Microbial nitrogen and phosphorus mineralization and microbial biomass stoichiometry as dependent on ratios of carbon, nitrogen and phosphorus in soils of temperate forests

Dissertation

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„Man darf nie an die ganze Straße auf einmal denken, verstehst du? Man muss nur an den nächsten Schritt denken, an den nächsten Atemzug, an den nächsten Besenstrich. Und immer wieder nur an den nächsten.
[...]
Dann macht es Freude; das ist wichtig, dann macht man seine Sache gut. Und so soll es sein. Auf einmal merkt man, dass man Schritt für Schritt die ganze Straße gemacht hat. Man hat gar nicht gemerkt wie, und man ist nicht außer Puste. Das ist wichtig.“

Beppo Straßenkehrer in „Momo“
von Michael Ende

Summary

This thesis focuses on the question of how different ratios of carbon (C), nitrogen (N) and phosphorus (P) in soils of temperate forests influence soil microbial C:N:P ratios as well as their net N and P mineralization. This addresses two gaps in the knowledge of the meaning of C:N:P stoichiometry, i.e. the relationship of C, N and P, in soil. Although microbial biomass C:N:P stoichiometry is thought to be globally constrained, it is unknown whether these constraints apply to fertilized soils. Moreover, the research on the relationship of organic layer C:N:P stoichiometry to microbial net N and P mineralization is fragmentary. The influence of organic layer C:N or C:P ratios on net N and P mineralization has most frequently been examined in fresh litter, thus ignoring Oe and Oa horizons of organic layers as well as soil N:P ratios. In the course of studying net mineralization, special attention was paid to net P mineralization. This is related to the current discussion on the impact of continuously increased atmospheric N depositions on the P demand of temperate forests, which sparked great interest in the details of P cycling in temperate forest soils.

Twelve temperate forests in Europe and the Eastern USA were sampled for this thesis. The inclusion of deciduous and coniferous forests with different soil N and P contents ensured variability in soil C:N:P stoichiometry. The constraints of microbial C:N:P stoichiometry were tested with respect to short-term and long-term changes of soil nutrient availability. In a short-term laboratory incubation experiment with full-factorial design, microbial C, N and P were determined after addition of easily available C, N and P to two exemplary soils (nutrient rich vs. poor). Moreover, microbial C, N and P was determined in long-term N fertilization experiments (> 25 years). In both cases, the chloroform-fumigation extraction method was used to measure microbial C, N and P. Net N and P mineralization were determined in different horizons (Oi, Oe, Oa, Oe+Oa) of the organic layers of all studied forests. Net N and P mineralization were derived from the increase of N and P concentrations over time in regularly prepared soil extracts during laboratory incubations (3 months). Net P mineralization was examined in more detail in two experiments. The microbial mineralization of a both C- and P-rich compound was analyzed in a short-term laboratory incubation of soil with either ^{14}C or ^{33}P labeled glucose-6-phosphate. Moreover, phosphatase activity as determined with a fluorogenic substrate was related to net P mineralization in the long-term N fertilization experiments.

The microbial biomass C:N:P stoichiometry of soils exposed to different element inputs was mostly constrained. There was only one case of increased variability of microbial C:N:P stoichiometry in a nutrient-poor B horizon treated with short-term C, N and P amendments. The relationship between soil C:N:P ratios and microbial net N and P mineralization was strong and depended on the decomposition state of organic matter. Net N mineralization occurred below certain threshold soil C:N ratios (28 - 40); in some cases there were also threshold soil N:P ratios (42 - 60). Below the thresholds, net N mineralization increased with decreasing soil C:N and, in a few instances, N:P ratios. Net P mineralization had both threshold soil C:P (1000 - 1400) and N:P ratios (40 - 44) below which it increased with decreasing C:P and N:P ratios. Oi horizons had higher threshold ratios and stronger increases of net N and P mineralization with decreasing C:N, C:P or N:P ratios than Oa horizons. No clear trends were found for the intermediate Oe horizons and long-term N fertilization of forests partly obfuscated stoichiometric relationships. Regarding net P mineralization, soil microorganisms recovered more C than P from glucose-6-phosphate (^{14}C recovery: 28 - 37%, ^{33}P recovery: 1 - 6%) thus P was released into the soil. Phosphatase activity increased (on average +260%) in N-fertilized organic layers, but this did not coincide with increased net P mineralization except in one case.

In conclusion, the constraints of microbial biomass C:N:P stoichiometry were robust to variance in soil C:N:P stoichiometry, whereas microbial net N and P mineralization in soils of temperate forests was largely determined by it. In the future, the occurrence and amount of net N or P mineralization may be assessed by simply determining C:N:P ratios of soils. However, reliable estimates will depend on a larger database than presented here. Especially, more research of coniferous and temperate forests is needed. Moreover, net P mineralization driven by the microbial C demand in temperate forest soils may be a common phenomenon that is beneficial for plant nutrition. The increase of phosphatase activity seems to indicate increased P demand in N-fertilized forests. Under this condition, additionally mineralized P due to the increased phosphatase activity is more likely to be consumed quickly by plants or microorganisms than to accumulate in soil.

Zusammenfassung

Diese Arbeit widmet sich der Frage wie unterschiedliche Verhältnisse von Kohlenstoff (C), Stickstoff (N) und Phosphor (P) in Böden die C:N:P-Verhältnisse der mikrobiellen Biomasse und die Netto-N- und -P-Mineralisierung in Waldböden der gemäßigten Breiten beeinflussen. Mikrobielle C:N:P-Verhältnisse gelten gemeinhin als stabil, aber ob diese Annahme auch bei zusätzlichen C-, N- und P-Einträgen in Böden zutrifft ist noch unklar. Auch die Zusammenhänge von Netto-N- und -P-Mineralisierung und der Stöchiometrie organischen Materials sind nur lückenhaft bekannt. Bis jetzt beschränkt sich die Forschung auf den Einfluss der C:N- und C:P-Verhältnisse frischer Laubstreu auf Netto-N- und -P-Mineralisierung. N:P-Verhältnisse werden in diesen Analysen vernachlässigt, ebenso wie stärker zersetztes organisches Material. Der Netto-P-Mineralisierung wird in dieser Studie besondere Aufmerksamkeit gewidmet, um das Verständnis des P-Kreislaufs zu vertiefen. Dies ist motiviert durch die aktuelle Vermutung, beständig erhöhte atmosphärische N-Deposition erhöhe den P-Bedarf von Waldökosystemen der gemäßigten Breiten.

Für die hier durchgeführten Versuche wurden die Böden von zwölf Waldökosystemen der gemäßigten Breiten Europas und Nordost-Amerikas beprobt. Um ein breites Spektrum der C:N:P-Stöchiometrie von Waldböden abzubilden, wurden sowohl Laub- als auch Nadelwälder mit Böden unterschiedlicher N- und P-Gehalte untersucht. Die Reaktion der mikrobiellen C:N:P-Stöchiometrie auf zusätzliche Stoffeinträge wurde für kurze und lange Zeiträume getestet. Zum einen wurden beispielhaft zwei Mineralböden (nährstoffreich bzw. nährstoffarm) im Rahmen eines Laborversuchs mit voll-faktoriellem Design drei Tage mit hohen Konzentrationen leicht verfügbaren C, N und P inkubiert. Zum anderen wurde die C:N:P-Stöchiometrie der mikrobiellen Biomasse in der organischen Auflage von Langzeit-N-Düngungsexperimenten untersucht. Mikrobieller C, N und P wurde mittels Chloroform-Fumigation und Extraktion bestimmt.

Netto-N- und -P-Mineralisierung wurden in L-, Of- und Oh-Horizonten der organischen Auflagen aller zwölf Wälder bestimmt. Dazu wurden die Böden für drei Monate im Labor inkubiert und in regelmäßigen Intervallen Bodenextrakte hergestellt. Der Anstieg der N- bzw. P-Konzentrationen in diesen Extrakten über den Inkubationszeitraum diente als Berechnungsgrundlage der Netto-N- und -P-Mineralisierung. Weiterhin wurden beispielhaft zwei Prozesse, die zur Netto-P-Mineralisierung in Böden beitragen, besonders berücksichtigt. Dies war zum einen der Abbau einer sowohl C- als auch P-reichen Substanz durch die mikrobielle Biomasse, der in einem Tracerversuch mit ^{14}C- bzw. ^{33}P-gelabeltem Glucose-6-Phosphat untersucht wurde. Darüber hinaus wurde die Netto-P-Mineralisierung in N-gedüngten Waldböden im Zusammenhang mit der Phosphataseaktivität dieser Böden untersucht. Dabei wurde die Phosphatase-Aktivität mittels eines fluorogenen Substrats bestimmt.

Die Experimente ergaben, dass zusätzliche Stoffeinträge auf die C:N:P-Stöchiometrie der mikrobiellen Biomasse kaum Einfluss hatten. Dies traf sowohl für kurzzeitige Veränderungen, als auch für Langzeitversuchen mit N-Düngung zu. Die einzige Ausnahme davon war ein nährstoffarmer B-Horizont, in dem die C:N:P-Stöchiometrie der mikrobiellen Biomasse nach kurzer Inkubation mit hohen Konzentrationen von C, N und P stark variierte. Darüber hinaus war die C:N:P-Stöchiometrie von Böden eng mit der Netto-N- und -P-Mineralisierung verbunden. Netto-N-Mineralisierung trat nur auf, wenn bestimmte C:N-Verhältnisse (28 - 40) unterschritten wurden, in einigen Fällen traf dies auch auf N:P-Verhältnisse zu (42 - 60). Sobald diese Grenzwerte unterschritten wurden, stieg die Netto-N-Mineralisierung mit weiter abnehmenden C:N- und teilweise auch N:P-Verhältnissen an. Netto-P-Mineralisierung trat ebenfalls nur unterhalb bestimmter stöchiometrischer

Verhältnisse innerhalb von Böden auf. Dies galt für C:P-Verhältnisse (1000 - 1400) in gleichem Maße wie für N:P-Verhältnisse (40 - 44). Wurden diese Grenzwerte unterschritten, nahm die Netto-P-Mineralisierung mit sinkenden C:P- bzw. N:P-Verhältnissen stetig zu. Die Zunahme von Netto-N- bzw. Netto-P-Mineralisierung bei abnehmenden C:N-, C:P- und N:P-Verhältnissen war in L-Horizonten stärker als in Oh-Horizonten. In Of-Horizonten trat weder diese Beziehung auf, noch konnten Grenzwerte ermittelt werden, genauso in den meisten N-gedüngten Böden. Die beiden Experimente zur P-Mineralisierung ergaben zum einen, dass Mikroorganismen mehr C als P aus Glucose-6-Phosphat nutzten (^{14}C-Wiederfindung: 28 - 37%, ^{33}P-Wiederfindung: 1 - 6%) und somit P in verfügbarer Form im Boden freigesetzt wurde. Zum anderen konnte festgestellt werden, dass eine erhöhte Phosphatase-Aktivität in N-gedüngten Böden (im Mittel +260%), von einer Ausnahme abgesehen, nicht mit einer Erhöhung der Netto-P-Mineralisierung einherging.

Zusammengefasst war die C:N:P-Stöchiometrie der mikrobiellen Biomasse von Waldböden der gemäßigten Breiten unempfindlich gegenüber Veränderungen der C:N:P-Stöchiometrie des Bodens durch verschiedene Stoffeinträge, während Netto-N- und -P-Mineralisierung stark von der C:N:P-Stöchiometrie des Bodens abhängig waren. Dieser Zusammenhang könnte in Zukunft für die Schätzung der Netto-N- bzw. -P-Mineralisierung eines Bodens anhand seiner C:N:P-Stöchiometrie genutzt werden. Dies setzt allerdings die Erweiterung der vorhandenen Datengrundlage voraus. Weiterhin bleibt zu klären, ob Netto-N- bzw. -P-Mineralisierung in Laub- und Nadelwälder unterhalb der gleichen C:N-, C:P- bzw. N:P-Verhältnisse einsetzt oder ob die Grenzwerte sich unterscheiden. In Bezug auf die Untersuchungen zum P-Kreislauf ergab sich, dass durch mikrobielle Abbauprozesse zur Deckung des mikrobiellen C-Bedarfs angetriebene Netto-P-Mineralisierung vermutlich der Waldvegetation zugutekommt. Im Gegensatz dazu hing die Phosphatase-Aktivität in organischen Auflagen nicht direkt mit der Netto-P-Mineralisierung zusammen. Vermutlich spiegelt die höhere Phosphatase-Aktivität in N-gedüngten Waldböden einen erhöhten pflanzlichen und mikrobiellen P-Bedarf. In diesem Fall wird zusätzlich mineralisierter P schnell immobilisiert und nicht in den Boden freigesetzt.

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1. Synopsis

1.1 Introduction

1.1.1 Stoichiometric theory

Stoichiometry in biological studies focuses on the balance of energy and chemical elements in organisms and ecosystems (Sterner & Elser, 2002) and therefore is a helpful tool to investigate element cycling. The elements carbon (C), nitrogen (N) and phosphorus (P) and their ratios are usually included in this kind of analysis, sometimes in combination with sulfur. The stoichiometric relationship between organisms and their food is of special interest because, most often, consumer organisms (e.g. herbivores, detritivores) have to grow on resources with elemental compositions very different to their biomass element ratios. This is because the elemental composition of plant residues like leaf or root litter differs substantially from microbial stoichiometry. Plant residues are C-rich substances containing comparatively small amounts of N and P, which is e.g. due to the high C content of structural compounds like lignin or cellulose. In contrast, microorganisms have higher N and P contents compared to their C content (Hessen *et al.*, 2013). Organisms differ in their potential to maintain certain elemental ratios. On the one hand, the stoichiometry of an organism can be resource dependent, either being identical to resource stoichiometry or deviating from it by a certain factor. On the other hand, the elemental composition of an organism can be strictly confined to a particular ratio, independently of resource stoichiometry (Sterner & Elser, 2002). The ability to maintain a certain elemental composition is called homeostasis.

The use of stoichiometric theory in biology has gained increasing popularity over the last years. Yet, the roots of stoichiometric analyses in ecology are found in Liebig's Law of the Minimum (Liebig, 1855) and Redfield's prominent studies (1934, 1958) of the C:N:P ratios of plankton and its relationship to the distribution of C, N and P in oceans and the atmosphere. The discovery of a constrained atomic C:N:P ratio of marine plankton (106:16:1), termed "Redfield Ratio" today, largely increased the implementation of stoichiometry in ecological studies. However, due to their origin, stoichiometric studies were long performed mainly in aquatic ecosystems (e.g. Goldman *et al.*, 1979; Elser & Hasset, 1994). Only the development of general stoichiometric models and the spread of the term "Ecological Stoichiometry" (Reiners, 1986; Sterner & Elser, 2002) stimulated the use of stoichiometric approaches in other biologic fields, leading to a broader understanding of global biogeochemical cycles.

1.1.2 Ecological Stoichiometry in terrestrial ecosystems

The discovery of the Redfield Ratio inspired many scientists to search for equally confined stoichiometric ratios in terrestrial ecosystems. By now, intensive analyses of the C:N:P ratios of green foliage and litter (McGroddy *et al.*, 2004), soil organic matter (Tipping *et al.*, 2016) and the soil microbial biomass (Cleveland & Liptzin, 2007; Hartman & Richardson, 2013; Xu *et al.*, 2013) revealed constrained stoichiometric ratios also for terrestrial ecosystems (Table 1). Yet, in spite of these constraints, stoichiometric ratios across terrestrial ecosystems are not invariable. For example, the variance of stoichiometric ratios can depend on the spatial scale. Sometimes stoichiometric ratios become less variable with decreasing scale, for example in the foliage of forest vegetation (McGroddy *et al.*, 2004), whereas in other cases variance decreases with increasing scale. This is the case for soil microbial biomass, which expresses a globally relatively constrained C:N:P stoichiometry (Cleveland & Liptzin, 2007; Xu *et al.*, 2013), but significant stoichiometric variation on the ecosystem scale (Hartman & Richardson,

2013). Other parameters that may induce stoichiometric variance in terrestrial ecosystems, e.g. fertilization, are neglected in research. Especially the soil microbial biomass stoichiometry may be susceptible to changed element inputs. This is indicated by the variance of bacterial C:N:P stoichiometry induced by different element addition treatments (Tezuka, 1990; Chrzanowski & Kyle, 1996; Makino *et al.*, 2003). However, this has not been tested experimentally with soil microbial communities.

Table 1 Mean C:N:P ratios of different compartments of terrestrial ecosystems

Ecosystem compartment	Global C:N:P ratio	Source
Foliage	1334:28:1	McGroddy *et al.*, 2004
Litter	3144:46:1	McGroddy *et al.*, 2004
Soil	186:13:1	Cleveland & Liptzin, 2007
Soil organic matter	52:5:1	Tipping *et al.*, 2016
Soil microbial biomass	60:7:1	Cleveland & Liptzin, 2007
	42:6:1	Xu *et al.*, 2013

There are more examples where stoichiometric analyses broadened the knowledge about terrestrial ecosystems. The resorption of the nutrients N and P by trees before litterfall is clearly reflected in increased C:N and C:P ratios in litter compared to green foliage (McGroddy *et al.*, 2004). Furthermore, the elemental composition of organic matter approximates that of soil microbial biomass, which could either be caused by microorganisms being the source of stable organic matter in soils (Kirkby *et al.*, 2011) or by preferential incorporation of certain decomposition products in organic matter (Tipping *et al.*, 2016). Further, the constrained stoichiometry of soil microbial biomass is reflected in a likewise constrained stoichiometry of hydrolytic exoenzyme activity (e.g. β-glucosidase, chitinase, phosphatase), illustrating boundaries of the influence of C, N or P availability on their acquisition (Sinsabaugh *et al.*, 2008). This selection of insights gained with the help of stoichiometric analyses highlights the utility of the approach in ecological studies. Here, it will mainly be used to shed light onto the resource dependency of microbial biomass C:N:P ratios and net mineralization processes in temperate forest soils.

1.1.3 Net C, N and P mineralization in organic layers of temperate forests

The decomposition of organic matter in forest ecosystems has been studied for decades, and many studies have investigated relations between organic matter stoichiometry and mass loss or net mineralization rates (Bosatta & Staaf, 1982; Hättenschwiler & Jørgensen, 2010; Manzoni *et al.*, 2010; Mooshammer *et al.*, 2012; Carrillo *et al.*, 2016). Net mineralization of C, N and P as terminal steps of organic matter decomposition releases these elements in their plant available forms. It is defined as the gross mineralization rate minus the net immobilization rate of a certain element. Yet, in spite of the many studies focusing on litter decay, our understanding of C, N and P net mineralization rates in organic layers is still incomplete.

On the one hand, this is because most studies consider only the net mineralization rates of one or two elements although strong relationships link the net mineralization of different elements. Net C mineralization is often coupled to net N mineralization because both elements are bonded to each other in many organic compounds, and thus are decomposed together (McGill & Cole, 1981; Parton *et al.*, 2007; Manzoni *et al.*, 2008). There are also hints that net C mineralization can coincide with net P mineralization if P-rich organic compounds are decomposed (Spohn & Kuzyakov, 2013). Further, net N and P mineralization may be

coupled because of N and P co-limitation of the microbial biomass in organic layers (Marklein *et al.*, 2016). These correlations underline that studies on net mineralization in soils will profit by considering more than one element.

On the other hand, most analyses of net mineralization rates are limited to one specific decomposition state of organic matter. For example, many studies on stoichiometry and mineralization concentrate on young litter (e.g. Gosz *et al.*, 1973; Berg & Staaf, 1981; Blair, 1988; Cortez *et al.*, 1996; Aerts, 1997; Berg & Matzner, 1997; Craine *et al.*, 2007; Hättenschwiler & Jørgensen, 2010; Moore *et al.*, 2011; Mooshammer *et al.*, 2012; Brandstätter *et al.*, 2013), whereas organic matter in later stages of decomposition, like in Oe and Oa horizons of organic layers, is less well studied (McClaugherty & Berg, 1987; Berg & Ekbohm, 1991; Berg & Matzner, 1997; Moore *et al.*, 2011). The C, N and P concentrations and the proportions of easily degradable and recalcitrant substances in organic matter strongly influence decomposition and thereby net mineralization. Nutrient-rich litter is known to decompose fast at first (Berg & McClaugherty, 2014), whereas high N concentrations often hinder further decomposition in later stages of organic matter decay (Berg & Ekbohm, 1991; Berg & Matzner, 1997; Berg & McClaugherty, 2014). In addition, high concentrations of easily degradable organic C, like sugars or carbohydrates, result in high decomposition rates, whereas high lignin concentrations usually cause slow decay (Blair, 1988; Scott & Binkley, 1997; Berg & McClaugherty, 2014). Stoichiometric ratios like C:N ratios or, sometimes, lignin:N ratios have also been related to decomposition (Taylor *et al.*, 1989; Cortez *et al.*, 1996; Zhang *et al.*, 2008).

It is assumed that the stoichiometry of organic matter and its divergence from microbial biomass stoichiometry strongly influence rate and occurrence of net mineralization. Microorganisms will only release N and P by net mineralization, if their demand of the respective element is satisfied and they are able to maintain their microbial biomass stoichiometry (Spohn, 2016). The turning point between microbial immobilization and microbial net mineralization of an element is thought to be characterized by certain C:N or C:P ratios of organic matter, called threshold element ratio (Bosatta & Staaf, 1982). Theoretical threshold C:N and C:P ratios for net N and P mineralization have been calculated as 10 - 30 and 80 - 160, respectively (Kaiser *et al.*, 2014; Spohn & Chodak, 2015). However, it is unclear whether these ratios can be derived from the relationship of organic matter stoichiometry and net N and P mineralization. Studies reporting empirical estimates of threshold C:N and C:P ratios are usually litterbag studies. These report the respective C:N or C:P ratio at the onset of net N or P mineralization, but without further investigation of the stoichiometric relationships between soil and net N and P mineralization. Model studies suggest net N and P mineralization in young litter to increase with further decreasing C:N or C:P ratios below threshold element ratios (Manzoni *et al.*, 2008, 2010). However, this has not yet been tested experimentally. Threshold C:N ratios derived from litterbag studies range from 20 to 40 (Gosz *et al.*, 1973; Blair, 1988; Parfitt *et al.*, 1998; Parton *et al.*, 2007; Moore *et al.*, 2011) and threshold C:P ratios from 300 to 1700 (Edmonds, 1980; Blair, 1988; Parfitt *et al.*, 1998; Saggar *et al.*, 1998; Moore *et al.*, 2011). N:P ratios have not been considered in the existing analyses. In addition, most thresholds were derived for fresh leave litter, thus it is not clear whether they also exist in more strongly decomposed organic matter, e.g. of Oa horizons.

1.1.4 The environmental significance of net P mineralization

P-related processes receive special attention in this study because, at present, phosphorus (P) cycling in temperate forest ecosystems is of particular interest for ecological research. Decreasing foliar P concentrations in forests across Europe have frequently been observed during the past decades (Houdijk & Roelofs, 1993; Flückiger & Braun, 1998; Duquesnay *et al.*, 2000; Ilg *et al.*, 2009; Jonard *et al.*, 2009; Prietzel & Stetter, 2010; Marschner *et al.*, 2011; Talkner *et al.*, 2015). Most often, this decrease in foliar P is seen as a consequence of long-lasting high N depositions caused by increased use of industrial fertilizer and fossil fuel burning. While the implementation of air pollution control measures was followed by a decline in N depositions (Xing *et al.*, 2013; Vet *et al.*, 2014; Li *et al.*, 2016), their magnitude is still problematic (Schlesinger, 2009) and likely altered both N and P cycling in temperate forests.

P cycling in forest ecosystems is largely driven by soil microbial activities. The plant available P pool is small and dependent on continuous replenishment because plants only take up inorganic P, i.e. phosphate (PO_4) (Hawkesford *et al.*, 2012). However, PO_4 is prone to be sorbed or precipitated in the mineral soil (Fox *et al.*, 2011) since aluminum and iron oxides, hydroxides and clay minerals have a high affinity to immobilize it (Parfitt & Atkinson, 1976; Lindsay, 1979; Hinsinger, 2001; Pierzynski *et al.*, 2005). Therefore, only small PO_4 concentrations < 1% of total soil P (Pierzinsky, 1991), are present in the soil solution and the continuous renewal of that pool via weathering of primary minerals (Rodríguez & Fraga, 1999), PO_4 desorption by organic acids (Fox, 1995) or mineralization of organic P (Stutter *et al.*, 2012) is crucial for the P nutrition of plants. Microbial activity plays an important role in all of these processes.

Besides being a P source itself, the microbial biomass is a major driver of the mineralization of organically bound P. This is mainly due to the microbial exudation of several types of phosphatase enzymes that are adapted to different substrates and environmental conditions. The most prominent group of phosphatases are phosphomonoesterases, which hydrolyze ester-phosphate bonds, hence releasing PO_4 into the soil (Nannipieri *et al.*, 2011). Phosphomonoesterases are further separated into acid and alkaline phosphatases due to their different pH optima of 4 and 10, respectively (Eivazi & Tabatabai, 1977; Dick *et al.*, 1983). Of these, acid phosphatase is produced by both plants and microorganisms, whereas alkaline phosphatase is only synthesized by microorganisms.

