or becomes more available, there will be a positive response of the variable of interest (Vitousek and Howarth, 1991). We found this to be true in both the barley pot experiment and in the soil chronosequence. However, what was not so predictable was that the effect of nutrient addition was not linear. The strength of the effect of nutrient addition was dependent on how much nutrient was already available and appeared to be governed by a critical value in both investigations. For example, in the chronosequence study (Chapter 3) an increase in SOC by a 0.5% increment had a great effect on microbial biomass carbon (MB-C, Fig. 3.2) and metabolic quotient (qCO_2, Fig. 3.4) in soils with less than 1% SOC. However, above this threshold, a similar incremental increase of 0.5% had a significantly reduced effect. Similarly, in the barley experiment, when total P fertilisation was lower than the critical value of 0.25 mmol, an increase in N of 1.9 mmol had no effect on biomass, yet when P was high, the same incremental increase in N had a huge effect on biomass (Fig. 2.2).

These results can be interpreted such that the response to changes in (or alleviation of) stoichiometric imbalance was dependent on the nutrient limitation status. This was well exemplified in the barley experiment, as there was a huge difference in response of biomass to N:P ratio between when P was limiting or not limiting growth, despite overlapping N:P ratios (Fig. 2.3). Similarly, in the chronosequence study, we could see that the soils with $< 1\%$ SOC were predominantly C limited as microbial biomass C was strongly correlated with SOC, yet above the 1% SOC, the dependence significantly decreased suggesting a reduced C limitation. Interestingly, we also observed a shift towards increased N limitation above the 1% SOC threshold as C limitation decreased (Fig. 3.2).

In their meta-analysis Sistla et al. (2015) found an effect of nutrient status on stoichiometric flexibility. They examined stoichiometric flexibility due to fertilisation in both aquatic and terrestrial systems and found that stoichiometric flexibility was highest when initial nutrient status was poor, e.g., in nutrient-poor environments and in nutrient limited organisms as defined by high C:nutrient ratios. They hypothesised that systems with low starting C:nutrient ratios were less able to take up more nutrients, which limited flexibility (i.e., closer to nutrient saturation limits), whereas high C:N ratios gave a higher potential for nutrient uptake. Our chronosequence data confirm this effect but further show that low nutrient status also led to disproportionate losses of C through raised maintenance respiration (qCO_2), which was alleviated on crossing the 1% SOC threshold (Fig. 3.4). This confirms observations from Hartman and Richardson (2013) in that microbial C:N and N:P ratios correlated with metabolic quotient.

There are two sides of the coin, on the one hand, when nutrient status is low (high limitation) there is a great potential to shorten C:nutrient ratios via nutrient uptake. Yet, it is also the case that when a system is very limited by a nutrient, this results in a decrease in carbon use-efficiency which leads to low biomass and high proportional losses (e.g., high metabolic quotient) (Sinsabaugh et al., 2013). As also demonstrated in the barley experiment, increased N availability did not translate to increased biomass when P was limiting (Fig. 2.2).

We interpreted the shift in responses of nutrient accrual and metabolic quotient in the chronosequence as shifts between stoichiometric stable states, or equilibria. Alternative states in ecology have been hotly discussed and usually used to understand population dynamics (Beisner et al., 2003; Kéfi et al., 2016; Petraitis, 2013; Schröder et al., 2005), but there is less discussion of alternative stable states in ecological stoichiometry (Andersen et al., 2004). Scheffer et al. (2012) hypothesised that critical transitions (regime shifts) between equilibria are often preluded by increased variation until a tipping point is reached and the system falls into a new stable state, which is something we observed in the chronosequence data (Chapter 3). As well as alternative stable states, alternative transitional states have also been debated, i.e., an intermediatory state undergoing transition to an alternative stable state (Fukami and Nakajima, 2011; Van Geest et al., 2007).