The phosphatase activity strongly depends on N and P concentrations in soils. High N availability, e.g. due to fertilization, often increases phosphatase activity (Marklein & Houlton, 2012). Partly, this is because high N availability facilitates the synthesis of the N-rich enzyme, partly, because high N availability often concurs with increased P demand, e.g. because of increased plant growth. In contrast, high P availability is often associated with decreased phosphatase activity due to a negative feedback of PO_4 on phosphatase production (Spiers & Mcgill, 1979; Olander & Vitousek, 2000). However, a high phosphatase activity must not necessarily translate into high net P mineralization. If there is little P available in a soil, e.g. due to a P-poor parent material or because plants and/or microorganisms have a high P demand and thus immobilize large amounts of P, net P mineralization and phosphatase activity may not be coupled. The net P mineralization rates of organic layers are often calculated from nutrient budgets, measured as an increase of available PO_4 during incubations or via ^{33}P dilution experiments. The amount of net P mineralization in organic layers is variable between ecosystems, for example it was up to 4.1 μg P g^{-1} d^{-1} in oak forests of central Spain (Turrión *et al.*, 2008) and 5.6 kg P ha^{-1} yr^{-1} in a North American hardwood forest (Yanai, 1992). .

1.2. Objectives of the study

This work was driven by the question of how net mineralization processes and microbial C:N:P ratios are influenced by the C:N:P stoichiometry of soils of temperate forest ecosystems. This topic was addressed in three case studies, the first focusing on (1) short-time adjustments of microbial C:N:P ratios to variances in resource stoichiometry and the relationship of net P mineralization and the microbial C demand, the second on (2) the influence of the C:N:P ratios of organic layers on the size and occurrence of microbial net C, N and P mineralization and the third on (3) the influence of long-term changes of soil C:N:P ratios by chronic N fertilization on microbial C:N:P ratios, net N and P mineralization and phosphatase activity.

The main hypotheses of these studies were:
(i) Microbial biomass C:N:P ratios are not affected by resource C:N:P stoichiometry.
(ii) The rate of net C, N and P mineralization is determined by the C:N:P ratios of the organic layer.
(iii) Net N and P mineralization occur only below threshold C:N, C:P and N:P ratios.
(iv) Microorganisms use organic phosphorylated compounds as C source and in doing so mineralize P.
(v) Increased phosphatase activity coincides with increased net P mineralization.

1.3. Materials and methods

1.3.1 Study sites

Net mineralization and microbial stoichiometry were examined in two sets of study sites, one offering variable soil C:N:P stoichiometry due to varying P contents (Table 2), the other due to different N contents (Table 3). Varying P contents were represented by eight German study sites. Six beech forests (*Fagus sylvatica*, Bad Brückenau, Conventwald, Lüss, Mitterfels, Steigerwald, Vessertal), one spruce forest (*Picea abies*, Waldstein) and one pine forest (*Pinus sylvestris*, Geißmann) were included in this site set. Except for Steigerwald, the beech sites represented a geosequence of P stocks decreasing in the order Bad Brückenau > Mitterfels > Vessertal > Conventwald > Lüss (Lang et al., 2017). The three additional forests were included to further broaden the spectrum of organic layer C:N:P stoichiometry. Most sites are situated at intermediate heights of central and southern German mountain ranges, only the site Lüss is located in northern lowlands. The main soil type of all sites was Cambisol, which developed from different parent materials.

The site set representing varying N contents (Table 3) encompassed four forests exposed to experimental long-term N additions (25 to 150 kg N ha-1 yr-1) for more than 25 years. Two of the experimental forests were hardwood forests (Harvard Forest, Bear Brook) dominated by American beech (*Fagus grandifolia*) and maple (*Acer saccharum*, *Acer rubrum*) and situated in the eastern USA. The other two experimental forests were spruce forests (Picea abies) in Denmark (Klosterhede) and Sweden (Gårdsjön). The Scandinavian sites formerly belonged to the NITREX project (Dise & Wright, 1992) that assessed forest development under different N deposition regimes.

Study I and Study II were conducted with samples from the German sites differing in soil C:N:P stoichiometry due to variance in P (Table 2), whereas Study III was conducted with samples of second site set focusing on variance in N (Table 3).

Table 2 Site characteristics of six beech forests and two coniferous forests (Geißmann and Waldstein) in Germany. The beech forests Bad Brückenau, Conventwald, Lüss, Mitterfels and Steigerwald represent a geosequence covering a wide range of total P stocks (Lang *et al.*, 2017).

Site	Geographical position	Elevation [m a.s.l.]	MAT [°C]	MAP [mm]	Dominating tree species	Parent material	Soil type (FAO)	Humus form
Bad Brückenau	N 50°21' E 009°55'	809	5.8	1031	*Fagus sylvatica*	Basalt	Dystric skeletic cambisol	Mull-like moder
Conventwald	N 48°01' E 007°57'	840	6.8	1749	*Fagus sylvatica, Picea abies*	Paragneiss	Hyperdystric skeletic folic cambisol	Mor-like moder
Geißmann	N 49°57' E 011°28'	360	8.4	634	*Pinus sylvestris*	Keuper	Cambisol	Mull
Lüss	N 52°50' E 010°16'	115	8.0	779	*Fagus sylvatica*	Sandy till	Hyperdystric folic cambisol	Mor-like moder
Mitterfels	N 48°58' E 012°52'	1023	4.5	1229	*Fagus sylvatica*	Paragneiss	Hyperdystric chromic folic cambisol	Moder
Steigerwald	N 49°52' E 010°27'	440	7.9	787	*Fagus sylvatica, Quercus robur*	Upper Keuper	Dystric Cambisol	Moder
Vessertal	N 50°36' E 010°46'	810	6.0	1200	*Fagus sylvatica*	Trachyandesite	Hyperdystric skeletic chromic cambisol	Moder
Waldstein	N 50°08' E 011°52'	765-785	5.3	1162	*Picea abies*	Porphyritic granites, phyllites, quartzite	Cambisol	Mor

MAT = mean annual temperature, MAP = mean annual precipitation

Table 3 Site characteristics of two North American hardwood forests (Harvard Forest, Bear Brook) and two Scandinavian coniferous forests (Gårdsjön, Klosterhede), which were exposed to > 25 years of experimental N fertilization.

Site	Geographical position	Elevation [m a.s.l.]	MAT [°C]	MAP [mm]	Dominating tree species	Parent material	Soil type (FAO)	Humus form	N addition [kg N ha^{-1} yr^{-1}]	Begin of N additions
Harvard Forest (USA)	N 42°32' W 072°10'	330	8.5	1240	*Quercus velutina, Quercus rubra*	Sandy till	Cambisol	Moder	+50 and +150 (NH_4NO_3)	1988
Bear Brook (USA)	N 44°52' W 068°06'	300-400	4.9	1400	*Fagus grandifolia, Acer sp.*	Quartzite, gneiss	Podzol	Moder	+25 ($(NH_4)_2SO_4$)	1989
Gårdsjön (Sweden)	N 58° 04' E 012° 01'	135-145	6.4	1100	Picea abies, Pinus sylvestris	Glacial till	Podzol	Mor	+40 (NH4NO3)	1991
Klosterhede (Denmark)	N 56° 29' E 008° 24'	27	9.0	860	Picea abies	Glacial sands	Podzol	Mor	+35 (NH4NO3)	1992

MAT = mean annual temperature, MAP = mean annual precipitation

1.3.2 Soil sampling and sample preparation

For Study I, bulk samples of the A and B horizons of the mineral soil at the sites Bad Brückenau and Lüss (Table 2) were collected in February 2014. At both sites, the organic layer was carefully removed and samples were taken with a shovel. For study II, the organic layer of the complete German site set (Table 2) was sampled in July 2015. The Oi, Oe and Oa horizons were separated with a shovel at four different points in the field. The four replicates per horizon were than combined into one composite sample per organic horizon. For study III, the organic layers of the N fertilization site set (Table 3) were sampled in July 2016 and April 2017 by collaborating researchers from the University of New Hampshire, the IVL Swedish Environmental Research Institute and the University of Copenhagen. At Harvard Forest and Bear Brook, the organic layers were sampled with a 20 x 20 cm frame and divided in the Oi and Oe+Oa horizon in the field. At Klosterhede, the organic layer was sampled by soil coring and at Gårdsjön samples were taken with a shovel and equally separated in Oi and Oe+Oa horizons. Control and fertilization treatments were sampled in six field replicates per treatment. All field moist samples of organic soils were homogenized by hand and stored in plastic bags at 5°C until further use. Moreover, the material of the Oi horizons of the *Fagus sylvatica* forests was cut into 1 - 2 cm^2 pieces. Mineral soil samples were sieved (2 mm) and stored in the same way.

1.3.3 Soil characteristics

Soil water contents and maximum water holding capacities (Naeth *et al.*, 1991) were determined gravimetrically. For the analysis of total C, N and P, subsamples of organic soils were dried at 60°C for 48 h and subsamples of mineral soils at 105°C for 24 h. Dried samples were ground in a ball mill. Soil C and N concentrations were measured with a CN analyzer (Vario MAX, Elementar) and P concentrations were determined with inductively coupled plasma-optical emission spectroscopy (ICP-OES, Vista-Pro radial, Varian) after pressure digestion in concentrated nitric acid (organic soils) or microwave digestion in nitric acid/perchloric acid/hydrochloric acid/hydrogen fluoride. All reported pH values were determined with a gel electrode (WTW) in a 1:5 (w/v) mixture of soil and deionized water.

1.3.4 Soil microbial C, N and P

Soil microbial biomass C, N and P concentrations were determined with the chloroform-fumigation extraction method (Brookes *et al.*, 1984, 1985; Vance *et al.*, 1987). For microbial C and N, an aliquot of soil was extracted with 0.5 M K_2SO_4 with a soil:solution ratio of 1:5 (w/v) as control and a second aliquot was fumigated with $CHCl_3$ for 24 h and extracted similarly. This was similar for microbial P, except soils were extracted with Bray-1 solution (0.03 M NH_4F + 0.025 M HCl, Bray & Kurtz, 1945) in a soil:solution ratio of 1:10 (w/v). Microbial C, N and P were calculated as the difference of C, N and P concentrations in the extracts of fumigated and control soil. The differences were than corrected by the conversion factor 2.22 for microbial C and N and by 2.5 for microbial P (Jenkinson et al., 2004). This method was used to determine the molar microbial C:N:P stoichiometry in the mineral A and B horizons of the sites Bad Brückenau and Lüss (Table 2) and in the Oe+Oa horizon of the long-term N fertilization experiments (Table 3). Further information is given in the methods of Study I.

1.3.5 Addition of available C, N and P to mineral soil

To assess the reaction of microbial C:N:P stoichiometry to short-term variations of C, N and P supplies, 2 mg g^{-1} glucose-C, 0.1 mg g^{-1} NH_4NO_3-N and 0.1 mg g^{-1} KH_2PO_4-P dissolved in 1 ml deionized water were added in a full factorial design to mineral A and B horizons of the sites Bad Brückenau and Lüss (Table 2). Controls received 1 ml of deionized water. The respective amendments were added to four replicates of 25 g dry-weight equivalents of soil and incubated for 65 h at 20°C before microbial biomass C, N and P was determined as above. In addition, the composition of bacterial and fungal communities in soils of the C, N and P addition experiment were analyzed with automated ribosomal intergenic spacer analysis (ARISA, Fisher and Triplett (1999)). For more details, please refer to the methods of Study I.

1.3.6 Tracer experiment with ^{33}P- and ^{14}C-labeled glucose-6-phosphate

The microbial respiration and uptake of C and P was tested with glucose-6-phosphate. 20 kBq ^{14}C-glucose-6-phosphate or 80 kBq ^{33}P-glucose-6-phosphate dissolved in 1 ml deionized water were added to 10 g dry-weight equivalents of mineral A and B horizons of the sites Bad Brückenau and Lüss (Table 2). Microbial C and P were determined via chloroform-fumigation extractions as above and the ^{33}P and ^{14}C activity in the extracts was determined with a scintillation counter. Moreover, the ^{14}C activity of respired CO_2, which was trapped in 2 ml of 1 M NaOH, was measured with a scintillation counter at five time points until 164 h after addition of the labeled substrates. The method is further described in Study I.

1.3.7 Net C, N and P mineralization

The net C mineralization was determined for the Oi, Oe and Oa horizons of the first set of sites (Table 2). The respiration of 0.5 g (Oi horizon), 1.0 g (Oe horizon) and 2.0 g soil (Oa horizon) was measured with a respirometer at 15°C for 50 days (Respicond V, Nordgren Innovations), which followed the increase of CO_2 concentrations in alkaline traps containing 10 ml 0.6 M KOH. The net C mineralization rate was calculated as the slope of the regression of the increase of CO_2-C concentrations with time.

For the determination of the net N and P mineralization, Material of the Oi, Oe and Oa horizons of the first set of sites (Table 2) and of the Oe+Oa horizons of the second set (Table 3) was incubated at 15°C for 50 or 76 days, respectively. Weekly to biweekly, 5 g dry-weight equivalent aliquots of soil were extracted with deionized water in a soil:solution ratio of 1:20. Ammonium (NH_4), nitrate (NO_3) and PO_4 concentrations were measured colorimetrically. NH_4 and NO_3 concentrations were determined by flow-injection analysis (FIA-Lab, MLE) and PO_4 concentrations either by a fluorescence spectrometer (UV 1800, Shimadzu) or by a microplate reader (M200 Pro, Tecan). Net P mineralization rates for the long-term N fertilization sites (Table 3) could not be determined from water extracts because increases in water-extracted PO_4-P over time were too small to be distinguished from noise. Therefore, two-point measurements of PO_4 in Bray-1 extracts (0.03 M NH_4F + 0.025 M HCl, soil:solution ratio 1:10 (w/v)) were used instead. Calculations were performed equally to net C mineralization. More details on the determination of net C, N and P mineralization can be found in the methods of Study II and Study III.

1.3.8 Exoenzyme activity

The potential activities of the exoenzymes cellobiohydrolase, chitinase and phosphatase were measured in the Oe+Oa horizons of the long-term N fertilization experiments (Table 3) using the fluorogenic substrates 4-methylumbelliferyl-β-D-cellobioside, 4-methylumbellifery-N-acetyl-β-D-glucosaminide and 4-methylumbelliferyl-phosphate (Marx et al., 2001; German et al., 2011). Fluorescence was corrected for the background fluorescence of soil and substrate as well as for fluoresence quenching by the soil (German et al., 2011). The ratios cellobiohydrolase-to-chitinase, cellobiohydrolase-to-phosphatase and chitinase-to-phosphatase were calculated according to Sinsabaugh et al (2008). The details of the method are reported in Study III.

1.3.9 Statistics

The threshold of significance was defined as $p < 0.05$ in all statistical analyses. Differences between three or more groups were tested by analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. Kruskal-Wallis tests followed by Dunn's multiple comparisons tests (Pohlert, 2014) were used if ANOVA assumptions were violated. This was tested with Shapiro-Wilk's normality test and the Levene's test for homogeneity of variance of the data. Differences between pairs were tested by t-tests or, if assumptions were not met, by Wilcoxon rank sum tests.

All correlation analyses were Spearman rank analyses to prevent interference of not-normally distributed data with the analysis. Linear regression analyses were followed by the analysis of their residuals for normal distribution and homoscedasticity. If least-squares regression was inapt, robust linear regressions (Yohai, 1987; Koller & Stahel, 2011) were performed. All statistical analyses were conducted in R (R Core Team, 2015), and details of the statistical analyses can be found in the "Statistics" section in the methods of each study.

1.4. Results and discussion of key findings

1.4.1 The influence of different element inputs on microbial biomass stoichiometry

The influence of long-term N fertilization on microbial stoichiometry

Microbial C:N, C:P and N:P ratios in the organic layers of temperate forests did not change due to more than 25 years of N fertilization (Table 4, site details see Table 3). Overall, microbial C:N ratios ranged from 5.9 to 8.0, microbial C:P ratios from 12.6 to 25.6 and microbial N:P ratios from 2.1 to 4.2. There were significantly positive relationships between microbial C and N ($R^2 = 0.86$, $p < 0.001$), microbial C and P ($R^2 = 0.44$, $p < 0.001$) as well as microbial N and P ($R^2 = 0.49$, $p < 0.001$).

Table 4 Microbial C:N, C:P and N:P ratios in the Oe+Oa horizons of long-term N fertilization experiments at the deciduous sites Harvard Forest and Bear Brook and the coniferous sites Klosterhede and Gårdsjön. Values are given as mean with standard deviation (n = 6).

Site	Treatment	C:N ratio	C:P ratio	N:P ratio
Harvard Forest	Control	7.3 ± 1.1	16.8 ± 2.2	2.3 ± 0.3
	N50	6.2 ± 0.4	15.1 ± 1.9	2.3 ± 0.5
	N150	6.6 ± 0.6	19.5 ± 5.9	2.9 ± 0.8
Bear Brook	Control	6.0 ± 0.4	12.6 ± 2.3	2.1 ± 0.5
	N	6.0 ± 0.5	15.7 ± 1.3	2.8 ± 0.4
Klosterhede	Control	8.0 ± 1.7	25.6 ± 2.7	3.3 ± 1.0
	N	7.9 ± 0.8	22.9 ± 3.1	2.9 ± 0.4
Gårdsjön	Control	5.9 ± 0.6	24.4 ± 5.2	4.2 ± 1.2
	N	6.2 ± 0.8	20.9 ± 2.7	3.4 ± 0.5

The influence of short-term variability of element inputs on microbial stoichiometry

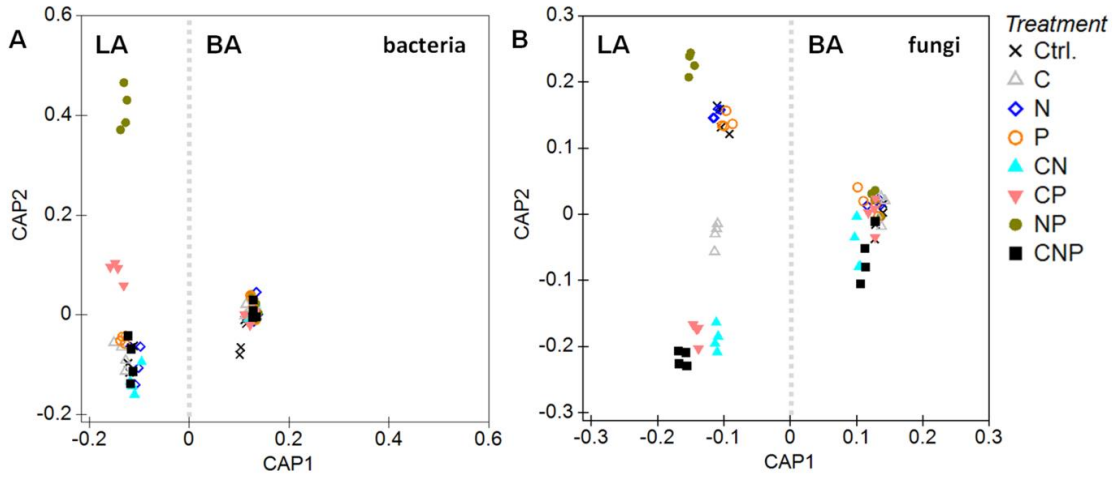
The microbial biomass C:N:P stoichiometry was barely altered by increased availability of C, N and/or P in soil, except in a nutrient-poor B horizon (Table 5, site details see Table 2). In both the A and B horizon of the nutrient-rich site Bad Brückenau, microbial C:N:P ratios were barely affected by the treatments. Significant variation occurred only in one case due to C amendment. Equally minor changes occurred in the nutrient-poor A horizon of the site Lüss due to C addition, both single or combined with N. In the B horizon of the site Lüss, microbial C:P increased significantly due to C addition (+540%, $p < 0.05$). Further, the microbial C:N ratios did not significantly differ from each other due to different C, N and/or P additions ($p > 0.05$). However, N addition clearly conincided with an increase in microbial N to a detectable level. The microbial N:P ratios in treatments including P addition were significantly lower than in treatments without P addition (-80%, $p < 0.001$). The microbial C, N and P concentrations used for the calculation of microbial C:N:P ratios are presented in Study I (Figures 1 - 3).

Table 5 Microbial C:N, C:P and N:P ratios in the A and B horizons of the sites Bad Brückenau (BA, BB) and Lüss (LA, LB) as respondent to full factorial additions of labile C, N and P. Values are given as mean with standard deviation (n = 4), significant differences are marked by asterisks. Levels of significance were * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. See also Study I.

Soil	Treatment	C:N ratio	C:P ratio	N:P ratio	Soil	Treatment	C:N ratio	C:P ratio	N:P ratio
BA	Control	4.7 ± 1.0	26.1 ± 9.6	5.4 ± 1.0	LA	Control	7.8 ± 0.2	31.7 ± 2.3	4.1 ± 0.2
	C	4.5 ± 1.1	26.5 ± 7.7	5.9 ± 0.5		C	47.1 ± 20.1***	94.3 ± 26.6*	2.3 ± 1.1
	N	6.3 ± 1.7	30.1 ± 8.3	5.1 ± 1.7		N	5.4 ± 0.5	39.0 ± 0.8	7.3 ± 0.8
	P	7.5 ± 1.9	36.5 ± 19.2	4.8 ± 2.2		P	7.6 ± 0.6	40.6 ± 20.4	5.3 ± 2.4
	CN	8.2 ± 0.6	43.2 ± 7.7	5.3 ± 1.1		CN	25.4 ± 6.3	178.3 ± 46.0***	7.1 ± 1.1
	CP	7.4 ± 0.2	38.8 ± 7.4	5.3 ± 1.1		CP	8.9 ± 1.6	33.7 ± 10.5	4.0 ± 1.9
	NP	8.1 ± 0.6	47.9 ± 23.5	5.8 ± 2.7		NP	4.5 ± 1.3	8.6 ± 5.8	1.9 ± 1.1
	CNP	9.3 ± 3.7*	53.6 ± 1.3	6.5 ± 2.6		CNP	11.6 ± 2.9	41.0 ± 40.5	3.5 ± 3.1
BB	Control	7.5 ± 0.1	18.0 ± 7.2	2.4 ± 0.9	LB	Control	-	79.3 ± 6.3	-
	C	5.6 ± 0.4	45.4 ± 0.0	8.1 ± 0.6*		C	-	510.9 ± 292.3*	-
	N	8.0 ± 1.7	7.9 ± 2.2	1.0 ± 0.1		N	12.0 ± 8.5	89.2 ± 9.6	10.8 ± 6.6
	P	8.2 ± 0.9	12.1 ± 0.8	1.5 ± 0.1		P	86.1 ± 72.9	6.9 ± 4.3	0.2 ± 0.2
	CN	8.9 ± 1.6	6.9 ± 3.4	0.8 ± 0.4		CN	32.9 ± 18.1	425.0 ± 372.2	11.8 ± 9.2
	CP	11.7 ± 1.6	10.7 ± 2.3	0.9 ± 0.2		CP	-	471.8 ± 131.2	-
	NP	10.3 ± 4.8	4.8 ± 1.2	0.7 ± 0.6		NP	8.2 ± 4.8	5.9 ± 0.8	1.0 ± 0.7
	CNP	8.9 ± 1.3	10.3 ± 2.2	1.2 ± 0.4		CNP	28.2 ± 8.2	110.5 ± 49.4	4.4 ± 2.5

The variations in microbial C:N:P ratios due to C, N and/or P additions suggested shifts in the microbial community compositions. An automated intergenic spacer analysis (ARISA) of the A horizons of Bad Brückenau and Lüss showed both site specific bacterial as well as fungal communities (Figure 1). Moreover, bacterial and fungal communities in soil LA differed depending on which elements were added in the experiment. The bacterial community composition was affected by CP and NP additions, and four different fungal communities could be distinguished after C, N and P addition. The first community included the control and the samples receiving labile N or P, the second one emerged due to NP addition, the third community formed due to CN, CP and CNP additions, and the fourth one exclusively occurred in samples with C addition.

Figure 1 Canonical analyses of principal components of the bacterial (A, mis-classification error = 67.2%) and



fungal (B, mis-classification error = 39.1%) communities of the soils BA and LA after addition of labile carbon (C), nitrogen (N) and phosphorus (P) in a full factorial design. Microbial communities were characterized by automated ribosomal intergenic spacer analysis (ARISA). See also Study I.

The small variability of microbial C:N:P stoichiometry and the significant correlations between microbial C, N and P despite large changes in element inputs is in accordance with the presumption of a globally constrained microbial biomass stoichiometry (Cleveland & Liptzin, 2007; Sistla & Schimel, 2012; Hartman & Richardson, 2013; Xu *et al.*, 2013; Li *et al.*, 2014). Neither long-term changes due to element inputs, for example decreased C:N and increased N:P ratios in N-fertilized soils, nor short-term alterations of available C, N and P in soil were able to remove these constraints. Very nutrient-poor soils, like the B horizon of the site Lüss, may be an exception to this finding.

The microbial C:N:P ratios reported here were largely comparable to the estimates of a global average of microbial C:N:P stoichiometry. The microbial C:N ratios matched global estimates well, whereas the presented microbial C:P and N:P ratios were smaller (Cleveland & Liptzin, 2007; Xu *et al.*, 2013). The soil microbial C:N:P stoichiometry of Bad Brückenau and Lüss was overall comparable to other ratios published for these sites (Lang *et al.*, 2017; Zederer *et al.*, 2017). Differences in microbial C:N:P stoichiometry on smaller scales, e.g. between ecosystems or soil depths, are frequent despite the overall constraints of microbial stoichiometry (Hartman & Richardson, 2013; Xu *et al.*, 2013).

Although the microbial biomass C:N:P stoichiometry appears to be largely constrained, it is not strictly homeostatic on the short-term, as shown by the significant variation of microbial C:N:P ratios due to the additions of labile C, N and/or P to a nutrient-poor soil (Table 5). This implies that stoichiometric plasticity might be higher if microorganisms experience stress, e.g. due to a lack of resources. The variability in microbial C:N:P ratios could be caused by shifts in the soil microbial community due to changed element inputs (Figure 1). Other studies also report shifts in microbial community composition after additions of C, N or P to forest and grassland soils (Allison *et al.*, 2007; Rooney & Clipson, 2009). The observed shifts in microbial communities cannot be further characterized here because the ARISA analysis does not allow for the identification of species. However, it is likely that in the soil from Lüss a community dominated by oligotrophic bacteria was replaced by a copiotrophic-dominated community. Fierer *et al.* (2007) described copiotrophic organisms as efficient in using high C concentrations, whereas oligotrophic species are specialized in limited C and nutrient supplies. C additions, which were followed by the strongest shifts in microbial communities, could have enabled copiotrophic organism groups to outcompete the previously dominant oligotrophic organisms.

Moreover, changes of microbial C:N:P ratios due to increased availability of C, N and/or P could be caused by excess uptake and storage of C, N and P. The storage of C, N or P in soil microorganisms could also influence microbial C:N:P ratios. C can be stored by bacteria in the forms of glycogen, starch and lipids (Wilkinson, 1963; Wilson *et al.*, 2010) up to an amount of 20 - 40% of bacterial dry weight, as determined under laboratory conditions (Wilkinson, 1963). Thus, soil microorganisms could have used the added glucose to build up C storage molecules in treatments that included C addition. P storage molecules can amount to 10 - 20% dry weight in yeast cells (Kornberg, 1995) and > 10% dry weight in some bacteria (Deinema *et al.*, 1985). In addition, high P availability was found to cause non-homeostatic behavior in microorganisms (Scott *et al.*, 2012). Both may account for changes in microbial P concentrations after P addition. However, microorganisms were probably unable to store large amounts of excess N in their cells because there is no known N storage form (Banham & Whatley, 1991; Mooshammer *et al.*, 2014).

Taken together, microbial C:N:P stoichiometry appears to be well constrained, even if confronted with massive element inputs both due to long-term N fertilization and short-term additions of available C, N and/or P. Moreover, it is very likely that the variability of microbial stoichiometry induced by C, N and/or P additions to nutrient-poor soil do not represent permanent changes. This is strongly suggested by the invariability of microbial stoichiometry in organic layers exposed to > 25 years of N fertilization.

1.4.2 Microbial net C, N and P mineralization as dependent on the C:N:P ratios of organic layers

Net C, N and P mineralization in natural and N-fertilized organic layers

In the organic layers of unfertilized German forests (site details see Table 2), net C, N and P mineralization rates decreased from Oi to Oa horizons and always followed the order net C mineralization > net N mineralization > net P mineralization (Figure 2). Net C mineralization ranged from 5.0 ± 0.8 to 128.2 ± 5.0 μmol C g^{-1} d^{-1} and net N and P mineralization ranged from 0 to 1.58 ± 0.51 μmol N g^{-1} d^{-1} and 0.49 ± 0.01 μmol P g^{-1} d^{-1}, respectively. The net N and P mineralization in coniferous Oi horizons were > 90% smaller than in beech Oi horizons ($p < 0.05$). In Oe and Oa horizons, net N and P mineralization did not differ significantly between forest types. Net C mineralization in coniferous Oi horizons was on average 65% higher than in beech Oi horizons ($p < 0.001$), whereas it was significantly lower in coniferous than in beech Oe and Oa horizons (Oe: -40%, Oe: -65%, $p < 0.05$; Figure 2, a+b). In the Oe and Oa horizons, net N and P mineralization did not differ between forest types. The net N and P mineralization in the Oe+Oa horizons of forests exposed to long-term N fertilization (site details see Table 3) were higher in deciduous than in coniferous organic layers. Net N mineralization ranged from 0.09 to 0.56 μmol N g^{-1} d^{-1} at the deciduous sites (Harvard Forest, Bear Brook) and between 0.02 and 0.05 μmol N g^{-1} d^{-1} at coniferous sites (Klosterhede, Gårdsjön; Figure 3). Net P mineralization was between 2.6 and 12.7 nmol P g^{-1} d^{-1} in deciduous Oe+Oa horizons and 0 and 0.4 nmol P g^{-1} d^{-1} in coniferous Oe+Oa horizons (Figure 4). Net N and P mineralization were significantly higher in deciduous than in coniferous forests ($p < 0.001$) and were only affected significantly by N fertilization in deciduous forests. At Harvard Forest, net N mineralization was unaffected in the N50 treatment and significantly increased in the N150 treatment (+290%, $p < 0.001$). At Bear Brook, net N mineralization increased similarly (+210%, $p < 0.01$). Net P mineralization only increased significantly due to N fertilization at Bear Brook (+400%, $p < 0.05$), whereas it significantly decreased in the N50 treatment at Harvard Forest (-60%, $p < 0.001$). The net N and P mineralization of both coniferous sites did not react to N fertilization.

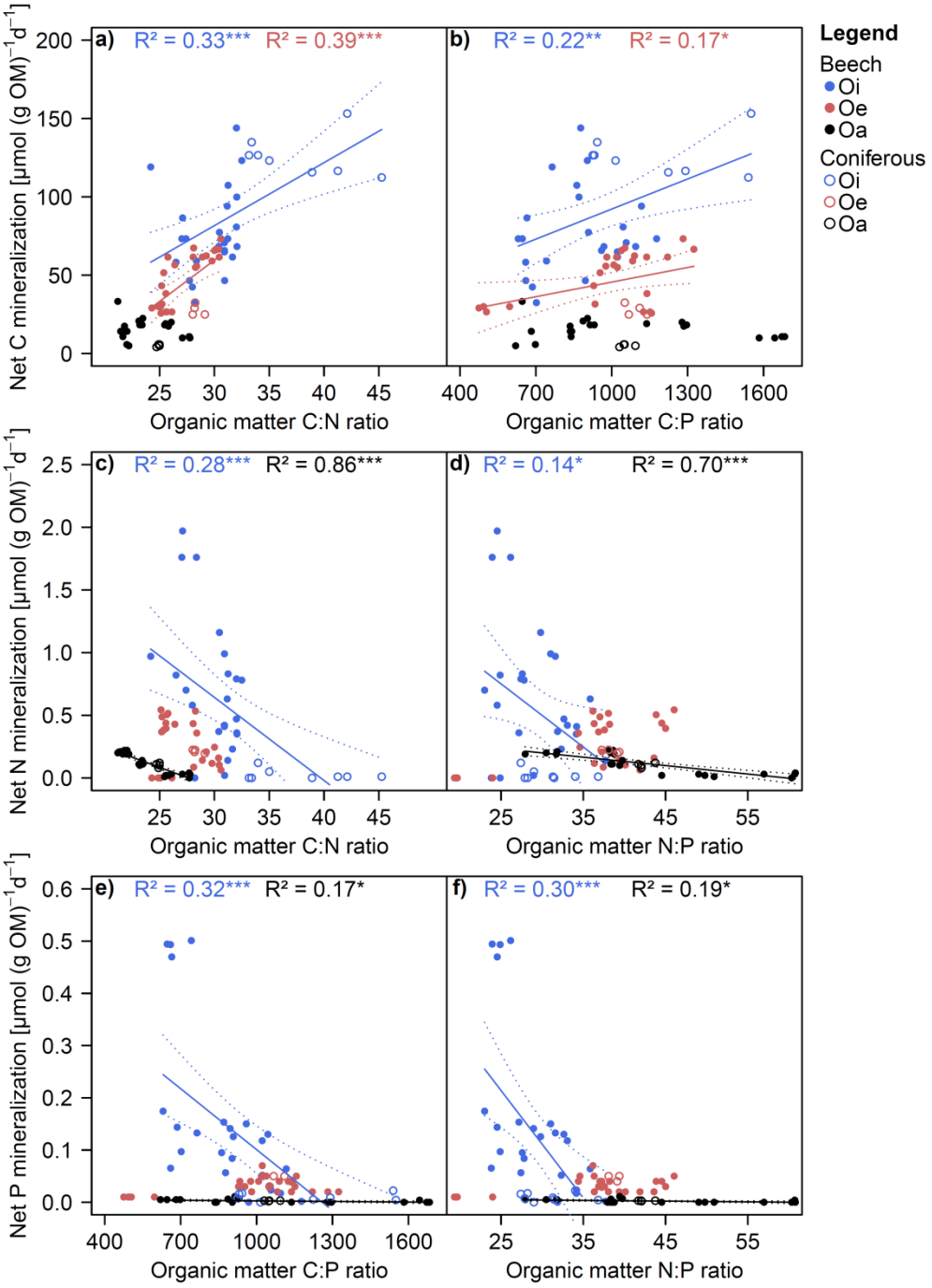


Figure 2 Relationship of net C mineralization rates and C:N and C:P ratios of organic matter (a+b), net N mineralization rates and C:N and N:P ratios of organic matter (c+d) and net P mineralization rates and C:P and N:P ratios of organic matter (e+f). Beech and coniferous samples were combined in regression analyses. Only significant linear regressions are presented with R^2 and 95% confidence intervals in the color corresponding to the respective horizon. Levels of significance were * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. See also Study II.

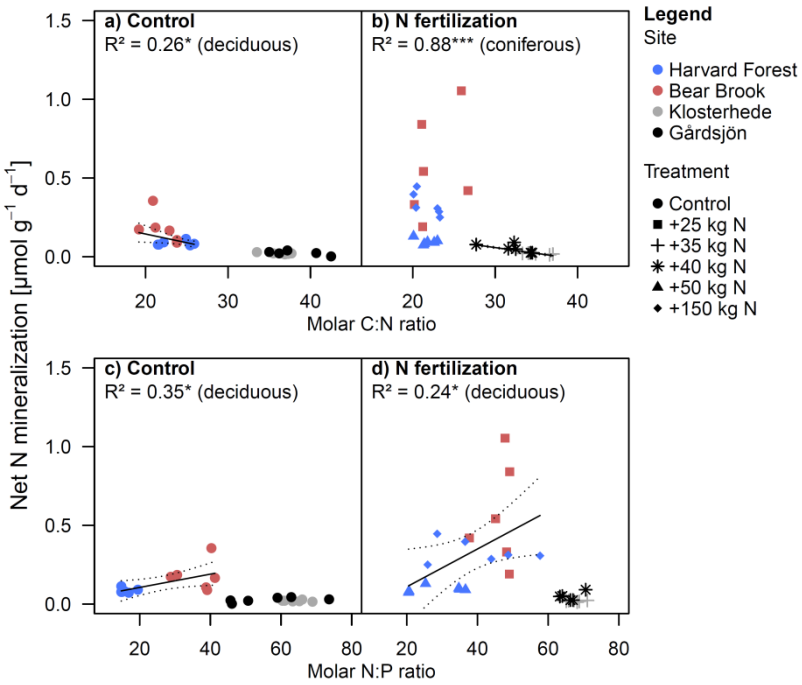


Figure 3 Relationship of net N mineralization and C:N (a+b) and N:P ratios (c+d) of organic layers of the controls and N fertilization treatments of four long-term N fertilization experiments. Different sites are indicated by color (blue: Harvard Forest, red: Bear Brook, grey: Klosterhede, black: Gårdsjön), treatments by symbol (point: control, square: +25 kg N, plus: +35 kg N, star: +40 kg N, triangle: +50 kg N, diamond: +150 kg N). Regressions were calculated separately for deciduous (Harvard Forest, Bear Brook) and coniferous sites (Klosterhede, Gårdsjön). Only significant linear regressions are presented with R^2 and 95% confidence intervals. Levels of significance were * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

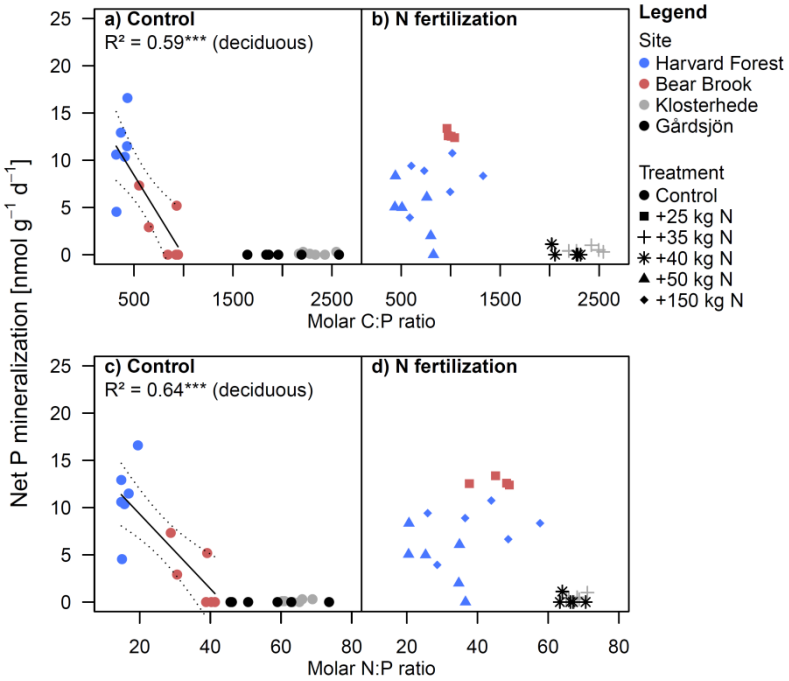


Figure 4 Relationship of net P mineralization and C:P (a+b) and N:P ratios (c, d) of organic layers of the controls and N fertilization treatments of four long-term N fertilization experiments. Different sites are indicated by color (blue: Harvard Forest, red: Bear Brook, grey: Klosterhede, black: Gårdsjön), treatments by symbol (point: control, square: +25 kg N, plus: +35 kg N, star: +40 kg N, triangle: +50 kg N, diamond: +150 kg N). Regressions were calculated separately for deciduous (Harvard Forest, Bear Brook) and coniferous

sites (Klosterhede, Gårdsjön). Only significant linear regressions are presented with R^2 and 95% confidence intervals Levels of significance were * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Relationships between net C, N and P mineralization

The existence and strength of correlations between net C,N and P mineralization rates differed between organic horizons (Table 6). Net C mineralization, which was only considered at the German sites (details see Table 2), was significantly positively correlated with both net N and P mineralization ($p < 0.05$), if organic layers were considered in total. There were no significant correlations in specific organic horizons except a negative relationship between net C and P mineralization in Oi horizons. Net N and P mineralization were strongly positively correlated, if organic layers were considered in total. Moreover, there were positive correlations in Oi and Oe horizons ($r > 0.60$, $p < 0.001$) and in N-fertilized Oe+Oa horizons ($r = 0.74$, $p < 0.001$).

Table 6 Spearman rank correlation coefficients of the relations between net C, N and P mineralization (Cmin, Nmin, Pmin) in the total organic layer (Oi+Oe+Oa horizons combined in one dataset) and each separate horizon (Oi, Oe, Oa) of the German study sites as well as in untreated (Ctr.) and N-fertilized (Fert.) Oe+Oa horizons of the N-fertilization experiments. Significant correlations are marked by asterisks, levels of significance were * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. See also Study II.

Organic horizon		Cmin	Nmin
Study II			
Oi+Oe+Oa	Nmin	0.27*	--
	Pmin	0.50***	0.64***
Oi	Nmin	-0.15	--
	Pmin	-0.47**	0.68***
Oe	Nmin	-0.11	--
	Pmin	-0.16	0.72***
Oa	Nmin	-0.05	--
	Pmin	-0.13	0.31
Study III			
Oe+Oa, Ctr.	Nmin	--	--
	Pmin	--	0.38
Oe+Oa, Fert.	Nmin	--	--
	Pmin	--	0.74***

The close relation of net N and P mineralization (Table 6) is in accordance with the finding of a recent meta-analysis (Marklein *et al.*, 2016). It is likely a result of the connection of N and P mineralization in microbial organic matter decomposition and immobilization of N and P for the build-up of biomass (anabolism), whereas C mineralization is part of the energy metabolism of microorganisms (catabolism). The tight relationship between net N and P mineralization is again contradicting the model of McGill and Cole (1981), which states that P mineralization is separated from both C and N mineralization because it is regulated only by microbial P demand. The mineral-soil-derived model may not be appropriate for processes in organic layers, for example because the availability of labile C in fresh organic matter in Oi horizons exceeds the availability of the nutrients N and P by far. The conditions in the Oa horizons likely still differ strongly from mineral soil, hence the expected correlation between net C and N mineralization was still missing, whereas the relationship between net N and P mineralization seems to fade with organic layer depth. In N-fertilized Oe+Oa horizons however, net N and P mineralization were strongly positively related, which might be explained by a facilitation of enzyme synthesis due to high inputs of labile N (Olander & Vitousek, 2000; Allison & Vitousek, 2005) that enhanced both net N and P mineralization.

Relationships between net C, N and P mineralization and organic layer C:N:P ratios

Net C, N and P mineralization rates and the organic layer stoichiometry were closely related. At the German study sites, net C mineralization increased significantly with increasing C:N and C:P ratios in the Oi and Oe horizons (Figure 2, a+b). Both the highest C mineralization rates and the highest C:N and C:P ratios occurred in coniferous organic layers. Net N mineralization was related to the C:N and N:P ratios of Oi and Oa horizons (Figure 2, c+d). Threshold C:N ratios, above which net N mineralization ceased, were 40 (Oi) and 28 (Oa). Further, the threshold N:P ratios for net N mineralization were 42 (Oi) and 60 (Oa). The relationship between C:N and N:P ratios of organic matter and net N mineralization was stronger in the Oa horizons ($R^2 \geq 0.70$) than in the Oi horizons ($R^2 < 0.30$) in both cases. Net P mineralization only decreased significantly with increasing C:P and N:P ratios of Oi horizons ($p < 0.001$, Figure 2, e+f). That resulted in a threshold C:P ratio for net P mineralization of about 1400, and a threshold N:P ratio of 40.

At long-term N fertilization experiments, net N and P mineralization significantly differed between deciduous and coniferous Oe+Oa horizons($p < 0.001$). Net N mineralization decreased significantly with the C:N ratios of Oe+Oa horizons in non-fertilized deciduous forests and N-fertilized coniferous forests, resulting in threshold C:N ratios of 33 and 38, respectively (Figure 3, a+b). However, there were no threshold N:P ratios for net N mineralization. In the coniferous forests, there was no significant relationship between net N mineralization and N:P ratios of Oe+Oa horizons. In deciduous forests, there was a significantly positive relationship. Net P mineralization was related significantly negatively to organic layer C:P and N:P ratios in non-fertilized deciduous forests ($p < 0.001$, Figure 4). The threshold C:P ratio was about 1000 and the threshold N:P ratio was 44.

The positive relationship between net C mineralization and C:N as well as C:P ratios of organic layers is well known (Taylor et al., 1989; Ohtonen, 1994; Gödde et al., 1996; Saggar et al., 1998; Michel & Matzner, 2002; Spohn, 2015; Spohn & Chodak, 2015). This relationship likely results from metabolic adjustments of soil microorganisms in environments providing high C, but low N and P concentrations. Microorganisms could either increase respiration above their actual energy need, thus expending excess C (overflow respiration; Russell & Cook, 1995; Schimel & Weintraub, 2003; Manzoni et al., 2008; Sinsabaugh et al., 2013), or they could increase their respiration to gain energy for the acquisition of nutrients from recalcitrant substrates (nutrient mining; Moorhead & Sinsabaugh, 2006; Craine et al., 2007). Both processes are dependent on high concentrations of easily available C as in Oi or Oe horizons. In the Oa horizons, no relationship between C mineralization and organic matter stoichiometry was found. This is likely because high concentrations of recalcitrant substances decreased C mineralization (McClaugherty & Berg, 1987; Berg & Matzner, 1997), and replaced organic matter stoichiometry as the principal rate determining parameter.

Similarly, the amounts of net N and P mineralization were related most strongly to organic layer stoichiometry in Oi horizons (Figure 2). Increases of net N and P mineralization with decreasing C:N or C:P ratios of organic layers as shown here (Figures 2 - 4) have been reported for the leaf litter of different plants (Mafongoya *et al.*, 2000; Parton *et al.*, 2007) and in model studies (Manzoni *et al.*, 2008, 2010). In Oi horizons, low C:N or C:P ratios probably indicate a higher availability of N or P, which is beneficial for microbial nutrition. Thus, as soon as microbial N and P demands are satisfied, increasing availabilities of N or P are likely to be mirrored in equally increased microbial net mineralization. Net P mineralization increased with decreasing N:P ratios, which is most likely a direct effect of increased P availability. In the Oe+Oa horizons of the N-fertilized forests (site details see Table 3), net N mineralization was probably found to decrease with decreasing N:P ratios because this represented decreasing N availability. Interestingly, the relationship was the other way

around in the site set comprised of German study sites (site details see Table 2). There, net N mineralization may have increased with decreasing N:P ratios of organic layers because less N had to be invested in P acquisition due to increased P availability.
In Oa and Oe+Oa horizons, respectively, the relationships between organic layer stoichiometry and microbial net mineralization were weaker than in Oi horizons or did not exist at all (Figures 2 - 4). In the more strongly decomposed Oa horizons, N and P acquisition was probably hard for microorganisms. Easily available sources of N and P are usually depleted; hence the strongly decomposed material mainly consists of recalcitrant compounds. N for example is often incorporated in hardly decomposable lignin (Berg & Matzner, 1997). As a consequence, microorganisms likely need to invest more resources to satisfy their nutrient demand than in Oi horizons, which would reduce the amount of spare N and P released by net mineralization. In soils, where the nutrient demand of microorganisms was still satisfied, this probably led to the smaller increase of net N and P mineralization with decreasing soil C:N, C:P or N:P. Where there was no relationship between organic matter stoichiometry and net N and P mineralization, it indicates that the microbial nutrient demand could no longer be satisfied. The amount of net N and P mineralization still present was likely because the efficiency of microbial nutrient recycling is confined.

The threshold C:N ratios of net N mineralization determined here are in accordance with the published range of threshold C:N ratios (20 - 40), based on litter from different plants (e.g. Gosz *et al.*, 1973; Blair, 1988; Parfitt *et al.*, 1998; Parton *et al.*, 2007; Moore *et al.*, 2011). However, our ratios partly exceeded threshold C:N ratios for net N mineralization derived from theoretical considerations that amount to 10 - 30 (Kaiser *et al.*, 2014; Spohn & Chodak, 2015). As to my knowledge, this is the first study to report threshold N:P ratios of net N and P mineralization. Thus, no comparisons with previous studies were possible.

The presented threshold C:P ratios for net P mineralization belong to the upper range of published threshold C:P ratios for net P mineralization (300 - 1700) (Edmonds, 1980; Blair, 1988; Parfitt *et al.*, 1998; Saggar *et al.*, 1998; Moore *et al.*, 2011). With one exception, the missing increase of net P mineralization with C:P ratios of the Oa horizons was likely because the available P barely exceeded the microbial P demand. Substantial net P mineralization has been found to occur only below organic matter C:P ratios of 100 - 300 (Cheshire & Chapman, 1996) and the lowest C:P ratios in the present study were > 400. These threshold ratios differed between organic layer horizons, thus it seems that they depend on the decomposition state of organic matter. For example, net N mineralization had higher threshold C:N ratios in the Oi than in Oa or Oe+Oa horizons, which was the same for net P mineralization and threshold C:P ratios. Likely, easily available C sources helped microorganisms to acquire both N and P in Oi horizons (Moorhead & Sinsabaugh, 2006; Craine *et al.*, 2007; Spohn, 2015). Therefore, microorganisms could afford to release N and P, even if the total concentrations of these elements were low compared to C concentrations. This was likely no longer the case in Oa horizons because of the absence of easily degradable C and the stronger incorporation of N and P in recalcitrant substances. Thus, lower threshold C:N and C:P ratios for net N and P mineralization represent the more unfavorable living conditions for microorganisms in the lower parts of organic layers.

Coniferous and deciduous organic layers could be integrated in a single analysis of the German study sites, but this was not the case for the N fertilization sites (Figures 2 - 4). Likely, low N and P concentrations in coniferous Oe+Oa horizons, maybe in combination with high recalcitrance, did not allow for microbial N and P demands to be satisfied. If so, microorganisms would be expected to immobilize any additionally available N or P instead. Hence, the low-level net N and P mineralization found in these coniferous organic layers likely represents a minimum of microbial N and P loss that cannot be prevented. The relationships and threshold ratios presented above do not apply under these conditions. These stoichiometric concepts are based on the assumption that the microbial nutrient demand in organic layers is fulfilled. Additionally, excess nutrient availability as caused by N fertilization interfered with the above stoichiometric concepts. High variability, induced both by different N fertilization rates and site-specific reactions of net N and P mineralization to it, obscured the relationship between organic layer stoichiometry and net N and P mineralization.