Coming back to the findings of Sistla et al. (2015), in which systems with initial high C:nutrient ratios exhibited high stoichiometric flexibility in response to fertilisation, this flexibility could be explained as the high variation inherent to a transition between stoichiometric states, i.e., from stoichiometrically imbalanced to a more balanced state. Moreover, an investigation into soil microbial C:N:P ratios on a precipitation gradient of the Tibetan Plateau showed marked differences in C:N:P variability between high SOC soils of lower latitude alpine meadows and low SOC soils of higher latitude alpine steppe soils (Chen et al., 2016). The alpine steppe soils had a mean SOC content $< 1\%$ SOC whereas the meadow soils were at least 3 times this. In line with our results, microbial biomass in alpine steppe soils exhibited high C:N and N:P ratios and much greater variation than the lower latitude meadow soils.

Similarly, in Chapter 4 we discussed the impact of a freeze-thaw perturbation on soil microbial biomass. In this case freeze-thaw caused significant decrease in microbial C:N (MB-C:MB-N, Fig. 4.1c) by enrichment of microbial N and disproportionate losses of C (qCO_2, Fig. 4.2b). In a subsequent investigation on the same chronosequence soils, Rosinger and Bonkowski (2021) could show that the largest decreases in microbial N arose from the 2nd freeze-thaw cycle but there was little change in total dissolved nitrogen to reflect this loss, which means that the N lost from biomass was likely transformed to N_2O. Therefore, a temporary enrichment in microbial N 18 hours after thawing, as shown in our results, later lead to large losses of N in subsequent freeze thaw cycles. This demonstrates the idea that the freeze-thaw event created a temporary instable stoichiometric state where the surviving microbial N was enriched, and in order to return to the stable stoichiometric state this required expulsion on N, which became apparent after subsequent freeze-thaw cycles. This gives evidence for a possible mechanism for N_2O emissions after soil freezing. There is already evidence that N_2O emissions after freeze-thaw is microbial derived (King et al., 2021). But our results show for the first time that the first step in this natural phenomenon may be the enrichment of N in the surviving microbial biomass after the first freeze-thaw cycle causing a stoichiometric imbalance.

The barley experiment results can also be explained by considering stable stoichiometric states. The plants were less able to deal with increases in N under low P. Here, it was also shown for the first time that the synergistic effect of N and P addition was only present when P was not limiting. This corresponds with Yan et al. (2015), who found that growth rate of *Arabidopsis thaliana* decreased with leaf N:P ratio when P limited, but increased when N limited.

The upper limits to stoichiometric flexibility are the theoretical limits imposed by physical laws (Sterner and Elser, 2017b), but these are seldomly realised (Varma et al., 1993). Upper stoichiometric thresholds have been considered, such as saturation levels of C and N, to model sequestration of C and N in soils (Sterner and Elser, 2017a). Similarly, eutrophication can be viewed as a regime shift between stable states due to high input of nutrient (Sirota et al., 2013). Yet, our data shows the importance for considering regime shifts that may occur at lower nutrient levels at the points where nutrient limitation is alleviated. A lower critical theshold could indicate the boundary between a stoichiometric inefficient state (when a particular nutrient is limiting) and a more efficient state.

Now that we are aware that these stoichiometric boundaries may exist, it should be considered when analysing and interpreting the stoichiometric data already available. For example, models for the prediction of when microbes may switch from net immobilisation to net mineralisation may not give accurate results when applied to soils that are far from a stoichiometric stable state (Wei et al., 2020). Moreover, it may not be possible to make direct comparisons between soils in studies if said eco-physical states, or distance from stoichiometric equilibrium are not comparable. More work looking at the extremes is necessary to show the boundaries of the observed effects and to find where the assumptions of a model breakdown.

This poses many challenges. If systems which are in an unbalanced state are inherently more variable, this causes more difficulty in decerning the true signal from the noise (Hillebrand et al., 2020). Soils containing $< 810\,\mathrm{mmol\ SOC\ kg^{-1}}$ ($<$ 1% SOC) are underrepresented in available meta-analyses (Cleveland and Liptzin, 2007; Hartman and Richardson, 2013; Xu et al., 2017, 2013), as are nutrient limited systems such as arable land. Also meta-analyses which consider changes in SOC often neglect to give starting and ending SOC levels for comparison (Chen et al., 2020; Guo and Gifford, 2002). Large datasets on systems with low nutrient status are required to overcome this statistical challenge, but this is still lacking.