The stoichiometry of organic layers explains the different reactions of net N mineralization to N fertilization in coniferous and deciduous forests. The C:N and N:P ratios of coniferous organic layers approached or exceeded the threshold C:N and N:P ratios determined here, below which net N mineralization occurs in both control and N-fertilized soil (Figure 4). Other published threshold C:N ratios were exceeded as well (Prescott *et al.*, 2000; Parton *et al.*, 2007). Contrary to this, deciduous organic layers had C:N and N:P ratios below these thresholds, thus the preconditions for net N mineralization were given. N fertilization strongly increased N availability in deciduous organic layers and caused a situation in which microorganisms had more N available than they needed for maintaining their biomass. This allows for microbial releases of inorganic N (Prescott *et al.*, 1992). In coniferous forests however, long-term N fertilization was likely not sufficient to exceed microbial N demand, resulting in similar net N mineralization in control and N-fertilized organic layers. Moreover, the efficiency of exoenzymes may have been reduced in coniferous organic layers, which would also fit the overall increased enzyme activities (Figure 6). This is likely because N fertilization can increase the recalcitrance of organic matter by changing chemical bond structures (Aber *et al.*, 1998; Nave *et al.*, 2009). Thus, N fertilization may have made it hard for microorganisms to cover their N demand, leading to few N release by net N mineralization in coniferous organic layers.

Similarly to the above, there was net P mineralization in deciduous organic layers, whereas it was barely measurable in coniferous organic layers (Figure 5). The different stoichiometry of deciduous and coniferous organic layers also explains the different magnitudes of net P mineralization. Threshold C:P and N:P ratios as determined here or in literature (e.g. Saggar *et al.*, 1998; Moore *et al.*, 2011), below which net P mineralization occurred, were exceeded only by coniferous Oe+Oa horizons. In contrast, the C:P and N:P ratios of deciduous organic layers were below the thresholds, allowing for net P mineralization to occur.

Other than expected, the increased phosphatase activity due to N fertilization was not mirrored in similarly increased net P mineralization rates (Figure 5, b+d, Figure 4 in Study III). The variety of reactions of net P mineralization to high N inputs indicates that the influence of long-term N fertilization on net P mineralization in organic layers may be strongly ecosystem-specific. The translation of increased phosphatase activity into increased net P mineralization only occurs if the microbial P demand is satisfied. Low concentrations of total and available P in coniferous Oe+Oa horizons imply that this may not be the case there (Table 2, Study III). Thus, P mineralized additionally due to the N-induced increased phosphatase activity is likely immobilized by microorganisms instead of being released into

the organic layers. Yet, it is also possible that the increased phosphatase activity was without effect because of a lack of substrates.
In contrast to both coniferous forests, the microbial P demand in deciduous Oe+Oa horizons seemed to be met by the P supply of the organic layers because net P mineralization occurred. Moreover, net P mineralization may have contributed to the significant depletion of the P stocks in the Oe+Oa horizons of both Harvard Forest and Bear Brook (Table 2, Study III) because the microbial release of available P into the soil may have been followed by plant P uptake or P leaching into the mineral soil. In coniferous organic layers, this was likely not the case because microbial P cycling did not release considerable amounts of available P.

1.4.3 Microbial mineralization of ^{14}C and ^{33}P labeled glucose-6-phosphate in mineral soils

The mineralization of both ^{14}C and ^{33}P labeled glucose-6-phosphate was tracked in the A and B horizons of the nutrient-rich site Bad Brückenau and the nutrient-poor site Lüss (site details see Table 2). The sum of ^{14}C recovered in microbial biomass and CO_2 was about 20% higher in soils from Lüss than in the soils from Bad Brückenau ($p < 0.001$, Figure 5). The respiration of > 20% of glucose-6-phosphate derived C during the first 24 h after tracer addition indicated a fast uptake and metabolization of glucose-6-derived C. The ^{33}P recovery in the microbial biomass was on average 11% higher than the ^{14}C recovery in microbial biomass. Compared to the sum of ^{14}C recovered in microbial biomass and in respired CO_2, the microbial ^{33}P recovery was about 5- to 40-times smaller. All major changes in microbial ^{14}C and ^{33}P recovery occurred in the first 66 h after application of the isotopic tracer (Figure 5). There was a higher ^{14}C recovery in CO_2 in the soils from Lüss than in soils from Bad Brückenau. On a net balance, P derived from glucose-6-phosphate was mineralized and left in the soil.

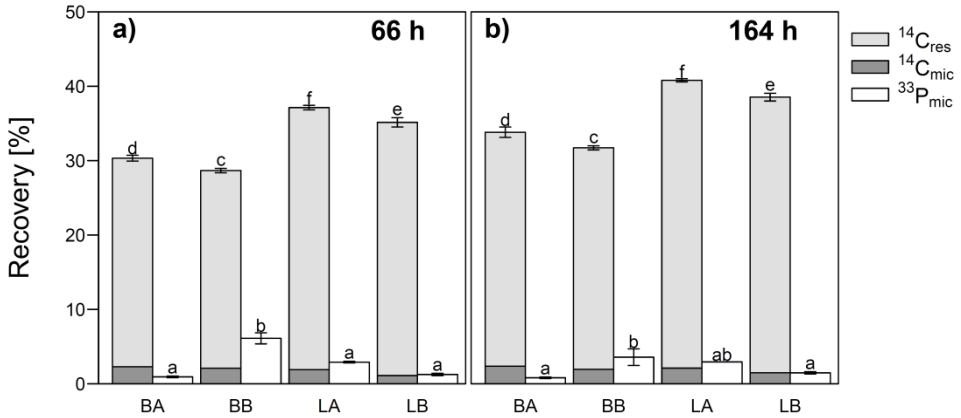


Figure 5 ^{14}C and ^{33}P recovery in the soil microbial biomass ($^{14}C_{mic}$, $^{33}P_{mic}$) and ^{14}C recovery in respired CO_2 ($^{14}C_{res}$) 66 h (a) and 164 h (b) after addition of the ^{14}C and ^{33}P labeled glucose-6-phosphate. Error bars show standard errors (n = 4) and significant differences ($p < 0.05$) are marked by lowercase letters. See also Study I.

Microorganisms used the added glucose-6-phosphate mainly as a C source and not as a P source, thus supporting the hypothesis that soil microorganisms are usually C limited. This is in contrast to a common model by McGill and Cole (1981) that claims a decoupled mineralization of organic P and C. The model states that the mineralization of P-esters is induced exclusively by microbial P demand and not related to C or N mineralization. However, other studies investigating microbial C and P mineralization in temperate forest also showed that both can be closely related (Spohn & Kuzyakov, 2013; Wang *et al.*, 2016). The C limitation of the soil microbial biomass indicates that the threshold C:P ratio of net P mineralization was undercut. This was the case for the C:P ratios of organic matter of both A horizons (Lang *et al.*, 2017). In mineral soils, organic matter C:N:P stoichiometry should be used as a proxy to assess net N and P mineralization because the mineral fraction of soil has no part in microbial net mineralization.

The net P mineralization found here could be catalyzed either by exoenzymes (Louche *et al.*, 2010; Plassard *et al.*, 2011) or by internal mineralization of glucose-6-uptake in cells and subsequent P release. However, it is unlikely that the latter happened on a large scale. Although bacteria are able to mineralize glucose-6phosphate internally (Winkler, 1973; Sonna *et al.*, 1988; Kadner *et al.*, 1994), it requires energy-consuming active transport. In general, the release of available P from organic phosphorylated compounds due to mineralization driven by microbial C demand likely contributes to the P supply of plants. It might be especially important in temperate forest soils with low P availability.

1.4.4 The influence of N fertilization on phosphatase activity and its relationship to net P mineralization

Cellobiohydrolase activity significantly increased at Bear Brook and Klosterhede, chitinase activity at Klosterhede and Gårdsjön and phosphatase activity significantly increased at Harvard Forest (N150), Bear Brook and Gårdsjön (Figure 6, a-c). On average, phosphatase activity increased more strongly (+260%) than cellobiohydrolase (+150%) or chitinase activity (+80%) due to N fertilization. Overall, phosphatase activity (4.8 - 57.6 μmol g^{-1} h^{-1}) was also much higher than cellobiohydrolase and chitinase activity (1.2 - 7.8, 4.1 - 11.0 μmol g^{-1} h^{-1}, respectively) in the Oe+Oa horizons. Enzyme activity ratios revealed a significant increase in phosphatase activity compared to cellobiohydrolase and chitinase activity due to N fertilization at Harvard Forest, but not at the other sites (Figure 6, e+f). The cellobiohydrolase-to-chitinase activity ratios were unaffected by N fertilization, except for a significant increase at Bear Brook.

Phosphatase activity was significantly correlated with total soil N, total soil P and the soil N:P ratio ($p < 0.001$, Figure 7Figure 7). An exponential model was fitted to the relationship between phosphatase activity and total P concentration in all soils ($R^2 = 0.42$, Figure 7, a), which approximated the decreasing phosphatase activity with increasing P concentrations in soil. Robust linear models were fitted to phosphatase activity as a function of total N concentrations and N:P ratios of the soil. The relationship between phosphatase activity and soil N:P ratios ($R^2 = 0.65$, Figure 7, b) was stronger than between phosphatase activity and total N concentrations ($R^2 = 0.26$, Figure 7, c).

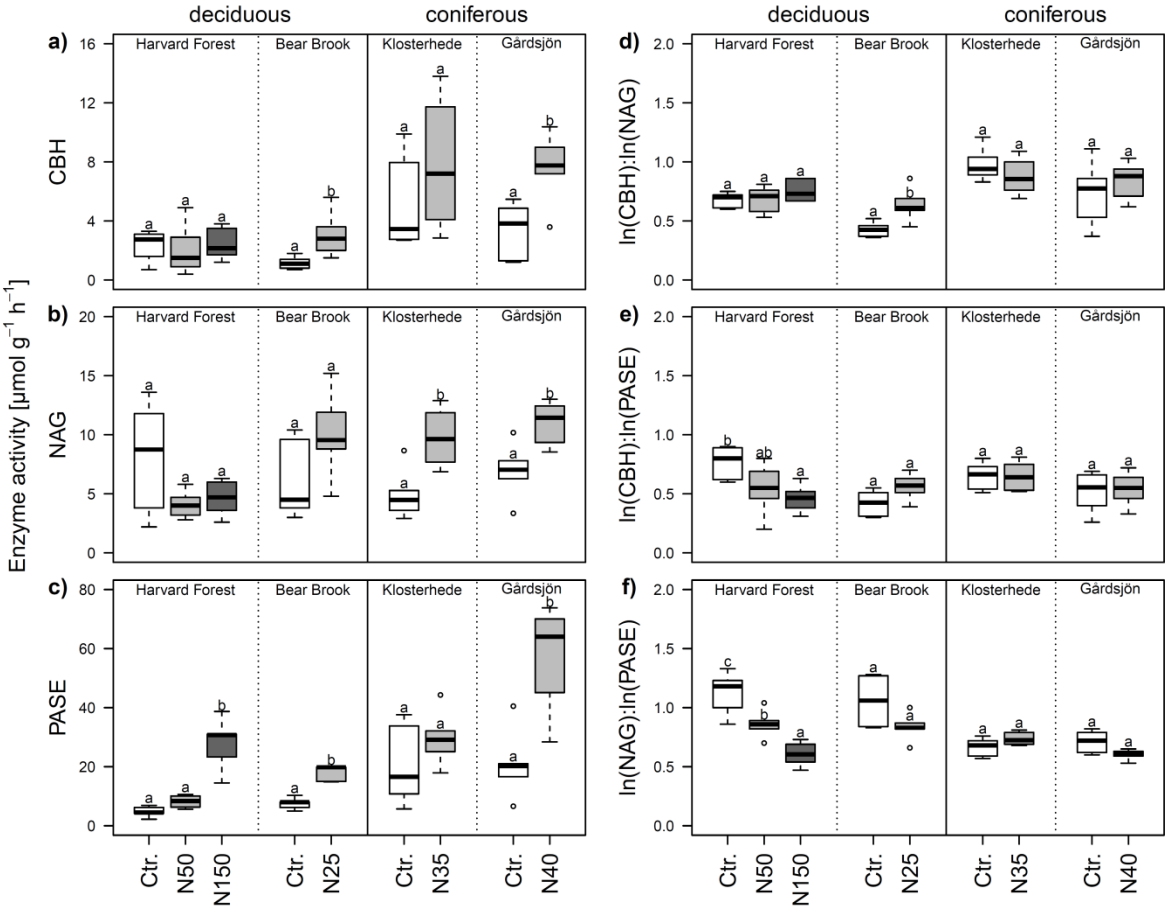


Figure 6 Activities of the enzymes cellobiohydrolase (CBH), chitinase (NAG), phosphatase (PASE) and ratios of the natural logarithms of specific enzyme activities (CBH:NAG, CBH:PASE, NAG:PASE) of controls and N fertilization treatments at the sites Harvard Forest, Bear Brook, Klosterhede and Gårdsjön. Different lowercase letters mark significant site-specific differences ($p < 0.05$). See also Study III.

The correlations of enzyme activities with different C, N and P fractions in the organic layer and organic layer C:N:P stoichiometry (Table S1, Study III) showed that cellobiohydrolase activity was correlated positively with total organic C concentrations ($r = 0.62$, $p < 0.001$), the C:P and N:P ratios ($r = 0.60$ and $r = 0.61$, $p < 0.001$) and negatively with total P ($r = -0.59$, $p < 0.001$). The same was true for phosphatase activity, which also had a positive relation with total N ($r = 0.56$, $p < 0.001$), and a negative relation with labile P ($r = -0.50$, $p < 0.001$). Chitinase activity was positively correlated with total C ($r = 0.43$, $p < 0.01$), total N ($r = 0.46$, $p < 0.001$) and available P concentrations ($r = 0.44$, $p < 0.05$) in soil. Overall, chitinase had much fewer and weaker correlations with the tested parameters than cellobiohydrolase and phosphatase.

Phosphatase activity increased more strongly due to long-term N fertilization than other exoenzymes (cellobiohydrolase, chitinase; Figure 6). This, in combination with the positive linear relationship between phosphatase activity and the total N concentration of the Oe+Oa horizons (Figure 7) confirms previous findings. Phosphatase activity frequently increases due to N fertilization (Naples & Fisk, 2010; Weand *et al.*, 2010; Marklein & Houlton, 2012), which is because the P demand of microorganisms and vegetation increases concomitantly with their N uptake, and also because a high N supply is beneficial for the N-expensive enzyme synthesis (Olander & Vitousek, 2000; Allison & Vitousek, 2005). The main allocation of additional N to phosphatase synthesis as compared to enzymes of the C and N

cycle indicates an increased P demand of plants and/or microorganisms in the organic layers. In coniferous organic layers, N fertilization also benefitted the synthesis of enzymes of the C and N cycle, albeit on a much smaller scale than phosphatases. Maybe this was due to a general nutrient scarcity in coniferous organic layers.

In addition, we observed an exponential decline of phosphatase activity with increasing total P concentrations as well as strong positive correlations of phosphatase activity and the C:P and N:P ratios of the organic layers (Figure 7, Table S1 in Study III). The soil P concentration influences the up- or down-regulation of phosphatase synthesis (Spiers & Mcgill, 1979; Marklein & Houlton, 2012), and a negative relation of phosphatase activity and phosphorus concentrations in soil has often been reported (Juma & Tabatabai, 1977, 1978; Olander & Vitousek, 2000; Moscatelli *et al.*, 2005; Marklein & Houlton, 2012). In this study, the combination of both increased N and partly decreased P concentrations probably boosted the increase in phosphatase activity. This is also indicated by the strong relationships of phosphatase activity with different forms of both N and P in the soil (Table S1 in Study III). Due to their simultaneous appearance, the influence of increased N and decreased P concentrations on phosphatase activity cannot be entangled here.

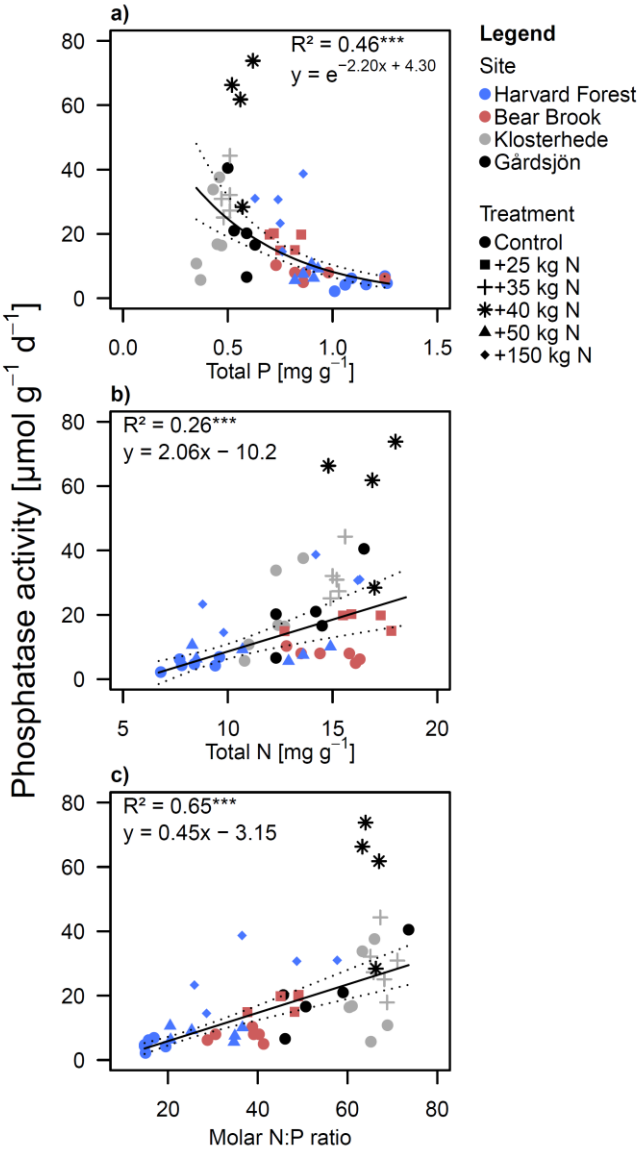


Figure 7 Relationship of phosphatase activity with a) total soil P concentrations, b) total soil N concentrations and c) molar N:P ratios. Sites are distinguished by colors (blue: Harvard Forest, red: Bear Brook, grey: Klosterhede, black: Gårdsjön) and treatments are distinguished by symbols (control: circle, +25 kg N: square, +35 kg N: plus, +40 kg N: star, +50 kg N: triangle, +150 kg N: diamond). An exponential model was fitted for a) and linear models for b) and d). Fitted lines are presented with 95%-confidence intervals, R^2 and the regression equations. Levels of significance are: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. See also Study III.

1.5. Conclusions

This thesis evaluated the influence of organic layer stoichiometry on microbial biomass stoichiometry and net C, N and P mineralization in temperate coniferous and deciduous forest soils. Moreover, it added details to the current research on P cycling in temperate forests by assessing processes potentially leading to net P mineralization in soils, i.e. microbial decomposition of P-rich organic compounds and P mineralization due to phosphatase activity in soils. The microbial C:N:P stoichiometry was unaffected by various changes of element inputs, both on the short- and on the long-term. This confirms the current assumption of microbial biomass stoichiometry in terrestrial ecosystems to be as constrained as in aquatic environments, which had not been tested for experimental manipulations of the stoichiometry of microbial resources. Stoichiometric variability induced by the additions of high concentrations of available C, N and P to a nutrient-poor soil represents an extreme situation, which is not to be expected in natural soils.

The invariability of microbial stoichiometry is probably closely connected to the dependency of both rate and occurrence of net C, N and P mineralization on organic layer stoichiometry. The microbial demand of an element needs to be satisfied before net mineralization of this element is expected to occur. Threshold C:N, C:P and N:P ratios of organic layers that were derived from the relationships between organic layer stoichiometry and net N and P mineralization designated the switch from microbial immobilization to net mineralization. The more C:N, C:P and N:P ratios decreased below the thresholds, the more net N or P mineralization increased. This effect was most pronounced in Oi horizons. The idea to use organic layer C:N:P stoichiometry as a proxy to estimate net N and P mineralization in forest soils is appealing because the determination of net mineralization is time consuming. However, there are limits of the stoichiometric approach to assess net N and P mineralization. Variability in the transition from little to strongly decomposed organic matter like in Oe horizons and manipulation of nutrient availability via N fertilization obscured the relationship between organic layer stoichiometry and net mineralization. Moreover, soil microbial N and P demands are not necessarily satisfied in all organic horizons. If soil microorganisms experienced N or P demand, microbial net mineralization did not increase above a minimum value that was independent of the organic layer C:N:P stoichiometry. This minimum net N and P mineralization likely represented the boundaries of the efficiency of microbial nutrient recycling.

Net P mineralization was of special interest here because, currently, the P demand of temperate forests is suspected to increase due to high atmospheric N depositions. It was shown that net P mineralization occurs as a byproduct of microbial mineralization processes that are driven by microbial C demand. This refutes a common model suggesting C and P mineralization in soil to be decoupled. Further, P released in this way has the potential to benefit plant P nutrition. On the contrary, increases in phosphatase activity due to N fertilization did not translate into increases in net P mineralization in organic layers. N fertilization may alter microbial nutrient demands, for example if increased N uptake needs to concur with increased P uptake due to stoichiometric constraints. Thus, increased phosphatase activity cannot be used as an indicator of increased available P in organic layers. Altogether, stoichiometric analyses proved to be a sound tool in assessing net mineralization in temperate organic layers, if its limitations are kept in mind. In future studies, it would be interesting to investigate net C, N and P mineralization in relation to organic layer stoichiometry in more coniferous Oi horizons of the temperate zone, which was not possible here, unfortunately. This could clarify whether the differences (as in Study III) or the

similarities (as in Study II) between deciduous and coniferous organic layers prevail and answer the question of whether threshold element ratios determined for deciduous and coniferous organic layers are transferrable. A meta-analysis of net N and P mineralization rates published together with organic layer stoichiometry may also suit this purpose.

1.6 References

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2. Manuscripts

Study I

2.1 Soil microbial biomass C:N:P stoichiometry and microbial use of organic phosphorus

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Soil microbial biomass C:N:P stoichiometry and microbial use of organic phosphorus

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ABSTRACT

Microbial mineralization and immobilization of nutrients strongly influence soil fertility. We studied microbial biomass stoichiometry, microbial community composition, and microbial use of carbon (C) and phosphorus (P) derived from glucose-6-phosphate in the A and B horizons of two temperate Cambisols with contrasting P availability. In a first incubation experiment, C, nitrogen (N) and P were added to the soils in a full factorial design. Microbial biomass C, N and P concentrations were analyzed by the fumigation-extraction method and microbial community composition was analyzed by a community fingerprinting method (automated ribosomal intergenic spacer analysis, ARISA). In a second experiment, we compared microbial use of C and P from glucose-6-phosphate by adding ^{14}C or ^{33}P labeled glucose-6-phosphate to soil. In the first incubation experiment, the microbial biomass increased up to 30-fold due to addition of C, indicating that microbial growth was mainly C limited. Microbial biomass C:N:P stoichiometry changed more strongly due to element addition in the P-poor soils, than in the P-rich soils. The microbial community composition analysis showed that element additions led to stronger changes in the microbial community in the P-poor than in the P-rich soils. Therefore, the changed microbial biomass stoichiometry in the P-poor soils was likely caused by a shift in the microbial community composition. The total recovery of ^{14}C derived from glucose-6-phosphate in the soil microbial biomass and in the respired CO_2 ranged between 28.2 and 37.1% 66 h after addition of the tracer, while the recovery of ^{33}P in the soil microbial biomass was 1.4–6.1%. This indicates that even in the P-poor soils microorganisms mineralized organic P and took up more C than P from the organic compound. Thus, microbial mineralization of organic P was driven by microbial need for C rather than for P. In conclusion, our experiments showed that (i) the microbial biomass stoichiometry in the P-poor soils was more susceptible to additions of C, N and P than in the P-rich soils and that (ii) even in the P-poor soils, microorganisms were C-limited and the mineralization of organic P was mainly driven by microbial C demand.

1. Introduction

At some temperate forest sites, the P concentrations in tree leaves and needles have decreased during the last decades (e. g. Houdijk and Roelofs, 1993; Flückiger and Braun, 1998; Duquesnay et al., 2000; Prietzel et al., 2008; Ilg et al., 2009; Braun et al., 2010). The reasons for these changes are not clear (Duquesnay et al., 2000; Prietzel et al., 2008; Ilg et al., 2009; Braun et al., 2010; Crowley et al., 2012), and thus require further investigations of P cycling in temperate forest ecosystems. Soil microorganisms play an important role in forest P nutrition as they both mobilize and immobilize P (Oberson and Joner, 2005), which is reflected in the stoichiometry of their biomass (Makino et al., 2003). By now, there is still little knowledge about the plasticity of soil microbial biomass stoichiometry under changing C and nutrient supplies and the microbial use of organic phosphorylated compounds.