Further experimental evidence is also required to investigate the role of nutrient status on stoichiometric flexibility and imbalance. Controlled C:nutrient and N:P ratios for different total levels of C and nutrient are required to test these hypotheses further.

5.1 Closing remarks

In this thesis, it was shown that responses of barley and soil microorganisms to changing nutrient availability were determined by nutrient status. We demonstrated in two independent studies that these changes in response were not gradual but were due to a critical level of nutrient availability. From these results, we further hypothesise that critical thresholds between states of stoichiometric stability and instability exist and are dependent on nutrient status of the system. It is not certain that threshold values are present in all systems, but it is still remarkable that critical values emerged in two independent systems as described in this thesis. Furthermore, we propose a mechanism for microbial derived N_2O emissions after freeze-thaw due to temporary stoichiometric imbalance in soil microbial biomass.

These results contribute to a better understanding of stoichiometric transformations in ecology and may lead the way to a more complete understanding of nutrient cycling and nutrient limitations in terrestrial ecosystems.

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I dedicate this work to my mum, Carol Clayton. My achievements in life are largely due to her sacrifices and for that I am eternally grateful. I also dedicate this thesis to my late grandfather, Harold Ormerod, former Head Engineer at Drax Power Station. Whom always had a keen interest in my scientific career, which began as early as my school days with explanations of statistics with many scruffy sketches of gaussian distributions over steaming mugs of tea.

7 Sub-publications and record of achievement

Chapter 2

(1,6,7,8,9) **Clayton** J et al. ”A two-way N×P fertilisation gradient experiment on barley reveals shifts from additive to synergistic N-P interactions at critical P fertilisation level under nutrient deficiency”. Unpublished manuscript.

Chapter 3

(1,3,5,8,9) **Clayton** J et al. (2021). ”Shifts in soil microbial stoichiometry and metabolic quotient provide evidence for a critical tipping point at 1% soil organic carbon in an agricultural post-mining chronosequence”. Biology and Fertility of Soils. DOI: 10.1007/s00374-020-01532-2

Chapter 4

(2,4,6,7,8,11) Rosinger C & **Clayton** J et al. (2022). ”Soil freezing-thawing induces immediate shifts in microbial and resource stoichiometry in Luvisol soils along a postmining agricultural chronosequence in Western Germany”. Geoderma DOI: 10.1016/j.geoderma.2021.115596

(1) The experimental design was planned by Prof. Dr. Michael Bonkowski and post-doc supervisor Dr. Kathleen Lemanski.
(2) The experimental design was planned by the author and Prof. Dr. Michael Bonkowski.
(3) Sampling was carried out by the author
(4) Sampling was planned and carried out by the author
(5) Laboratory work was carried out by the author
(6) The author contributed to the laboratory analysis
(7) Laboratory work was overseen and supervised by the author
(8) Data analysis and statistical modelling was carried out by the author
(9) The Manuscript was written by the author with guidance from Prof. Dr. Michael Bonkowski and Prof. Dr. Vicky M. Temperton.
(10) The manuscript was written by the author with guidance from Prof. Dr. Michael Bonkowski.

(11) The manuscript was co-written with author Dr. Christoph Rosinger with guidance from Prof. Dr. Michael Bonkowski.