Ecological stoichiometry deals with the C:N:P ratios of organisms and substrates as a tool to gain insight into the cycling of these elements (Sterner and Elser, 2002). In addition, it provides a helpful

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tool to determine the nutritional state of single organisms or communities. One of the questions of ecological stoichiometry is whether organisms are homeostatic or non-homeostatic. An organism is homeostatic if it maintains a certain C:N:P ratio independent of the chemical composition of its food resources. Non-homeostatic organisms show changing C:N:P ratios with changing elemental composition of resources (Sterner and Elser, 2002). Heterotrophic organisms, like most soil microorganisms, are assumed to exhibit stronger homeostatic behavior than autotrophic organisms (Andersen and Hessen, 1991; Sterner et al., 1998). Constrained C:N:P ratios have also been found for whole soil microbial communities. A mean global microbial biomass C:N:P ratio of 60:7:1 was reported for soils (Cleveland and Liptzin, 2007), similar to the Redfield ratio described for planktonic biomass (Redfield, 1958). Yet, these concepts have been contradicted by studies showing stoichiometric variability in the soil microbial biomass (between 11:1:1 and 93:10:1; Tischer et al., 2014) or leaf litter (between 77:7:1 and 175:13:1; Fanin et al., 2013).

Variability in the stoichiometry of the soil microbial biomass can have various reasons. First, shifts in the microbial community composition due to changing environmental conditions can alter stoichiometric ratios since species differ in C:N:P ratios (Quigg et al., 2003; Hall et al., 2010; Fanin et al., 2013). Second, microorganisms are able to take up resources in excess and to store them in the form of e.g. glycogen or polyphosphates, leading to changes in their biomass C:N:P ratio (Wilkinson, 1963; Kornberg, 1995; Wilson et al., 2010; Achbergerová and Nahálka, 2011).

The stoichiometry of soil microbial biomass is of interest because important ecosystem fluxes of C, N and P, such as mineralization or immobilization, are controlled by soil microorganisms (van der Heijden et al., 2008; Mooshammer et al., 2012). P can be rapidly immobilized by soil microorganisms under P limiting conditions (Bünemann et al., 2012), and the P concentration in the soil solution is strongly influenced by microbial P immobilization (Frossard et al., 2000). Microbial N immobilization can also strongly affect N availability and might even cause N deficiency in trees when litter decomposition is slow (Attiwill and Adams, 1993). Microbial immobilization of N and P might result in changes in microbial biomass stoichiometry.

Microbial C:N:P stoichiometry also affects mineralization of organic C, N and P in soils (Mooshammer et al., 2012). Organic C, N, and P in soils are mineralized by enzymes released by soil microorganisms and plants. Mineralization of organic P is catalyzed by phosphatases, excreted as exoenzymes by plants or soil microorganisms (Nannipieri et al., 2011). Organic P mineralization has been viewed mostly as being controlled by plant or microbial P demand (McGill and Cole, 1981; Olander and Vitousek, 2000; Nannipieri et al., 2011). However, since organic P compounds also contain C, it seems likely, that organic P mineralization is also controlled by microbial C demand. Organic P mineralization driven by microbial need for C has recently been found in temperate forest soils (Spohn and Kuzyakov, 2013). Besides, the respiration rates of soil microorganisms per unit microbial biomass have been found to increase with soil and litter carbon to nutrient ratios and to underlie stoichiometric controls (Spohn, 2014; Spohn and Chodak, 2015). Thus, understanding the stoichiometry of the soil microbial biomass can be important for the assessment of larger processes in forest ecosystems.

In this study, we tested the following hypotheses. (i) The C:N:P ratio of the soil microbial biomass is not strictly homeostatic but changes depending on element availabilities. These changes are expected to be stronger in a P poor than in a P rich soil. (ii) The limiting element for soil microbial biomass is C, even at temperate forest sites where plants are likely P limited. (iii) Microorganisms use organic phosphorylated compounds as C source and in doing so mineralize P. To assess these hypotheses, C, N and P were added to soil samples in a full factorial design and microbial C (C_{mic}), N (N_{mic}) and P (P_{mic}) were determined by chloroform-fumigation-extraction. The microbial communities were investigated by a community fingerprinting analysis after element additions. Microbial uptake of C and P from organic compounds was examined in an incubation experiment with either ^{14}C or ^{33}P labeled glucose-6-phosphate.

2. Materials and methods

2.1. Sites and soil sampling

Soil samples were taken at two sites in Germany with contrasting P availability. The site Bad Brueckenau is situated in the Rhoen Mountains (N 50° 21.38′, E 9° 55.71′) at 825 m above sea-level. The mean annual rainfall is 1025 mm and the mean annual temperature is 5.8 °C. The parent material is tertiary volcanic rock. The soil type is Eutric Cambisol (FAO) and the prevailing tree species is European beech (*Fagus sylvatica* L.). The site Unterluess (Luess) is located in the Lueneburg Heath (N 52° 50.32′, E 10° 16.06′) at 115 m above sea-level. The mean annual rainfall amounts to 779 mm and the mean annual temperature is 8 °C. The soil type is a Spodic Cambisol which developed from Pleistocene sediments. The vegetation is formed by European beech (*F. sylvatica* L.). The P availability is higher at the site Bad Brueckenau in both soil horizons compared to the site Luess (Table 1). In February 2014 bulk samples of A and B horizons were collected with a shovel after removal of the organic layers at both sites. The samples were sieved (2 mm) and pre-incubated at 20 °C and 50% water holding capacity for 3 weeks in plastic bags. In the following, the samples from the A and B horizon at Bad Brueckenau are called soil BA and BB and the A and B horizon from the site Luess are called soil LA and LB.

2.2. Element addition experiment

25 g dry weight equivalent of each pre-incubated field moist soil horizon (ca. 50% water holding capacity) was put into 180 ml plastic beakers with screw caps. After another pre-incubation in the beakers (3–8 days) the elements C, N and P were added in a full factorial design as 2 mg g^{-1} glucose-C, 0.1 mg g^{-1} NH_4NO_3–N and 0.1 mg g^{-1} KH_2PO_4–P dissolved in 1 ml distilled water (Demoling et al., 2007). 1 ml water was added to the controls. Samples were thoroughly mixed with the pipette tip, which was then kept in the incubation vessel. All treatments were conducted with 4 replicates. The incubation lasted for 65 h at 20 °C in the dark.

The C_{mic}, N_{mic} and P_{mic} concentrations were analyzed by the chloroform fumigation-extraction method (Brookes et al., 1982; Vance et al., 1987) after 65 h of incubation. The soil in the beakers was split into two parts, and one half was placed in a desiccator with chloroform. The desiccator was evacuated until the $CHCl_3$ had boiled for 2 min and samples were fumigated for 24 h at room

Table 1
Chemical properties of the soils from Bad Brueckenau (BA, BB) and Luess (LA, LB). Given are total carbon contents, total nitrogen contents as well as total phosphorus contents, molar C:N:P ratios and pH value (Lang et al., 2014; in prep.).

Soil	Depth [cm]	C [mg g^{-1}]	N [mg g^{-1}]	P [mg g^{-1}]	C:N:P ratio	pH (H_2O)
BA	0–10	151.4	9.8	3.1	125.9: 7.0: 1	3.9
BB	10–20	85.2	6.0	3.3	66.6: 4.0: 1	4.4
LA	0–10	73.0	2.9	0.2	941.3: 32.1: 1	3.7
LB	10–20	15.0	0.6	0.1	386.8: 13.3: 1	3.9

temperature. The other half of the soil was extracted directly after incubation. For C_{mic} and N_{mic} analysis (multi N/C 2100, Analytik Jena) the samples were extracted with 0.5 M K_2SO_4 with a soil:solution ratio of 1:5 (Joergensen et al., 1995), for P_{mic} analysis (FIA-LAB, MLE Dresden) a Bray-1 solution (0.03 M NH_4F – 0.025 M HCl) with a soil:solution ratio of 1:10 was used (Bray and Kurtz, 1945; Aponte et al., 2010). The sorption of P by the solid phase during the extraction was assessed by a P spike for soil BB (Brookes et al., 1982). For this purpose, 5 g dry weight equivalent of non-incubated soil were extracted with Bray-1 solution to which a spike of 50 µg KH_2PO_4–P, dissolved in 1 ml distilled water, was added.

To calculate C_{mic}, N_{mic} and P_{mic}, the concentrations of organic C, organic N and inorganic P extracted from non-fumigated soil were subtracted from the concentrations extracted from fumigated soil. These differences were corrected by the conversion factors 2.22 for C_{mic} as well as N_{mic} and 2.5 for P_{mic} (Jenkinson et al., 2004). Extracted P concentrations of soil BB were corrected for P sorption during the extraction before calculating P_{mic} (Brookes et al., 1982). C:N:P ratios were calculated from molar C_{mic}, N_{mic} and P_{mic} concentrations.

2.3. C and P tracer experiment

Uptake and respiration of C and P from glucose-6-phosphate by soil microorganisms was analyzed using ^{14}C-glucose-6-phosphate and ^{33}P-glucose-6-phosphate (Spohn and Kuzyakov, 2013). 10 g dry weight equivalent of soil were put into 180 ml beakers with screw-caps and pre-incubated for 2 weeks at 50% water holding capacity and at 20 °C. After 2 weeks, either 20 kBq ^{14}C-glucose-6-phosphate or 80 kBq ^{33}P-glucose-6-phosphate dissolved in 1 ml distilled water were added to each sample. C and P were added in trace concentrations, not exceeding 2.6 nmol glucose-6-phosphate per g soil. All treatments were conducted with 4 replicates. C_{mic} and P_{mic} were measured using chloroform fumigation extraction 66 and 164 h after addition of the labeled glucose-6-phosphate (see above). The ^{33}P and ^{14}C activity in the extracts was measured by a scintillation counter (Packard). Additionally, ^{14}C activity in the respired CO_2 was measured 24, 49, 69, 93 and 164 h after addition of the labeled glucose-6-phosphate on 4 replicates. For this purpose, the respired CO_2 was trapped in 2 ml of a 1 M NaOH solution. ^{14}C activity in the NaOH was measured by a scintillation counter (Packard). The percentage recovery of ^{33}P and ^{14}C in the microbial biomass and respired CO_2 was related to the total applied radioactivity and calculated as

$$recovery\ [\%] = \left(\frac{r}{R}\right) \cdot 100 \quad (1)$$

with r as radioactivity recovered in corrected microbial biomass and R as total applied radioactivity (Fardeau, 1996). The relative specific activity of ^{33}P and ^{14}C in the microbial biomass and respired CO_2 was calculated as

$$rel.specific\ activity\ [\%] = \left(\frac{r}{R}\right) \Big/ Q \quad (2)$$

with Q being the P_{mic} and C_{mic} concentration, respectively, according to Bünemann et al. (2004).

2.4. Nucleic acids purification and ARISA

Metagenomic DNA was extracted from soil using the NucleoSpin® Soil kit (Macherey–Nagel, Düren, Germany) from ca. 250–500 mg soil material using lysis buffer 1 (SL1). Nucleic acids were eluted from the NucleoSpin® Soil column in 50 µl elution buffer SE, pre-heated to 80 °C. All other purification procedures were performed as recommended by the manufacturer.

Automated ribosomal intergenic spacer analysis (ARISA; Fisher and Triplett, 1999) was adopted to analyze bacterial and fungal communities in parallel as follows. Ribosomal intergenic spacers/internal transcribed sequences were PCR-amplified in two separate reactions using bacteria-specific primers (ITSF and ITSReub; Cardinale et al., 2004) and fungi-specific primers (ITS1F-Z; Weig et al., 2013 and ITS2; White et al., 1990), respectively. 5 ng metagenomic DNA was used in a 12.5 µl PCR volume as previously described (Weig et al., 2013). The ITSF forward primer was labeled with fluorescent dye BMN-6 and the ITS1F-Z forward primer was labeled with fluorescent dye BMN-5 (Biomers, Ulm, Germany). Bacterial and fungal specific amplification products were combined and separated on a GenomeLab GeXP Genetics Analysis System (Beckman–Coulter, Krefeld, Germany; now: AB Sciex, Darmstadt, Germany) and a primary data matrix (sample vs. peak size) of absolute peak height of the bacterial (BMN-6) and fungal (BMN-5) fragments was obtained using a bin width of one nucleotide.

2.5. Statistics

In the element addition experiment, the effect of C, N and P addition on C_{mic}, N_{mic} and P_{mic} was analyzed by multiple linear regressions for each soil and horizon. The correspondent regression equation was

$$y = a + b_C \cdot C + b_N \cdot N + b_P \cdot P \quad (3)$$

where y was C_{mic}, N_{mic} or P_{mic} as dependent variable, a was the intercept and b_C, b_N and b_P were the regression coefficients of the C, N or P addition, respectively. The differences between single element addition treatments and microbial C:N:P ratios were investigated by ANOVA followed by Tukey's multiple comparison test, after homogeneity of variances was tested with Bartlett's test. In the tracer experiment, the differences between ^{14}C and ^{33}P recoveries in soils were also tested by ANOVA followed by Tukey's multiple comparison tests. These statistical analyses were conducted in R, version 2.15.2 (R Core Team, 2012).

For statistical analysis of the ARISA, only bacterial fragments larger than 100 bp and fungal fragments larger than 150 bp, respectively, were used. Raw data were analyzed by the PRIMER 6 software (Plymouth Routines In Multivariate Ecological Research, v. 6.1.15, PRIMER-E Ltd., United Kingdom) and raw data were transformed by the square-root algorithm to reduce the weight of dominating features. Resemblance matrices (Bray–Curtis similarity coefficient) were separately calculated from the abundance matrix of bacterial and fungal ARISA fragments, respectively. Canonical Analysis of Principal coordinates (CAP) (Anderson and Willis, 2003) was performed on the resemblance matrix using a combination of the two factors 'soil' and 'treatment'. Differences between microbial community signatures of different samples were tested by ANOSIM. The C_{mic}, N_{mic} and P_{mic} concentrations were compared to the ARISA profile by Spearman rank correlations. For this purpose, microbial element concentrations of the different sampling sites were log-transformed (natural logarithm) and missing values were filled with values calculated using the expectation maximum likelihood algorithm (1000 iterations, 0.001 min change) available in the PRIMER 6 software. A resemblance matrix using Euclidian distance was calculated from normalized values und compared to the resemblance matrices of bacterial and fungal ARISA data, respectively, using the RELATE algorithm of the PRIMER 6 software by Spearman correlation using 999 permutations.

3. Results

3.1. Element addition experiment

Addition of C either alone or in combination with other elements led to significantly increased C_{mic} concentrations in soils LA and LB (Fig. 1), but the size of the increase varied between soils and treatments. C_{mic} significantly increased by a factor of 4 compared to the control after C as well as CN addition in soil LA ($p < 0.01$ and $p = 0.001$, respectively). In soil LB, C_{mic} strongly increased after addition of C in combination with N (by a factor of 5) and in combination with P (by a factor of 30 compared to the control). Increases in C_{mic} due to CP and CNP addition were statistically significant ($p = 0.001$). Multiple linear regression supported that C addition had a greater influence on C_{mic} in the soils LA and LB than on C_{mic} in the soils BA and BB (Table 2).

Addition of N combined with C addition affected N_{mic} concentrations more strongly than single N addition (Fig. 2). In soil LA, N_{mic} concentrations after N and CN addition were 1.2- and 1.4-times higher than the N_{mic} concentration in the control, respectively. Multiple linear regression showed that N addition had only a significantly positive effect on N_{mic} in the soil LA, while C addition had a significantly positive influence on N_{mic} in the soils BA, BB and LB (Table 2). The N_{mic} concentration in the control of soil LB was below the detection limit. For N_{mic}, the only statistically significant differences between treatments were found in soil LA and LB. The N_{mic} concentration after C addition was significantly lower than after N or CN addition ($p < 0.001$) in soil LA. In soil LB, CNP addition led to the largest increase in N_{mic} concentrations, significantly different ($p < 0.05$) from all other treatments except CN addition.

The P_{mic} concentrations only significantly increased after C, N and P addition in the soils BB and LB (Fig. 3). CN ($p < 0.001$) and CP

Table 2
Summary of the multiple regression analyses for the variables C, N and P addition predicting C_{mic}, N_{mic} and P_{mic} for all four soils. b_C, b_N and b_P are regression coefficients of the variables C, N and P addition. The soils are BA: Bad Brueckenau, A horizon; BB: Bad Brueckenau, B horizon; LA: Luess, A horizon; LB: Luess, B horizon.

		Coefficient			
Soil		b_C	b_N	b_P	Adjusted R^2
BA	C_{mic}	415.2^{**}	131.5	262.3	0.26
	N_{mic}	63.6^{*}	-54.6^{*}	−43.8	0.26
	P_{mic}	49.7	-158.9^{***}	-93.4^{*}	0.41
BB	C_{mic}	424.9^{**}	−216.7	−80.6	0.24
	N_{mic}	31.8^{**}	−16.9	-19.5^{*}	0.39
	P_{mic}	88.9^{*}	51.6	7.4	0.25
LA	C_{mic}	568.0^{***}	−34.7	-441.7^{**}	0.52
	N_{mic}	−5.9	12.3^{**}	−7.3	0.24
	P_{mic}	−3.0	9.8	20.6^{*}	0.22
LB	C_{mic}	1459.5^{***}	-641.4^{*}	879.8^{**}	0.60
	N_{mic}	18.0^{*}	20.0	17.3^{*}	0.50
	P_{mic}	−12.1	−5.4	39.8^{***}	0.54

* significant at $p < 0.05$, ** significant at $p < 0.01$, *** significant at $p < 0.001$.

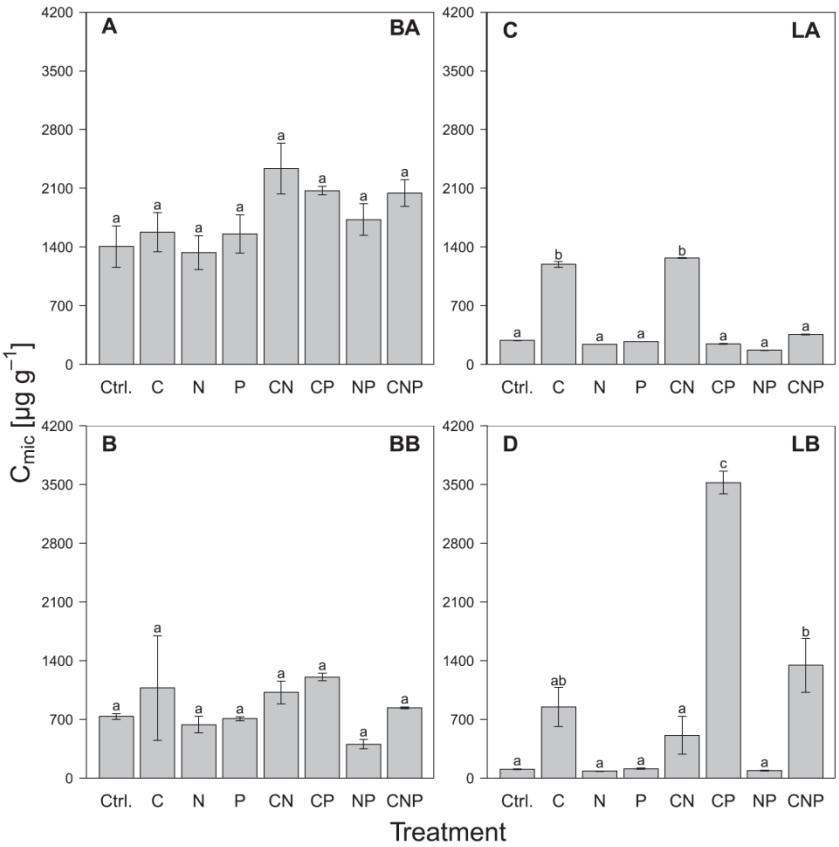

Fig. 1. Microbial biomass carbon (C_{mic}) concentrations of the four studied soils after addition of carbon (C), nitrogen (N) and phosphorus (P) in a full factorial design. Error bars show standard errors ($n = 4$) and significant differences are marked by lowercase letters.

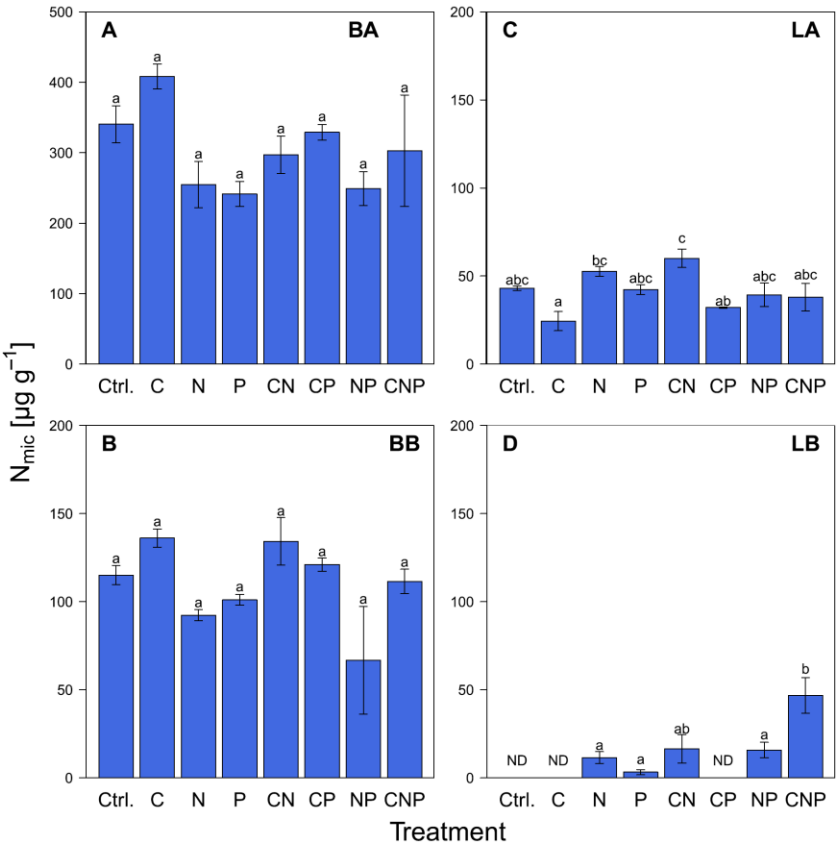

Fig. 2. Microbial biomass nitrogen (N_{mic}) concentrations of the four studied soils after addition of carbon (C), nitrogen (N) and phosphorus (P) in a full factorial design. ND: N_{mic} concentrations were below the detection limit (12.5 µg P_{mic} g^{-1}). Error bars show standard errors (n = 4) and significant differences are marked by lowercase letters. Please note the different scales of the y-axes.

addition ($p < 0.01$) to soil BB led to significantly higher P_{mic} concentrations compared to the control. In soil LB, all treatments that included P addition led to increased P_{mic} concentrations, but the changes were only significant for the addition of P ($p < 0.05$), NP ($p < 0.001$) and CNP ($p < 0.01$). The maximum increases in P_{mic} amounted to 3-times the control in the soil LA (after NP addition) and 13-times the control in the soil LB (after P addition). Multiple regression also showed a significant positive influence of P addition on P_{mic} in the soils from Luess (Table 2).

Taken together, increases in C_{mic} concentrations did not go along with high increases in P_{mic} or N_{mic} concentrations in all studied soils. In soils from the site Luess, increases in N_{mic} concentrations after N addition were even larger when N addition was coupled with C addition. Multiple regression analysis explained variations in C_{mic}, N_{mic} and P_{mic} found after C, N and P addition best in the soil LB from Luess, as shown by the higher adjusted R^2 values for this soil (Table 2).

The microbial biomass C:N:P stoichiometry of both soils from Bad Brueckenau did barely change significantly due to element addition, in contrast to the soils from Luess (Table 3). In the soil BA, the C:N:P ratio differed significantly from the control only after CNP addition. Soil BB showed significantly larger C:N:P ratios after C and P addition compared to all other treatments. The changes in soil microbial biomass stoichiometry in the soils LA and LB were similar, but soil LB showed the most pronounced changes after element additions. In soil LA, the C_{mic}:P_{mic} and C_{mic}:N_{mic} ratios increased statistically significant after C addition. N addition led to decreased C_{mic}:N_{mic} ratios as well as increased N_{mic}:P_{mic} ratios. CN addition significantly increased the C:N:P ratio. Moreover, C_{mic}:P_{mic} and N_{mic}:P_{mic} ratios were decreased after NP addition, but not after P or CP addition. P addition had a stronger impact on soil LB than on soil LA, leading to decreased C_{mic}:P_{mic} and N_{mic}:P_{mic} ratios after P as well as NP addition.

The results of the ARISA showed that bacterial as well as fungal communities differed between the two sites, as represented by the distance between dots (Fig. 4). Four fungal community signatures could be distinguished in soil LA depending on element additions. The first group included the control and the samples receiving N or P addition, the second one was found in the samples amended with NP, the third community in samples with CN, CP and CNP additions, and the fourth one exclusively formed in samples with C addition. Thus, the fungal communities in soil LA differed depending on which elements were added to the soils. In contrast, bacterial community signatures were affected only by CP and NP additions. All other combinations of added elements did not result in community signatures different from the control.

The Spearman rank correlations of C_{mic}, N_{mic} and P_{mic} concentrations and bacterial and fungal communities were highly significant. The variability of the bacterial community was best explained when C_{mic}, N_{mic} and P_{mic} were considered together ($R = 0.731$, $p < 0.001$), while the variability of the fungal community was best explained by N_{mic} and P_{mic} ($R = 0.719$, $p < 0.001$; Table 4).

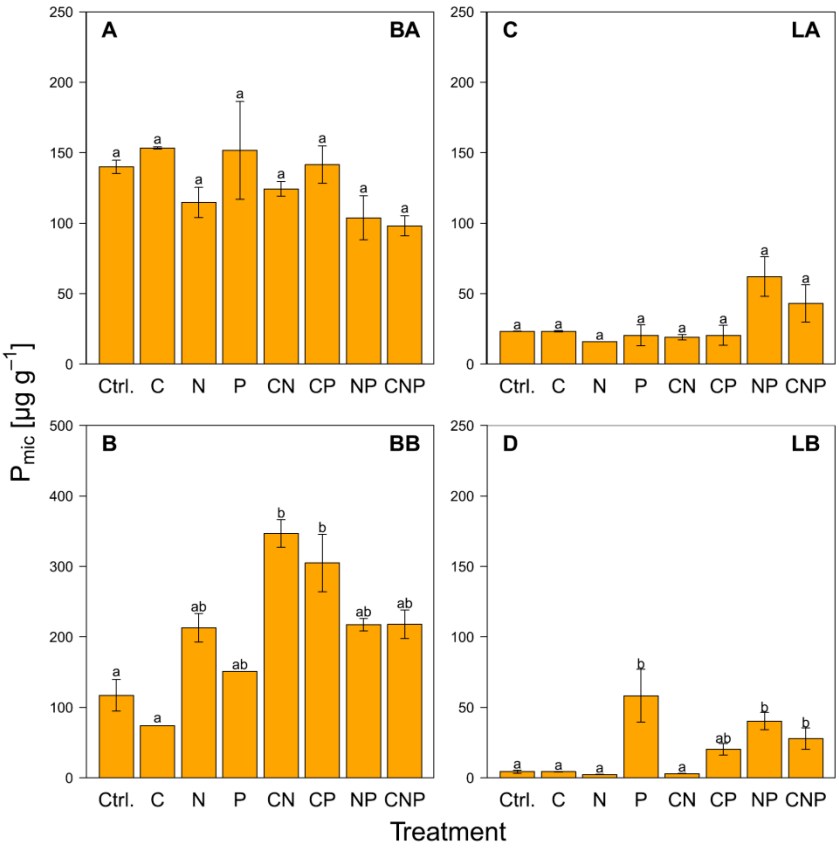

Fig. 3. Microbial biomass phosphorus (P_{mic}) concentrations of the four studied soils after addition of carbon (C), nitrogen (N) and phosphorus (P) in a full factorial design. Error bars show standard errors (n = 4) and significant differences are marked by lowercase letters. Please note the different scales of the y-axes.

Table 3
Molar C:N:P ratios of the microbial biomass of the four soils after addition of carbon (C), nitrogen (N) and phosphorus (P) in a full factorial design. The soils are BA: Bad Brueckenau, A horizon; BB: Bad Brueckenau, B horizon; LA: Luess, A horizon; LB: Luess, B horizon. Ratios are given with 90% confidence intervals and lowercase letters (online) mark significant differences between element addition treatments in each soil.

Treatment	Soil			
	BA	BB	LA	LB
Ctrl.	26.1 ± 8.0: 5.4 ± 0.8: 1a	18.0 ± 5.9: 2.4 ± 0.8: 1a	31.7 ± 1.9: 4.1 ± 0.2: 1a	79.3 ± 5.2: nd[a]: 1abc
C	26.5 ± 6.3: 5.9 ± 0.4: 1a	45.4 ± 0.0: 8.1 ± 0.5: 1b	94.3 ± 22.0: 2.3 ± 0.9: 1b	510.9 ± 241.1: nd[a]: 1c
N	30.1 ± 6.9: 5.1 ± 1.4: 1ab	7.9 ± 1.8: 1.0 ± 0.1: 1a	39.0 ± 0.7: 7.3 ± 0.7: 1a	89.2 ± 7.9: 10.8 ± 5.4: 1abc
P	36.5 ± 15.8: 4.8 ± 1.8: 1ab	118.3 ± 45.2: 14.3 ± 5.2: 1c	40.6 ± 16.8: 5.3 ± 2.0: 1a	6.9 ± 3.5: 0.2 ± 0.2: 1b
CN	43.2 ± 6.4: 5.3 ± 0.9: 1ab	6.9 ± 2.8: 0.8 ± 0.3: 1a	178.3 ± 38.0: 7.1 ± 0.9: 1c	425.0 ± 307.0: 11.8 ± 7.6: 1c
CP	38.8 ± 6.1: 5.3 ± 0.9: 1ab	10.7 ± 1.9: 0.9 ± 0.2: 1a	33.7 ± 8.7: 4.0 ± 1.6: 1a	471.8 ± 108.2: nd[a]: 1c
NP	47.9 ± 19.4: 5.8 ± 2.2: 1ab	4.8 ± 1.0: 0.7 ± 0.5: 1a	8.6 ± 4.8: 1.9 ± 0.9: 1a	5.9 ± 0.7: 1.0 ± 0.6: 1a
CNP	53.6 ± 1.1: 6.5 ± 2.2: 1b	10.3 ± 1.8: 1.2 ± 0.3: 1a	41.0 ± 33.4: 3.5 ± 2.5: 1a	110.5 ± 40.7: 4.4 ± 2.1: 1abc

[a] N_{mic} concentrations were below the detection limit (12.5 µg P_{mic} g^{-1} soil).

3.2. C and P tracer experiment

The microorganisms immobilized a higher percentage of the added C than of the added P from glucose-6-phosphate in all four soils (Fig. 5), and more than 20% of the glucose-6-phosphate derived C was respired during the first 24 h after the tracer addition (Fig. 6), indicating a fast uptake and metabolization of the glucose-6-derived C. The ^{33}P recovery in the microbial biomass was on average 1.3-times larger than the ^{14}C recovery in the microbial biomass and about 5- to 40-times smaller than the sum of ^{14}C recovered in the biomass and in the respired CO_2 of all 4 soils after 66 h as well as 164 h incubation time. Thus, on a net balance, P derived from glucose-6-phosphate was mineralized and left in the soil.

All major changes in ^{14}C and ^{33}P recovery happened in the first 66 h of incubation after application of the isotopic tracer (Fig. 5). The ^{14}C recovery in microbial biomass and CO_2 was about one third higher in soils from Luess than in soils from Bad Brueckenau. The sum of ^{14}C recovered in microbial biomass and CO_2 in both soil LA and soil LB was significantly higher than in soil BA and soil BB ($p < 0.001$).

The specific activity of ^{33}P in the microbial biomass was much higher than the specific activity of ^{14}C (Fig. 5, C and D). After 66 h,

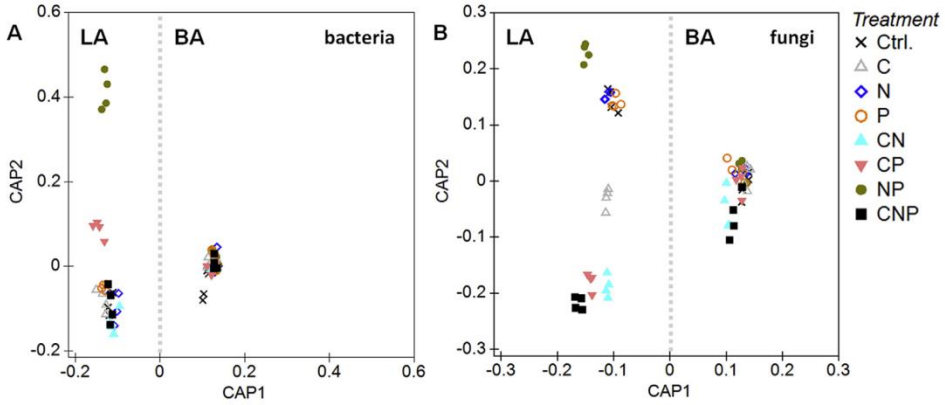

Fig. 4. Canonical analyses of principal components (CAP) of the bacterial (A, mis-classification error = 67.2%) and fungal (B, mis-classification error = 39.1%) communities of the soils BA and LA after addition of carbon (C), nitrogen (N) and phosphorus (P) in a full factorial design. Microbial communities were characterized by automated ribosomal intergenic spacer analysis (ARISA).

Table 4
Spearman's correlation coefficient (R) for the correlations between the resemblance matrices (Euclidian distance) of soil microbial communities and microbial biomass C, N and P concentrations.

Variables	Bacteria	Fungi
C_{mic}	0.520, $p < 0.001$	0.462, $p < 0.001$
N_{mic}	0.705, $p < 0.001$	0.699, $p < 0.001$
P_{mic}	0.641, $p < 0.001$	0.631, $p < 0.001$
C_{mic}, N_{mic}	0.697, $p < 0.001$	0.666, $p < 0.001$
C_{mic}, P_{mic}	0.705, $p < 0.001$	0.670, $p < 0.001$
N_{mic}, P_{mic}	0.721, $p < 0.001$	0.719, $p < 0.001$
C_{mic}, N_{mic}, P_{mic}	0.731, $p < 0.001$	0.707, $p < 0.001$

the specific activity of ^{33}P was between 15- and 125-times larger than the specific activity of ^{14}C. The specific activities of ^{14}C and ^{33}P were higher in soils from Luess than in soils from Bad Brueckenau. At both sites, the specific activities were higher in the B horizons.

The ^{14}C recovery in the respired CO_2 showed the same course in all four soils (Fig. 6). The ^{14}C recovery increased during the whole incubation with a strong increase in the first 24 h, and slower increase afterwards. The soils LA and LB showed a higher ^{14}C recovery in the CO_2 than the soil BA and BB.

4. Discussion

4.1. Microbial stoichiometry and community composition

The mean molar soil microbial C:N:P ratio of the control samples was 39:4:1. This was in agreement with the mean soil microbial C:N:P ratio of 32:3:1 measured in tropical soils (Tischer et al., 2014). However, this result deviates from the mean soil microbial C:N:P ratio 74:9:1 of forest soils calculated from a global dataset (Cleveland and Liptzin, 2007). If the C:N:P ratios of all element addition treatments are included in the calculation of a mean soil microbial C:N:P ratio, it accounts to 79:4:1. This result is closer to the findings by Cleveland and Liptzin (2007).

The P_{mic} concentrations could explain the differences between the microbial C:N:P ratios of this study and previous studies. The P_{mic} concentrations found in this study were very high. A reason for this could be the higher P extraction efficiency of the Bray-1 solution used here compared to the classically used extractant 0.5 M $NaHCO_3$ (Chen and He, 2004). Besides the better extraction efficiency, the Bray-1 solution was used because less organic substances are dissolved in the extracts and so the colorimetric PO_4-measurement is less biased.

The significant variations of the molar C:N:P ratios showed that the soil microbial biomass did not react strictly homeostatically to element addition treatments. The microbial C:N:P ratios of the soils LA and LB varied more strongly than the ratios of the soils BA and BB. At both sites more significant differences between C:N:P ratios were found in B horizons. This might be caused by the higher C, N and P availability in the upper soil horizons compared to the lower soil horizons as well as the higher C, N and P availability at the site Bad Brueckenau (Table 1).

The variations of microbial C:N:P ratios and the non-homeostatic behavior could be related either to shifts in the soil microbial community or excess uptake and storage of the added C, N and P. Shifts in microbial community composition as a cause of varying microbial C:N:P ratios will be discussed first. In soil LA, a shift in the bacterial as well as in the fungal community occurred after various element additions. This finding is supported by changed abundances of fungal species in this soil after element additions. A shift towards r-strategists could have caused the close microbial C:N:P ratios after NP addition in the soils LA and LB. R-strategists take up disproportionately high amounts of P to synthesize RNA (Elser et al., 2000a,b). Another possible shift could have been from oligotrophic to copiotrophic bacteria. Fierer et al. (2007) described copiotrophic organisms as specialized in using high C concentrations, while oligotrophic species can still grow when exposed to limited carbon and nutrient supplies. As the amounts of available C, N and P in soils from the site Luess were limited, it is probable that the original microbial community was dominated by oligotrophic organisms. Additional C, N and P input could have enabled copiotrophic organism groups to outcompete the previously dominant oligotrophic organisms.

The bacterial and fungal communities of the site Luess reacted differently to the element addition treatments. The ARISA of soil LA showed that the fungal community could be clearly divided into four groups depending on the treatment (1. N addition, P addition, control, 2. NP addition, 3. nutrient additions coupled with C addition, 4. C addition). Shifts in the structure of fungal communities in litter and soil of boreal forests after increasing N or C deposition in the field has also been found by Allison et al. (2007). In contrast, the bacterial community only changed after addition of P in combination with either C or N. Phosphate additions have been found to alter bacterial as well as fungal communities in grassland soil microcosms (Rooney and Clipson, 2009). The observed increases in

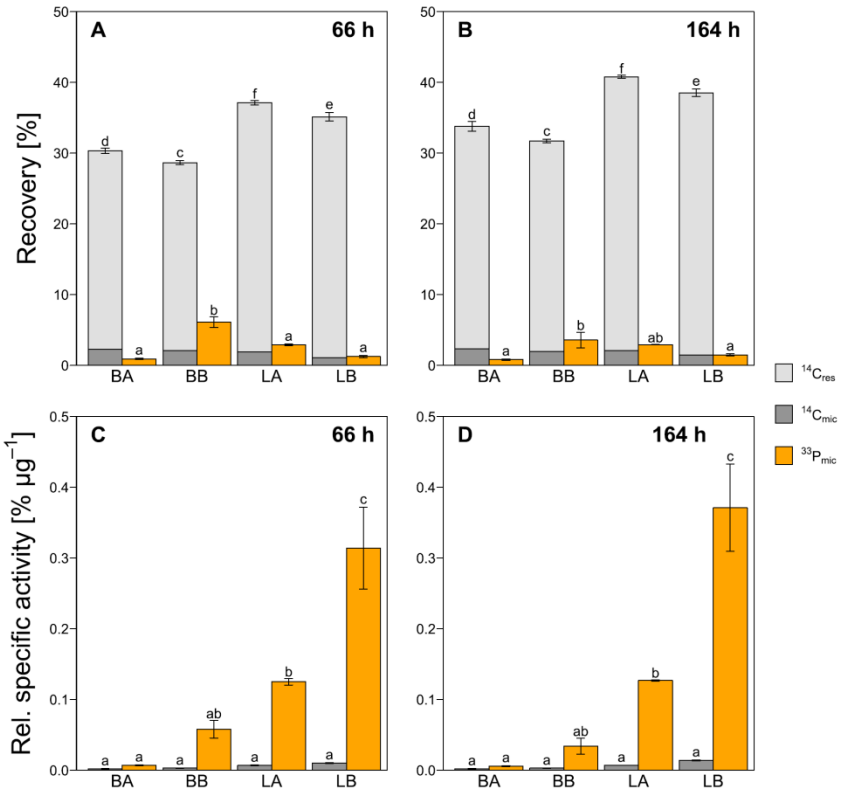

Fig. 5. ^{14}C and ^{33}P recovery in the soil microbial biomass ($^{14}C_{mic}$, $^{33}P_{mic}$) and ^{14}C recovery in respired CO_2 ($^{14}C_{res}$) 66 h (A) and 164 h (B) after addition of the ^{14}C and ^{33}P labeled glucose-6-phosphate, and specific activities of ^{14}C and ^{33}P in the soil microbial biomass 66 h (C) and 164 h (D) after addition of the isotopic tracers. Error bars show standard errors (n = 4), significant differences between soils are marked with lowercase letters.

C_{mic}:N_{mic} ratios might be caused by increases in the ratio of fungal-to-bacterial biomass because fungal C:N ratios often account to 4.5–15 and bacterial C:N ratios to 3–5 (Paul and Clark, 1996). However, this cannot be tested based on our data since the ARISA does not allow the calculation of fungal-to-bacterial biomass ratios because fungal and bacterial DNA are determined with different pairs of primers.

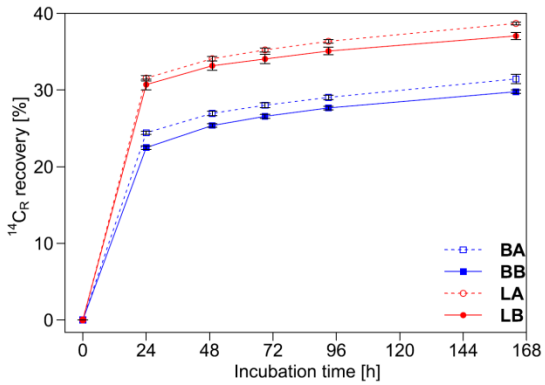

Fig. 6. Cumulative ^{14}C recovery in CO_2 respired by soil microorganisms ($^{14}C_R$) of the four studied soils during the first 166 h after addition of ^{14}C-glucose-6-phosphate. Error bars show standard errors (n = 4).

The variations of the microbial C:N:P ratios were statistically related to the shifts in the soil microbial community, as revealed by Spearman rank correlations. Species favored by element additions likely had a different elemental composition than the previously dominating species, and thus the C:N:P ratios varied depending on the treatment. The bacterial community in the soils BA and LA was best explained by microbial biomass C, N and P, while microbial biomass N and P explained most of the variation of the fungal community after element addition. C addition caused most significant differences in microbial biomass ratios in all soils. It has to be taken into account, that the C:N:P ratios referring to the total soil microbial biomass were connected to microbial community analyses referring to either bacteria or fungi. The correlation might even be better if microbial C, N and P concentrations could be determined separately for fungi and bacteria. Moreover, it is possible, that the appearing changes level out after a few days as we only conducted a short time incubation with measurement of microbial C, N and P after 66 h.

The BA soil did not show significant variations in soil microbial C:N:P ratios nor in microbial communities as have been found in the other soils. The available C, N and P concentrations probably met the need of the microorganisms, so that further element additions had only very little effect on both microbial biomass stoichiometry and community composition. It is also possible that the soil microbial community would have undergone further shifts if a longer incubation time after element addition were chosen. The results presented here highlight only the specific situation at one time point. Since fungi are assumed to be growing slower than

bacteria (Six et al., 2006), the reaction of the fungal communities to element addition treatments might not have been completed.

Besides shifts in the microbial community, the uptake of C, N or P in excess as well as storage of these elements in soil microorganisms could influence molar microbial biomass C:N:P ratios.

Bacteria are known to store C in form of glycogen, starch or lipids depending on C availability (Wilkinson, 1963; Wilson et al., 2010). The amount of C stored in bacteria can be as high as 20–40% of their dry weight, as determined under laboratory conditions (Wilkinson, 1963). As C was added in the form of easily available glucose, it is likely that soil microorganisms used this substrate to build up C storage molecules, causing high C_{mic} concentrations.

In addition, microorganisms are able to take up P efficiently in their cells. High P availability causes non-homeostatic behavior in microorganisms (Scott et al., 2012), while P limitation promotes homeostasis (Makino et al., 2003). P storage molecules can amount to 10–20% dry weight in yeast cells (Kornberg, 1995) and >10% dry weight in some bacteria (Deinema et al., 1985). Excess P uptake could explain the increase in P_{mic} independent of C_{mic} observed in soil LA and LB.

N_{mic} concentrations also increased after N addition, but the effect was smaller than for C or P addition. Soil microorganisms probably also used the added easily available N, but since there is no known N storage form (Banham and Whatley, 1991; Mooshammer et al., 2014), they were unable to keep high amounts of excess N in their cells. The P_{mic} concentrations in the studied soils were often almost equal to or even higher than N_{mic} concentrations, leading to the low N_{mic}:P_{mic} ratios. Similarly low N_{mic}:P_{mic} ratios have been found in German forest soils before (Khan and Joergensen, 2012).

4.2. Microbial element limitation

Carbon appears to be the element limiting soil microbial biomass at both sites, Bad Brueckenau and Luess, confirming our second hypothesis. This is indicated by increases in the C_{mic} concentrations after addition of C alone or in combination with other elements. Our findings corroborate many other studies on the C limitation of soil microorganisms in temperate forest soils (e. g. Joergensen et al., 1990; Aldén et al., 2001; Ekblad and Nordgren, 2002; Demoling et al., 2007). N or P addition had no positive effect on C_{mic} in the soils of either site, suggesting that these elements are not the major limiting elements. Still each of them appeared to be second limiting in one or two of the examined soils. In the soils BA and LA, CN addition led to the highest C_{mic} concentrations. Therefore, N seems to be the second limiting nutrient in these soils. In the soil LB, the second limiting nutrient was likely P. Thus, although the available P concentrations in the Luess soils appear to be scarce, the growth of soil microbial biomass did not seem to be impaired by them in the first place. Still, the soil microbial biomass of the soils LA and LB incorporated high amounts of P after P containing element addition treatments. Previous studies also found N or P as second limiting nutrients of the soil microbial biomass when the C limitation was eliminated, while single N or P addition usually had no impact on microbial growth (Allen and Schlesinger, 2004; Demoling et al., 2007; Kamble and Bååth, 2014).

The soil microorganisms at the site Bad Brueckenau did hardly adapt their biomass stoichiometry after element addition, as can be seen from the small changes between C:N:P ratios in soils BA and BB, which were not significant in most cases (Table 3). The soil microbial biomass at the site Luess reacted more strongly to element additions by taking up C, N and P, which is mainly to be seen in the high variability of the C:N:P ratios between the treatments in the soil LB. The differences between the sites might occur because the soil microbial C and nutrient demand was already satisfied by the resources available in the soils of Bad Brueckenau.

4.3. Microbial use of organic phosphorylated compounds

The results of the tracer experiment support the hypothesis that the soil microorganisms were C limited. The added glucose-6-phosphate was mainly used as a C source and not as a P source. It could be that glucose-6-phosphate was taken up as an intact molecule and that most of the P was later released by the microorganisms. It might also be that the organic P was mineralized outside the cells by exoenzymes and that C was taken up faster than the P by the microorganisms. In any case, the results of the tracer experiment indicate that the microorganisms used more C than P from the glucose-6-phosphate, indicating that organic P mineralization was driven by microbial need for C. This is in contrast to a common theory by McGill and Cole (1981) that claims decoupled organic P and C mineralization. The theory states that mineralization of P-esters is induced exclusively by microbial need for P, while C and N are mineralized because C is needed as an energy source.

Although glucose-6-phosphate can be taken up as an intact molecule by bacteria with the Uhp transport system (Winkler, 1973; Sonna et al., 1988; Kadner et al., 1994), it is usually enzymatically hydrolyzed before uptake (Louche et al., 2010; Plassard et al., 2011). As the soil microbial biomass in this study was mainly C limited, P mineralization seems to have been activated by microbial need for C, and P limitation was not the driving force for P mineralization as suggested by McGill and Cole (1981). Hence, the larger fraction of mineralized P remained in the soil. Spohn and Kuzyakov (2013) also found P mineralization in temperate forest soils not being driven by microbial P deficiency, but by C limitation in a study using ^{14}C and ^{33}P as isotopic tracers. Microbial mineralization of organic P caused by microbial C demand most likely contributes to the P supply of plants. This might be especially important in temperate forest soils with low P availability.

4.4. Conclusions

We found that the soil microorganisms reacted not strictly homeostatically to element additions, especially at the P-poor site. The variations seem to be partly caused by shifts in the community of soil microorganisms, although storage of C or P by microorganisms could also have occurred. In addition, we found that soil microorganisms used glucose-6-phosphate mainly as source for C, leaving inorganic P in the soil. This mechanism might increase P availability to plants at sites where P is limiting for plants, but not for microorganisms.

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Corrigendum to Heuck et al. (2015) 'Soil microbial biomass C:N:P stoichiometry and microbial use of organic phosphorus', Soil Biology & Biochemistry 85, 119-129

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The authors regret to inform that the values of the ^{14}C and ^{33}P recovery from labeled glucose-6-phosphate in the soil microbial biomass were calculated incorrectly in Figure 5A and B. This leads to the following changes in Section 3.2 "*C and P tracer experiment*".

- The recovery of ^{33}P in the soil microbial biomass was about 2- to 80-times smaller than the sum of the recovery of ^{14}C in the microbial biomass and respired CO_2 of all four soils after 66 h as well as 164 h incubation time.
- The ^{14}C recovery in microbial biomass and CO_2 was on average 14% higher in the A horizons than the B horizons of both sites. This difference was significant for the site Luess after 66 h and for both Bad Brueckenau and Luess after 164 h.

In addition, the C:N:P ratio of the soil BB (Bad Brueckenau, B horizon) was calculated wrongly for the P addition treatment (one line of Table 3). The corrected figure and table are attached below. To improve the clarity of the microbial biomass stoichiometry data, we also added a table of microbial C:N, C:P and N:P ratios (Table 5).

These corrections neither impact the discussion nor the conclusions of the study. The authors apologize for any inconvenience.