8 Declaration for the doctoral thesis

Erklärung zur Dissertation

Gemäß §7 Absatz 8 der Promotionsordnung vom 12.März 2020

”Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation selbstständig und ohne die Benutzung anderer als der angegebenen Hilfsmittel und Literatur angefertigt habe. Alle Stellen, die wörtlich oder sinngemäß aus veröffentlichten und nicht veröffentlichten Werken dem Wortlaut oder dem Sinn nach entnommen wurden, sind als solche kenntlich gemacht. Ich versichere an Eides statt, dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie – abgesehen von unten angegebenen Teilpublikationen und eingebundenen Artikeln und Manuskripten – noch nicht veröffentlicht worden ist sowie, dass ich eine Veröffentlichung der Dissertation vor Abschluss der Promotion nicht ohne Genehmigung des Promotionsausschusses vornehmen werde. Die Bestimmungen dieser Ordnung sind mir bekannt. Darüber hinaus erkläre ich hiermit, dass ich die Ordnung zur Sicherung guter wissenschaftlicher Praxis und zum Umgang mit wissenschaftlichem Fehlverhalten der Universität zu Köln gelesen und sie bei der Durchführung der Dissertation zugrundeliegenden Arbeiten und der schriftlich verfassten Dissertation beachtet habe und verpflichte mich hiermit, die dort genannten Vorgaben bei allen wissenschaftlichen Tätigkeiten zu beachten und umzusetzen. Ich versichere, dass die eingereichte elektronische Fassung der eingereichten Druckfassung vollständig entspricht.”

Teilpublikationen

Clayton J, Lemanski K, Solbach M, Temperton VM, and Bonkowski M. ”Two-way N×P fertilisation experiment on barley reveals shift from additive to synergistic N-P interactions at critical P fertilisation level”. (To be submitted to New Phytologist)

Rosinger C, **Clayton** J, Baron K, and Bonkowski M. ”Soil freezing-thawing induces immediate shifts in microbial and resource stoichiometry in Luvisol soils along a postmining agricultural chronosequence in Western Germany”. Geoderma 408 (2022) 115596

Clayton J, Lemanski K, Bonkowski M. ”Shifts in soil microbial stoichiometry and metabolic quotient provide evidence for a critical tipping point at 1% soil organic carbon in an agricultural post-mining chronosequence”. Biology and Fertility of Soils (2021) 57:435 – 446

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STUDIUM UND SCHULAUSBILDUNG

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PUBLIKATIONEN

Nov 2021	Rosinger C, Clayton J, Baron K, Bonkowski M. „Soil freezing-thawing induces immediate shifts in microbial and resource stoichiometry in Luvisol soils along a postmining agricultural chronosequence in Western Germany". Geoderma (2021). https://doi.org/10.1016/j.geoderma.2021.115596
Jan 2021	Clayton J, Lemanski K, Bonkowski M. „Shifts in soil microbial stoichiometry and metabolic quotient provide evidence for a critical tipping point at 1% soil organic carbon in an agricultural post-mining chronosequence". Biology and Fertility of Soils (2021). https://doi.org/10.1007/s00374-020-01532-2

TAGUNGEN UND BEITRÄGE

Mai 2020	Mündlicher Vortrag (Online webinar): „R for Reproducible Data Analysis" in dem Shortcourse „Handling your data efficiently from planning to reuse". European Geosciences Union Online, 2020. https://www.youtube.com/watch?v=tWn-0riN3oI
Feb 2020	Schriftlicher Inhalt für den Workshop: „R for Bloody Beginners" am BonaRes (Boden als nachhaltige Ressource für die Bioökonomie, BMBF Förderinitiative) Status Seminar, Leipzig 2020
Feb 2019	Poster: „The effect of a fertilisation gradient and arbuscular mychorrhizal fungi on biomass allocation and stoichiometry in barley" am BonaRes (Boden als nachhaltige Ressource für die Bioökonomie, BMBF Förderinitiative) Status Seminar, Leipzig 2019.
Apr 2018	Mündlicher Vortrag: „Changes in Nutrient Content and Stoichiometry in Soil and Soil Microbial Biomass over a 52 year Chronosequence from Post-Mining Arable Land" am European Geosciences Union, Vienna 2018.
Feb 2018	Poster: „Changes in Soil and Microbial Nutrient Content and Stoichiometry over a 52 year Chronosequnece from Post-Mining Arable Land" am BonaRes (Boden als nachhaltige Ressource für die Bioökonomie, BMBF Förderinitiative) International Conference, Berlin 2018.

FORT- UND WEITERBILDUNGEN

Scavetta Academy	Statistical Literacy (2021), Data visualisation in R (2020), R basics (2017)

STIPENDIUM, AUSZEICHNUNGEN, MITGLIEDSCHAFTEN

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SPRACHKENNTNISSE

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Englisch	Muttersprache

Köln, 03.01.2022