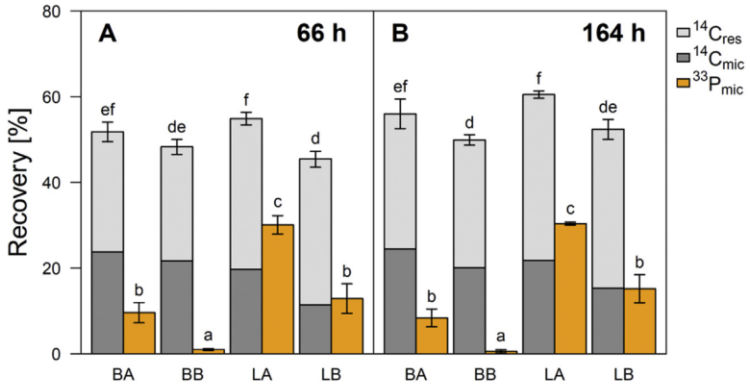

Fig. 5 (corrected) ^{14}C and ^{33}P recovery in the soil microbial biomass ($^{14}C_{mic}$, $^{33}P_{mic}$) and ^{14}C recovery in respired CO_2 ($^{14}C_{res}$) 66 h (A) and 164 h (B) after addition of the ^{14}C or ^{33}P labeled glucose-6-phosphate to four mineral soils. The soils are BA: Bad Brueckenau, A horizon; BB: Bad Brueckenau, B horizon; LA: Luess, A horizon; LB: Luess, B horizon. Error bars show standard errors (n = 4) and significant differences between all columns are marked with different lowercase letters.

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Table 3. Molar C:N:P ratios of the microbial biomass of the four soils after addition of carbon (C), nitrogen (N) and phosphorus (P) in a full factorial design. The soils are BA: Bad Brueckenau, A horizon; BB: Bad Brueckenau, B horizon; LA: Luess, A horizon; LB: Luess, B horizon. Ratios are given with 90% confidence intervals and different lowercase letters mark significant differences between element-addition treatments tested separately for each soil **(Corrected values: soil BB, P treatment).**

Treatment	Soil			
	BA	BB	LA	LB
Ctrl.	26.1 ± 8.0: 5.4 ± 0.8: 1a	18.0 ± 5.9: 2.4 ± 0.8: 1a	31.7 ± 1.9: 4.1 ± 0.2: 1a	79.3 ± 5.2: nd*: 1abc
C	26.5 ± 6.3: 5.9 ± 0.4: 1a	45.4 ± 0.0: 8.1 ± 0.5: 1b	94.3 ± 22.0: 2.3 ± 0.9: 1b	510.9 ± 241.1: nd*: 1c
N	30.1 ± 6.9: 5.1 ± 1.4: 1ab	7.9 ± 1.8: 1.0 ± 0.1: 1a	39.0 ± 0.7: 7.3 ± 0.7: 1a	89.2 ± 7.9: 10.8 ± 5.4: 1abc
P	36.5 ± 15.8: 4.8 ± 1.8: 1ab	12.1 ± 0.6: 1.5 ± 0.1: 1a	40.6 ± 16.8: 5.3 ± 2.0: 1a	6.9 ± 3.5: 0.2 ± 0.2: 1b
CN	43.2 ± 6.4: 5.3 ± 0.9: 1ab	6.9 ± 2.8: 0.8 ± 0.3: 1a	178.3 ± 38.0: 7.1 ± 0.9: 1c	425.0 ± 307.0: 11.8 ± 7.6: 1c
CP	38.8 ± 6.1: 5.3 ± 0.9: 1ab	10.7 ± 1.9: 0.9 ± 0.2: 1a	33.7 ± 8.7: 4.0 ± 1.6: 1a	471.8 ± 108.2: nd*: 1c
NP	47.9 ± 19.4: 5.8 ± 2.2: 1ab	4.8 ± 1.0: 0.7 ± 0.5: 1a	8.6 ± 4.8: 1.9 ± 0.9: 1a	5.9 ± 0.7: 1.0 ± 0.6: 1a
CNP	53.6 ± 1.1: 6.5 ± 2.2: 1b	10.3 ± 1.8: 1.2 ± 0.3: 1a	41.0 ± 33.4: 3.5 ± 2.5: 1a	110.5 ± 40.7: 4.4 ± 2.1: 1abc

*N_{mic} concentrations were below the detection limit (12.5 µg N_{mic} g^{-1} soil).
Additional material.

Table 5 Microbial C:N, C:P and N:P ratios in the A and B horizons of the sites Bad Brueckenau (BA, BB) and Luess (LA, LB) as respondent to full factorial additions of labile C, N and P. Values are given as mean with standard deviation (n = 4), significant differences are marked by asterisks. Levels of significance were * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Soil	Treatment	C:N ratio	C:P ratio	N:P ratio	Soil	Treatment	C:N ratio	C:P ratio	N:P ratio
BA	Control	4.7 ± 1.0	26.1 ± 9.6	5.4 ± 1.0	LA	Control	7.8 ± 0.2	31.7 ± 2.3	4.1 ± 0.2
	C	4.5 ± 1.1	26.5 ± 7.7	5.9 ± 0.5		C	47.1 ± 20.1***	94.3 ± 26.6*	2.3 ± 1.1
	N	6.3 ± 1.7	30.1 ± 8.3	5.1 ± 1.7		N	5.4 ± 0.5	39.0 ± 0.8	7.3 ± 0.8
	P	7.5 ± 1.9	36.5 ± 19.2	4.8 ± 2.2		P	7.6 ± 0.6	40.6 ± 20.4	5.3 ± 2.4
	CN	8.2 ± 0.6	43.2 ± 7.7	5.3 ± 1.1		CN	25.4 ± 6.3	178.3 ± 46.0***	7.1 ± 1.1
	CP	7.4 ± 0.2	38.8 ± 7.4	5.3 ± 1.1		CP	8.9 ± 1.6	33.7 ± 10.5	4.0 ± 1.9
	NP	8.1 ± 0.6	47.9 ± 23.5	5.8 ± 2.7		NP	4.5 ± 1.3	8.6 ± 5.8	1.9 ± 1.1
	CNP	9.3 ± 3.7*	53.6 ± 1.3	6.5 ± 2.6		CNP	11.6 ± 2.9	41.0 ± 40.5	3.5 ± 3.1
BB	Control	7.5 ± 0.1	18.0 ± 7.2	2.4 ± 0.9	LB	Control	–	79.3 ± 6.3	–
	C	5.6 ± 0.4	45.4 ± 0.0	8.1 ± 0.6*		C	–	510.9 ± 292.3*	–
	N	8.0 ± 1.7	7.9 ± 2.2	1.0 ± 0.1		N	12.0 ± 8.5	89.2 ± 9.6	10.8 ± 6.6
	P	8.2 ± 0.9	12.1 ± 0.8	1.5 ± 0.1		P	86.1 ± 72.9	6.9 ± 4.3	0.2 ± 0.2
	CN	8.9 ± 1.6	6.9 ± 3.4	0.8 ± 0.4		CN	32.9 ± 18.1	425.0 ± 372.2	11.8 ± 9.2
	CP	11.7 ± 1.6	10.7 ± 2.3	0.9 ± 0.2		CP	–	471.8 ± 131.2	–
	NP	10.3 ± 4.8	4.8 ± 1.2	0.7 ± 0.6		NP	8.2 ± 4.8	5.9 ± 0.8	1.0 ± 0.7
	CNP	8.9 ± 1.3	10.3 ± 2.2	1.2 ± 0.4		CNP	28.2 ± 8.2	110.5 ± 49.4	4.4 ± 2.5

Study II

2.2 Carbon, nitrogen and phosphorus net mineralization in organic horizons of temperate forests: stoichiometry and relations to organic matter quality

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Carbon, nitrogen and phosphorus net mineralization in organic horizons of temperate forests: stoichiometry and relations to organic matter quality

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Abstract The rates of mineralization processes influence C sequestration and soil fertility, but despite their importance for ecosystem functioning, C, N and P net mineralization rates are seldom investigated together. Hence, we studied the relationships between net mineralization rates and organic matter stoichiometry in an 8-week incubation experiment with Oi, Oe and Oa horizon material of six beech, one spruce and one pine site. We determined C, N and P net mineralization rates, organic C quality and C:N:P stoichiometry. Net N mineralization only occurred below molar organic matter C:N ratios of 40 (Oi) or 28 (Oa) and N:P ratios of 42 (Oi) or 60 (Oa), and increased with decreasing C:N and N:P ratios. Net P mineralization only occurred below C:P ratios of 1400 (Oi) and N:P ratios of 40 (Oi), and increased with decreasing C:P and N:P ratios. Net N and P mineralization were strongly positively correlated with each other ($r = 0.64$, $p < 0.001$), whereas correlations of both net N and net P mineralization with C mineralization were weak. The average C:N:P stoichiometry of net mineralization was 620:4:1 (beech, Oi), 15,350:5:1 (coniferous, Oi), 1520:8:1 (Oe) and 2160:36:1 (Oa). On average, ratios of C:N net mineralization were higher, and ratios of N:P net mineralization lower than organic matter C:N and N:P ratios. This difference contributed to the decrease of C:N ratios and increase of N:P ratios from the Oi to the Oa horizons. In conclusion, the study shows that C, N and P net mineralization rates were closely correlated with the organic matter stoichiometry and that these correlations were modified by the degree of decomposition of the organic matter.

Keywords Critical ratio · Ecological stoichiometry · Forest floor · Nutrient cycling · Respiration · Threshold element ratio

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Introduction

Decomposition of organic matter in forest ecosystems has been studied for decades, and many studies have investigated relations between organic matter stoichiometry and mass loss or mineralization rates (e.g. Bosatta and Staaf 1982; Hättenschwiler and Jørgensen 2010; Manzoni et al. 2010; Mooshammer et al. 2012; Carrillo et al. 2016). Nevertheless, our understanding of carbon (C), nitrogen (N) and phosphorus (P) net

mineralization rates in organic layers is incomplete because in most studies, only net mineralization rates of one or two elements are considered. Moreover, most analyses of net mineralization rates are limited because they consider only ratios of elements but not of the different mineralization rates. Further, processes are mostly studied in the complete organic layer or in one organic horizon, i.e. in one specific decomposition state. Many studies on stoichiometry and mineralization concentrate on young litter (Gosz et al. 1973; Berg and Staaf 1981; Blair 1988; Cortez et al. 1996; Aerts 1997; Berg and Matzner 1997; Craine et al. 2007; Hättenschwiler and Jørgensen 2010; Moore et al. 2011; Mooshammer et al. 2012; Brandstätter et al. 2013) while organic matter in later states of decomposition, such as Oe and Oa horizons of organic layers, is less well studied (McClaugherty and Berg 1987; Berg and Ekbohm 1991; Berg and Matzner 1997; Moore et al. 2011). Taken together, there are gaps in the otherwise extensive variety of topics covered by studies on decomposition, which we seek to address here.

Net mineralization of C, N and P is the terminal step of organic matter decomposition. The net mineralization rate of a given element is defined as the gross mineralization rate minus the net immobilization rate. Net C, N and P mineralization are strongly interrelated with each other. N and P can be mineralized along with C in nutrient rich organic matter (McGill and Cole 1981; Parton et al. 2007; Manzoni et al. 2008; Spohn and Kuzyakov 2013; Heuck et al. 2015), and net N and P mineralization may be coupled because of N and P co-limitation of the microbial biomass in organic layers (Marklein et al. 2016).

C, N and P concentrations and organic matter quality strongly influence decomposition and thereby net mineralization. Nutrient-rich litter is known to decompose fast at first (Berg and McClaugherty 2014), but high N concentrations often hinder decomposition in later stages (Berg and Ekbohm 1991; Berg and Matzner 1997; Berg and McClaugherty 2014). In addition, high concentrations of easily degradable organic C, such as sugars or carbohydrates, result in high decomposition rates, while high lignin concentrations usually cause slow decay (Blair 1988; Scott and Binkley 1997; Berg and McClaugherty 2014). Ratios like lignin:N or C:N ratios can also be related to decomposition (Taylor et al. 1989; Cortez et al. 1996; Zhang et al. 2008). However currently, there is no consensus on whether stoichiometry or organic C chemistry is a better predictor for decomposition (Swift et al. 1979; Heal et al. 1997; Zhang et al. 2008; Hättenschwiler and Jørgensen 2010; Mooshammer et al. 2012).

Mineralization of C, N and P is largely catalyzed by microorganisms, which are affected by organic matter chemistry. There is usually a large difference between the average soil microbial biomass C:N:P stoichiometry of on average 60:7:1–42:6:1 (Cleveland and Liptzin 2007; Xu et al. 2013) and the stoichiometry of temperate broadleaf (1702:29:1) and coniferous (2353:26:1) litter (McGroddy et al. 2004). Above certain C:N or C:P ratios of organic matter, net N and P mineralization rates cease because the microbial biomass immobilizes all available N and P under nutrient-poor conditions to maintain its biomass stoichiometry (Spohn 2016). The ratios at which microbial net N or P mineralization become larger than net immobilization or vice versa are called threshold ratios (Bosatta and Staaf 1982). Theoretical threshold C:N and C:P ratios are often calculated as 10–30 and 80–160, respectively (Kaiser et al. 2014; Spohn and Chodak 2015). Net mineralization is expected to increase with decreasing ratios, but this has not yet been tested experimentally. Empirical threshold ratios derived from case studies, mostly litterbag experiments, were between 20 and 40 for C:N ratios (Gosz et al. 1973; Blair 1988; Parfitt et al. 1998; Parton et al. 2007; Moore et al. 2011) and between 300 and 1700 for C:P ratios (Edmonds 1980; Blair 1988; Parfitt et al. 1998; Saggar et al. 1998; Moore et al. 2011), while threshold N:P ratios for net N and P mineralization are missing in the literature.

The aim of this study was to investigate net C, N and P mineralization in organic matter in different states of decomposition, focusing on the relationships between net mineralization rates, C:N:P stoichiometry and the quality of organic matter. Based on laboratory incubations, we tested the hypotheses (i) that the C:N:P stoichiometry of organic matter determines the rate of net C, N and P mineralization in Oi, Oe and Oa horizons of organic layers, (ii) that net N and P mineralization occurs only below threshold C:N, C:P and N:P ratios, and (iii) that the C:N:P stoichiometry of net C, N and P mineralization rates corresponds to the development of the C:N:P stoichiometry of organic matter with increasing depth in organic layers.

Materials and methods

Study sites and sampling

We sampled six beech forest sites, one spruce and one pine site in Germany in summer 2015 (Table 1). The inclusion of coniferous sites broadened the range of organic matter C:N:P stoichiometry covered by the experiment. At each site, one field—representative composite sample of the Oi, Oe and Oa horizon was taken by combining material of four sampling points into one composite sample per horizon, which was immediately transferred to the laboratory. Composite samples were homogenized by hand, pieces of wood and bark, fruits, stones and larger organisms were removed, and the beech leaves of the Oi horizons were cut into 1–2 cm^2 pieces using scissors. Until further processing, samples were stored at 5 °C. Water content and maximum water holding capacity were determined gravimetrically.

Determination of organic matter characteristics

All analyses were conducted in four replicates drawn from each composite sample taken in the field. Aliquots of the samples were dried at 60 °C for 48 h, and ground in a ball mill (Retsch) for the determination of main chemical characteristics of organic matter. C and N concentrations were measured by a CN analyzer (Vario MAX, Elementar) and P concentrations were determined by inductively coupled plasma-optical emission spectroscopy (ICP-OES, Vista-Pro radial, Varian) after pressure digestion in concentrated nitric acid. Total carbohydrate concentrations were determined after hydrolysis of 50 mg organic matter with sulfuric acid according to Kögel-Knabner (1995). Concentrations of soluble phenolic-like compounds were determined by the Folin–Ciocalteau method in 100 mg of ground samples (Bärlocher and Graça 2007). The quality of dissolved organic C was assessed in cold water extracts of moist organic matter, equivalent to 5 g dry weight and extracted with an organic matter: solution ratio of 1:40 (w/v). The humification index (HIX) of dissolved organic C in the water extracts was calculated according to Zsolnay et al. (1999) from fluorescence emission spectra (SFM 25, BIO-TEK Instruments). Specific UV absorbance at 280 nm (SUVA) was taken as a measure for the aromaticity of dissolved organic carbon (McKnight et al. 1997; Kalbitz et al. 2003) and determined with a spectrophotometer (UV 1800, Shimadzu).

Determination of net C, N and P mineralization rates and microbial biomass

To determine net N and P mineralization, 120 g dry-weight-equivalent of each sample were adjusted to a water content of 60% of the maximum water holding capacity and pre-incubated at 15 °C for two weeks in 2 l PE bottles. For each horizon, four replicates were prepared from the composite sample. Subsamples of 5 g dry-weight-equivalent were removed from each replicate and extracted in distilled water in a ratio of 1:40 (w/v) after 0, 5, 15, 22, 36, and 50 days. Extracts were filtered using 0.45 µm cellulose-acetate filters (Sartorius) and stored at 2 °C until analysis. NH_4 and NO_3 concentrations in the extracts were measured with flow-injection analysis (FIA-Lab, MLE Dresden) and PO_4 was determined spectrophotometrically (UV 1800, Shimadzu) following the method of Murphy and Riley (1962).

C mineralization was continuously measured over the incubation period of 50 days with a respirometer (Respicond V, Nordgren Innovations). Aliquots of 0.5 g (Oi horizons), 1.0 g (Oe horizons) and 2.0 g (Oa horizons) dry-weight-equivalent were adjusted to approximately 60% of the maximum water holding capacity in 250 ml plastic beakers and pre-incubated as above. Respired CO_2 was captured in traps filled with 10 ml 0.5 M KOH and CO_2 respiration was calculated from the change of conductance in KOH solutions over time. To prevent erroneous CO_2 measurements due to decreasing absorption capacity of the KOH solution with increasing CO_2 saturation, KOH solutions were renewed when 50% of the CO_2-absorption capacity was reached.

After 40 days of incubation, subsamples of 1 g dry-weight-equivalent were taken from each replicate to determine its microbial biomass by substrate induced respiration (Anderson and Domsch 1978). The optimal amount of added glucose and incubation time of organic matter treated with glucose solution was identified for the Oi, Oe and Oa horizons separately in preliminary tests.

Data analyses and statistics

All element ratios were calculated on a molar basis. Net N mineralization, net P mineralization and net C

Table 1 Description of the general characteristics of the eight study sites Bad Brueckenau (BBR), Conventwald (CON), Geißmann (GEI), Unterluess (LUE), Mitterfels (MIT), Steigerwald (STW), Vessertal (VES) and Waldstein (WST)

Site	Geographical position	Elevation (m a.s.l.)	Mean annual temperature (°C)	Mean annual precipitation (mm)	Dominating tree species	Parent material	Soil type (FAO)	Humus form	Organic horizon	Depth (cm)
BBR	N50°21′E 009°55′	809	5.8	1031	*Fagus sylvatica* L.	Basalt	Eutric Cambisol	Mull-like moder	Oi	3
									Oe	12
CON	N48°01′E 007°57′	884	6.8	1749	*Fagus sylvatica* L. *Picea abies* L.	Gneiss	Dystric Cambisol	Mull-like moder	Oi	3
									Oe	5
									Oa	5
GEI	N49°57′E 011°28′	360	8.4	634	*Pinus sylvestris* L.	Keuper	Cambisol	Mull	Oi	1
LUE	N52°50′E 010°16′	115	8.0	779	*Fagus sylvatica* L.	Glacial sandy material	Spodic Cambisol	Mor-like moder	Oi	4
									Oe	5
									Oa	4
MIT	N48°58′E 012°52′	1033	4.5	1229	*Fagus sylvatica* L.	Gneiss	Dystric Cambisol	Mor-like moder	Oi	4
									Oe	3
									Oa	1
STW	N49°52′E 010°27′	440	7.9	787	*Fagus sylvatica*, L. *Quercus robur* L.	Upper Keuper	Dystric Cambisol	Moder	Oi	1
									Oe	1.5
									Oa	0.5
VES	N50°36′E 010°46′	810	6.0	1200	*Fagus sylvatica* L.	Trachyandesite	Dystric Cambisol	Moder	Oi	5
									Oe	6
									Oa	3
WST	N50°08′E 011°52′	765–785	5.3	1162	*Picea abies* L.	Porphyritic granites, phyllites, quartzite	Cambisol	Mor	Oi	0.5
									Oe	5
									Oa	3

mineralization rates were calculated as the slope of the linear regressions of the concentrations of NH_4-N + NO_3-N, PO_4-P and CO_2-C over time.

We tested the chemical characteristics of organic matter for differences in respect to (i) vegetation type and (ii) organic horizon in two separate analyses. In the first analysis, data was pooled according to vegetation type so differences between beech and coniferous organic matter could be identified by Welch tests. If data were not normally distributed, Wilcoxon rank sum tests were used instead. In the second analysis, data was pooled by vegetation type and organic horizon and analyzed with Kruskal–Wallis tests. For significant results, the multiple comparisons test according to Dunn (Pohlert 2014) was used as post hoc test. These tests were chosen because most of the data was not normally distributed.

Relations among net C, N and P mineralization rates were assessed by Spearman rank correlations. Correlations of the mineralization rates were calculated (i) separately for the Oi, Oe and Oa horizons and (ii) for the complete organic layer. Regressions were calculated for (i) net N mineralization as a function of C:N and C:P ratios of organic matter, and (ii) net P mineralization as a function of C:P and N:P ratios of organic matter. The threshold element ratio, below which net mineralization of the respective nutrient occurred, was calculated as the intersection of each regression line with the x-axis. To obtain this value, the regression equations were solved for y (net mineralization rate = 0). The residuals of each regression were tested for normal distribution and homoscedasticity. Robust linear regression (MM-estimation with bisquare weighting, Yohai 1987; Koller and Stahel 2011) was computed instead of ordinary least squares regression when residuals were not normally distributed due to outliers using the R package "robustbase" (Rousseeuw et al. 2015). The standard errors of the regression coefficients were calculated by a robust method (White estimator, Zeileis 2004) to determine p values, when residuals showed heteroscedasticity using the R package "sandwich" (Zeileis 2004). The relationship between chemical characteristics of organic matter and C, N and P net mineralization was analyzed by a principal component analysis. All statistical analyses were conducted in R (R Core Team 2015) with $p < 0.05$ considered as the threshold for significance.

Results

Organic matter quality

Organic matter quality differed significantly between the Oi, Oe and Oa horizons (Table 2). C and P concentrations decreased from the Oi to the Oa horizons by 20 and 30%, respectively, in beech organic layers, and by 30 and 20%, respectively, in coniferous organic layers, while N concentrations stayed constant. N and P concentrations in most horizons across beech and coniferous litter were correlated (Oi: $r = 0.69$, $p < 0.001$, Oe: $r = 0.13$, $p = 0.5$, Oa: $r = 0.62$, $p < 0.001$, total organic layer: $r = 0.38$, $p < 0.001$), and there was also a strong positive correlation between C and N concentrations except in the Oi horizons (Oi: $r = -0.32$, $p = 0.08$, Oe: $r = 0.80$, $p < 0.001$, Oa: $r = 0.90$, $p < 0.001$, total organic layer: $r = 0.40$, $p < 0.001$). In contrast, C and P concentrations were only weakly correlated. The C:N ratios of organic matter decreased from 31.8 in the Oi horizons to 24.2 in the Oa horizons, while the N:P ratios increased from 29.5 in the Oi horizons to 43.1 in the Oa horizons. The C:P ratios of organic matter remained constant throughout the organic layer (Fig. 1).

Concentrations of carbohydrates and soluble phenolics decreased from the Oi to the Oa horizons by 40 and 60%, respectively, in beech, and 70 and 60%, respectively, in coniferous organic layers, while the specific UV absorption and humification index of dissolved organic C increased with depth by 7 and 30%, respectively, in beech, and by 160 and 500%, respectively, in coniferous organic layers. The size of the microbial biomass declined from the Oi to the Oa horizons by 80 and 90%, respectively, in beech and coniferous organic layers.

The chemical composition of the coniferous Oi horizons differed from the beech Oi horizons (Table 2). Compared to beech, concentrations of total C and carbohydrates in the coniferous Oi horizons were 10 and 30% higher, and concentrations of soluble phenolics were doubled. The humification index and specific UV absorption at 280 nm were lower in beech than in coniferous organic matter by 60 and 70%, respectively. However, N concentrations in coniferous litter were 15% lower than in beech litter. No pronounced differ-

Table 2 Characteristics of the Oi, Oe and Oa horizons of the beech forest sites Bad Brueckenau (BBR), Conventwald (CON), Unterluess (LUE), Mitterfels (MIT), Steigerwald (STW), Vessertal (VES) and the coniferous forest sites Geissmann (GEI) and Waldstein (WST). Specific UV absorption and humification index were measured in cold-water extracts of initial samples. Capital letters mark significant differences between the Oi, Oe and Oa horizons. One analysis was conducted for beech and one for coniferous sites. Significance was presumed at $p < 0.05$

Vegetation type	Organic horizon	Site	Total C (mg g^{-1})	Total N (mg g^{-1})	Total P (mg g^{-1})	Total soluble phenolics (μg g^{-1})	Total carbohydrates (mg g^{-1})	Specific UV absorption (280 nm)	Humification index	Microbial biomass (mg g^{-1})
			A	A	A	A	A	A	A	A
Beech	Oi	BBR	430.5	18.0	1.66	203.2	87.0	27.6	9.1	30.9
Beech	Oi	CON	459.5	17.1	1.07	341.6	93.8	30.7	9.6	41.4
Beech	Oi	LUE	422.0	17.1	1.24	238.2	95.7	33.6	12.9	37.9
Beech	Oi	MIT	469.8	17.5	1.20	423.4	97.3	30.7	9.0	33.6
Beech	Oi	STW	442.6	16.2	1.30	278.7	101.8	29.1	13.2	47.4
Beech	Oi	VES	449.2	19.2	1.71	276.7	98.4	35.4	10.9	27.5
			B	A	B	B	B	B	B	B
Beech	Oe	BBR	333.0	15.5	1.67	93.6	59.7	30.3	12.6	22.8
Beech	Oe	CON	464.3	18.0	0.97	228.5	88.7	34.7	12.1	34.1
Beech	Oe	LUE	392.3	15.6	0.95	160.6	79.1	32.9	15.4	36.5
Beech	Oe	MIT	469.9	21.5	1.06	257.6	83.7	33.9	11.1	10.8
Beech	Oe	STW	412.9	17.4	1.06	163.7	74.5	35.6	15.8	32.2
Beech	Oe	VES	417.6	19.2	1.14	201.1	75.8	31.4	15.1	23.1
			C	A	B	C	C	B	C	C
Beech	Oa	CON	426.1	19.3	0.88	159.4	62.1	32.9	14.2	8.3
Beech	Oa	LUE	358.0	15.1	0.56	133.1	63.1	34.4	13.5	4.9
Beech	Oa	MIT	425.0	22.7	1.31	143.8	55.5	37.8	16.1	7.4
Beech	Oa	STW	266.3	13.3	0.76	102.5	51.6	40.2	16.5	9.0
Beech	Oa	VES	276.4	14.9	1.08	60.4	37.9	21.6	10.7	1.8
			A	A	A	A	A	A	A	A
Coniferous	Oi	GEI	490.7	13.7	0.91	437.6	135.5	10.4	1.3	27.1
Coniferous	Oi	WST	482.7	16.6	1.31	809.1	106.3	15.6	4.4	34.9
			AB	B	A	AB	AB	AB	AB	AB
Coniferous	Oe	WST	463.3	19.0	1.10	364.9	85.1	27.3	10.0	10.8
			B	AB	A	B	B	B	B	B
Coniferous	Oa	WST	353.7	16.6	0.87	160.3	52.8	33.6	17.7	4.1

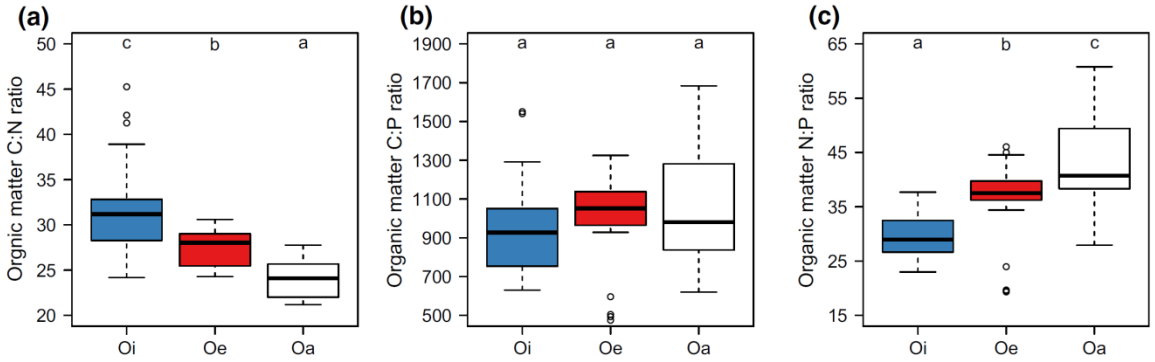

Fig. 1 Boxplots of molar C:N (**a**), C:P (**b**) and N:P ratios (**c**) of organic matter of the Oi, Oe and Oa horizons. *Lowercase letters* indicate significant differences as tested by Kruskal–Wallis tests followed by Dunn's tests. Significance was presumed at $p < 0.05$

ences between coniferous and deciduous Oe and Oa horizons were observed.

Net mineralization rates

C, N and P net mineralization rates decreased from the Oi to the Oa horizons (Figs. 2, 3; Online Resource 1) and always followed the order net C mineralization > net N mineralization > net P mineralization. Net C mineralization ranged from 5.0 ± 0.8 to 128.2 ± 5.0 µmol C g^{-1} d^{-1} and net N and P mineralization ranged from 0 to 1.58 ± 0.51 µmol N g^{-1} d^{-1} and 0.49 ± 0.01 µmol P g^{-1} d^{-1}, respectively. Net N mineralization rates in coniferous Oi horizons amounted only to 4% of the rates determined in beech Oi horizons. Similarly, net P mineralization rates in coniferous Oi horizons amounted only to 7% of the rates in beech Oi horizons. In contrast, net C mineralization was on average 65% higher than in beech (Fig. 3). As for organic matter quality parameters, differences between coniferous and beech organic matter only existed in the Oi horizons.

The strength of the correlations between C, N and P net mineralization rates differed between organic horizons (Table 3; Fig. 2). Net N and P mineralization rates correlated in the Oi horizons, the Oe horizons and the complete organic layer. This was the strongest relationship found for the measured net mineralization rates ($r > 0.60$). Net C mineralization correlated moderately with net P mineralization in the Oi horizons and the total organic layer ($r = 0.50$). The correlation between net C and N mineralization was the weakest ($r < 0.30$) and was only found for the total organic layer. None of the net mineralization rates correlated significantly in the Oa horizons (Table 3).

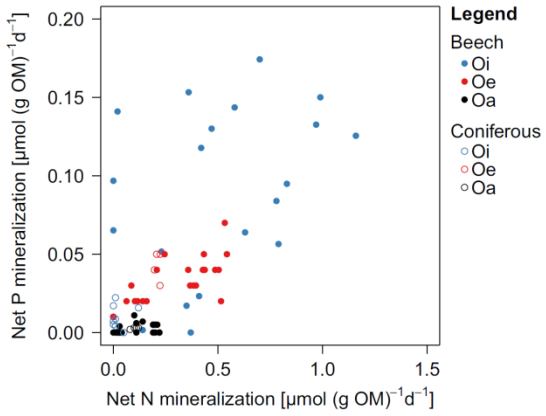

Fig. 2 Relationship of net N and P mineralization rates in the Oi, Oe and Oa horizons of beech and coniferous forest sites

Relations of net mineralization rates and organic matter C:N:P stoichiometry

Net C mineralization increased significantly with increasing C:N and C:P ratios of organic matter in the Oi and Oe horizons but not in the Oa horizons (Fig. 3a, b; Table 4). The relationship of net C mineralization and C:P ratios of organic matter was weaker than between net C mineralization and C:N ratios of organic matter, and it diminished with increasing depth of the organic layers (Table 4). Both the highest C mineralization rates and the highest C:N and C:P ratios of the organic matter appeared in coniferous samples.

Threshold C:N and N:P ratios, above which net N mineralization almost ceased, existed in the Oi and Oa horizons (Fig. 3c, d; Table 4), whereas in the Oe

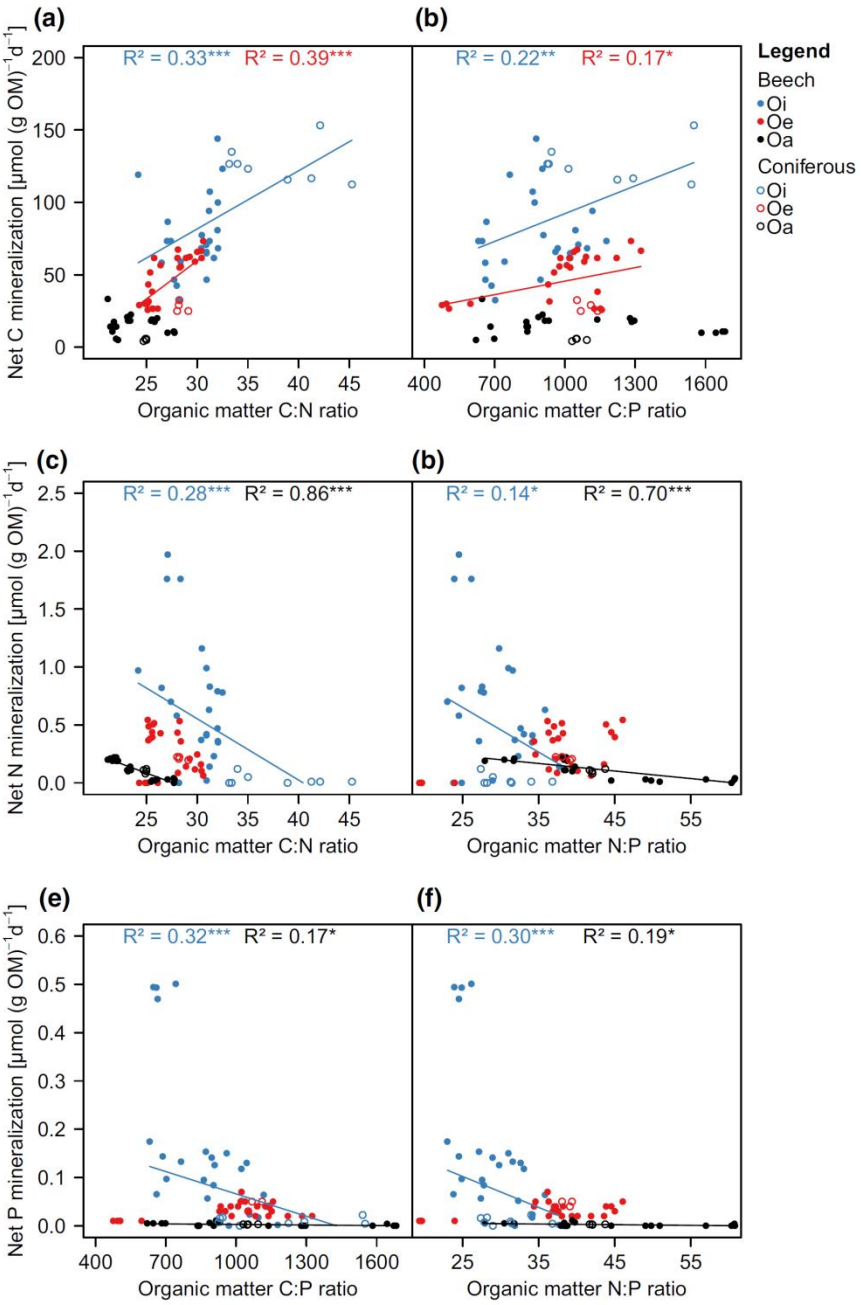

Fig. 3 Relationship of net C mineralization rates and C:N and C:P ratios of organic matter (**a**, **b**), net N mineralization rates and C:N and N:P ratios of organic matter (**c**, **d**), and net P mineralization rates and C:P and N:P ratios of organic matter (**e**, **f**). Beech and coniferous samples were combined for the analysis. Only significant linear regressions are shown (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) and R^2 are given in the *color* corresponding to each horizon

horizons no reliable regression could be calculated. The threshold C:N ratios for net N mineralization were 40 (Oi horizons) and 28 (Oa horizons), while the threshold N:P ratios for net N mineralization were 42 (Oi horizons) and 60 (Oa horizons). The relationship between C:N and N:P ratios of organic matter and net N mineralization was stronger in the Oa horizons ($R^2 \geq 0.70$) than in the Oi horizons ($R^2 < 0.30$) in both cases, but the regression slope was steeper in the Oi horizons (Table 4).

A major decrease in net P mineralization with increasing C:P and N:P ratios of organic matter

Table 3 Spearman rank correlation coefficients of the relationships between net C, N and P mineralization in the total organic layer (Oi, Oe and Oa horizons combined in one dataset) and each separate horizon (Oi, Oe, Oa). Significant correlations are marked by asterisks

	Net C mineralization	Net N mineralization
Total organic layer		
Net N mineralization	0.27*	
Net P mineralization	0.50***	0.64***
Oi horizon		
Net N mineralization	−0.15	
Net P mineralization	−0.47**	0.68***
Oe horizon		
Net N mineralization	−0.11	
Net P mineralization	−0.16	0.72***
Oa horizon		
Net N mineralization	−0.05	
Net P mineralization	−0.13	0.31

Levels of significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Table 4 Results of the regression analysis of the relationships between net C, N and P mineralization rates and organic matter C:N:P stoichiometry. Given are the regression equation, R^2, level of significance and the threshold C:N, C:P and N:P ratios of net N and net P mineralization. No threshold ratios are given for net P mineralization in the Oa horizons because of the very shallow slope of the linear regressions. Only significant regressions are presented

Net mineralization rate	Stoichiometric ratio of organic matter	Organic horizon	Linear equation	R^2	Threshold ratios
C	C:N ratio	Oi	$Cmin = -38.881 + 4.018 \times CN$	0.33***	–
C	C:N ratio	Oe	$Cmin = -98.340 + 5.265 \times CN$	0.39***	–
C	C:P ratio	Oi	$Cmin = 28.271 + 0.064 \times CP$	0.22**	–
C	C:P ratio	Oe	$Cmin = 14.532 + 0.031 \times CP$	0.17*	–
N	C:N ratio	Oi	$Nmin = 2.141 - 0.053 \times CN$	0.28***	40
N	C:N ratio	Oa	$Nmin = 0.896 - 0.033 \times CN$	0.86***	28
N	N:P ratio	Oi	$Nmin = 1.616 - 0.039 \times CP$	0.14*	42
N	N:P ratio	Oa	$Nmin = 0.401 - 0.007 \times CP$	0.70***	60
P	C:P ratio	Oi	$Pmin = 0.220 - 1.54 \cdot 10^{-4} \times CP$	0.32***	1400
P	C:P ratio	Oa	$Pmin = 0.006 - 3.73 \cdot 10^{-6} \times CP$	0.17*	–
P	N:P ratio	Oi	$Pmin = 0.267 - 0.007 \times NP$	0.30***	40
P	N:P ratio	Oa	$Pmin = 0.008 - 1.36 \cdot 10^{-4} \times NP$	0.22**	–

Levels of significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

appeared only in the Oi horizons (Fig. 3e, f; Table 4). There, the threshold C:P ratio for net P mineralization was about 1400, and the threshold N:P ratio was 40. For the Oe horizons no threshold element ratio was calculated because the regressions in this horizon were not significant. In the Oa horizons, threshold C:P and N:P ratios could not be calculated despite significant regressions because the mineralization rates were very low, and were not dependent on the soil C:P or N:P ratio.

Ratios of net mineralization rates

Ratios of C:P and N:P net mineralization rates significantly increased with depth of the organic layer, while the ratios of C:N net mineralization did not differ between the Oi, Oe and Oa horizons (Fig. 4). The ratios of C:P net mineralization in the Oi horizons were 40% smaller than in the Oe and Oa horizons, and ratios of N:P net mineralization in the Oi and Oe horizons were 80% lower than in the Oa horizons.

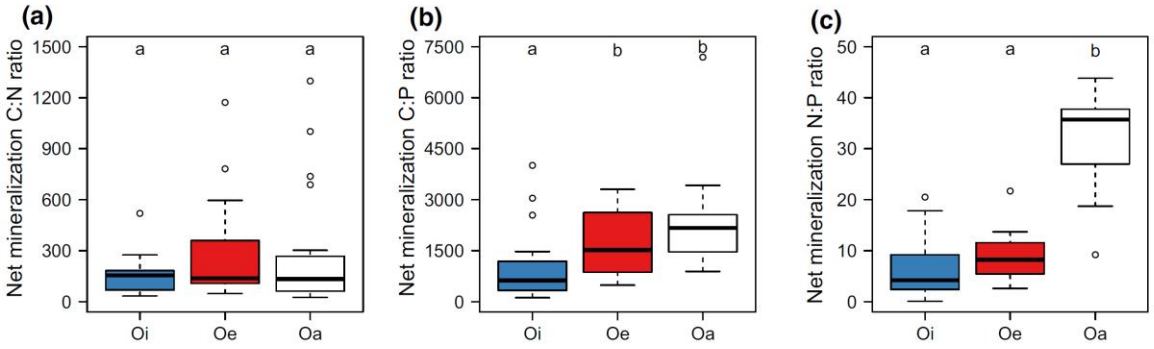

Fig. 4 Boxplots of molar C:N (**A**), C:P (**B**) and N:P ratios (**C**) of net C, N and P mineralization rates in the Oi, Oe and Oa horizons. C:N and C:P ratios of coniferous Oi horizons were excluded because they exceeded the plot limits (both >15,000). *Lowercase letters* indicate significant differences as tested by Kruskal–Wallis tests followed by Dunn's tests. Significance was presumed at $p < 0.05$

Taken together, the overall C:N:P stoichiometry of net mineralization rates in the beech Oi horizons was 620:4:1, and 115,350:5:1 in the coniferous Oi horizons (Fig. 4). As the characteristics of the coniferous Oe and Oa horizons did not differ from beech Oe and Oa horizons, one overall C:N:P ratio was calculated for each horizon. It was 1520:8:1 in the Oe horizons and 2160:36:1 in the Oa horizons.

The stoichiometry of net mineralization rates differed from organic matter stoichiometry (Figs. 1, 4). Ratios of C:N net mineralization were on average 500% higher than C:N ratios, while ratios of C:P net mineralization were 30% smaller than C:P ratios of the Oi horizons but 50 and 100% higher in the Oe and Oa horizons, respectively. Ratios of N:P net mineralization were on average 20% smaller than N:P ratios of the Oi and Oe horizons, but were similar to N:P ratios of the Oa horizons.

Principal component analysis of organic matter chemistry and net C, N and P mineralization

A principal component analysis showed that net N and P mineralization were closely related with each other but clearly separated from C mineralization (Fig. 5). More than 40% of the variability of the whole dataset was explained by principal component 1, which was related to the concentrations of C, carbohydrates and soluble phenolic-like substances in organic matter as well as the humification index and specific UV absorption at 280 nm. Principal component 2 explained 24.9% of the variability, and was determined by organic matter C:N:P stoichiometry and N and P concentrations. C mineralization was closely and positively connected to C concentrations and the concentrations of carbohydrates and soluble phenolics, while it linked negatively to specific UV absorption at 280 nm and the humification index of dissolved organic C in water extracts of organic matter.

Net N and P mineralization were positively associated with P concentrations and negatively with N:P ratios of organic matter, but showed no link to organic matter C quality. The principal component analysis also revealed that data of the same organic horizons were clustered together. The Oi horizons formed one group for beech and one for coniferous sites, both of which were clearly different from the Oe and Oa horizons.

Discussion

Here we reported that organic matter C:N:P stoichiometry strongly affected net C, N and P mineralization in organic layers of temperate forest soils. C mineralization increased with the C:N and C:P ratios of organic matter, and net N and P mineralization ceased beyond threshold C:N, C:P and N:P ratios. Moreover, N and P mineralization were closely related, while C mineralization was largely independent of net N and P mineralization.

Relationships between organic matter C:N:P stoichiometry and net mineralization rates

Organic matter C:N:P stoichiometry was closely related to the net C, N and P mineralization rates. C

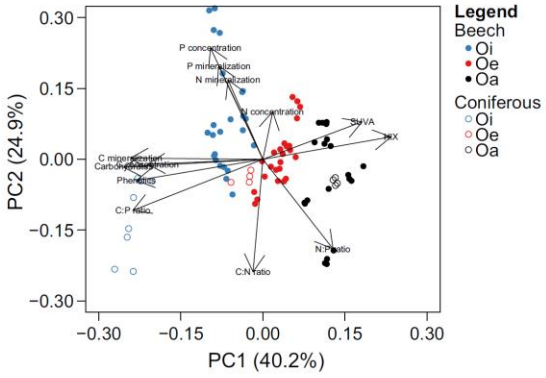

Fig. 5 Biplot of the principal component analysis between net C, N and P mineralization rates and organic matter characteristics of the Oi, Oe and Oa horizons. All organic matter characteristics were determined for dried and ground organic matter samples except SUVA (specific UV absorption at 280 nm) and HIX (humification index), which were measured in cold-water extracts of organic matter

mineralization in the Oi and Oe horizons increased with increasing C:N and C:P ratios of organic matter (Fig. 3a, b) as reported in previous studies (Taylor et al. 1989; Ohtonen 1994; Gödde et al. 1996; Saggar et al. 1998; Michel and Matzner 2002; Spohn 2015; Spohn and Chodak 2015). The increase is probably caused by metabolic adjustments of the soil microorganisms that are necessary due to the difference between the C:nutrient ratios of the organic matter and the soil microbial biomass. Confronted with high C:nutrient ratios, microorganisms could respire excess C (overflow respiration; Russell and Cook 1995; Schimel and Weintraub 2003; Manzoni et al. 2008, 2010; Sinsabaugh et al. 2013) or increase their respiration to gain energy which they can use to acquire nutrients from recalcitrant substrates (nutrient mining; Moorhead and Sinsabaugh 2006; Craine et al. 2007). Both processes would lead to the observed positive relationship between C mineralization and C:nutrient ratios of the organic matter. In the Oa horizons, no relationship between C mineralization and organic matter stoichiometry was found. This is likely because high concentrations of recalcitrant substances decreased C mineralization (McClaugherty and Berg 1987; Berg and Matzner 1997), and replaced organic matter stoichiometry as the principal rate determining parameter.

Net N and P mineralization increased with decreasing C:N, C:P and N:P ratios of organic matter, which is consistent with previous studies (Mafongoya et al. 2000; Parton et al. 2007; Manzoni et al. 2008). The reason for this seems to be that beyond a given C:nutrient ratio, more nutrients are mineralized during the decomposition of organic matter than needed by the soil microorganisms. Therefore, the amount of N and P immobilized by soil microorganisms does not increase with increasing amounts of N and P in the organic matter, but below a given C:nutrient ratio, net nutrient mineralization occurs. In the Oa horizons, net N mineralization increased less strongly with decreasing C:N and N:P ratios of the organic matter than in the Oi horizons (Fig. 3). This might be because organic N in the Oa horizons is often incorporated in lignin and other recalcitrant substances (Berg and Matzner 1997), which makes it harder and more N-expensive for microorganisms to access than in the other two horizons.

Threshold ratios of net N and net P mineralization

Both net N and P mineralization only occurred below threshold C:N, C:P or N:P ratios of the organic matter (Fig. 3). The threshold C:N ratios of net N mineralization determined here are in accordance with the threshold C:N ratios of 20–40, which was determined based on litter from different plants (e.g. Gosz et al. 1973; Blair 1988; Parfitt et al. 1998; Parton et al. 2007; Moore et al. 2011). However, our ratios partly exceeded threshold element ratios for net N mineralization derived from theoretical considerations that amount to 10–30 (Kaiser et al. 2014; Spohn and Chodak 2015).

This is the first study to report threshold N:P ratios of net N and P mineralization, so no comparisons with previous studies are possible.

The threshold C:P ratio reported here for net P mineralization (1400) was at the upper range of published threshold C:P ratios for P mineralization (300–1700) (Edmonds 1980; Blair 1988; Parfitt et al. 1998; Saggar et al. 1998; Moore et al. 2011). We did not find a threshold C:P ratio for P mineralization in the Oa horizons, which was most likely because substantial P mineralization only occurs below C:P ratios of organic matter of 100–300 (Cheshire and Chapman 1996), and the lowest C:P ratios in the present study were still >400.

The threshold ratios determined here were affected by the horizon, i.e. the decomposition state of the

organic matter. Net N mineralization had higher threshold C:N and N:P ratios in the Oi than the Oa horizons. The reason for this could be that high concentrations of easily available C in the Oi horizons are respired by the microbial biomass to gain energy for N acquisition (Moorhead and Sinsabaugh 2006; Craine et al. 2007; Spohn 2015), leading to higher threshold C:N ratios than in more strongly decomposed horizons of the organic layer.

There is an ongoing debate about whether organic C quality or C:N:P stoichiometry of litter is the most important parameter determining decomposition (Zhang et al. 2008; Hättenschwiler and Jørgensen 2010; Bradford et al. 2016). In this context, our study showed that net C, N and P mineralization rates were closely correlated with the organic matter stoichiometry and the correlations were modified by the degree of decomposition of the organic matter in the different horizons.

Relationships among net mineralization rates

Net N and P mineralization rates were strongly positively correlated with each other, while correlations of both net N and P mineralization with C mineralization were weak. The close relation of net N and P mineralization (Fig. 2, Table 3) is in accordance with the finding of a recent meta-analysis (Marklein et al. 2016). The reason for this correlation is most likely that C mineralization is part of the energy metabolism of microorganisms (catabolism), while net N and P mineralization is a result of organic matter decomposition and immobilization of N and P for the built-up of biomass (anabolism). The tight relationship between net N and P mineralization seems to contradict the popular model of McGill and Cole (1981), which states that P mineralization is separated from net C and N mineralization because it is regulated by microbial P demand, whereas C and N mineralization are related by microbial energy demand.

The stoichiometry of organic matter and net mineralization rates

Here we found that C:N ratios of the organic matter decrease strongly from the Oi to the Oa horizon (Fig. 1) as reported previously (Gosz et al. 1973; Albers et al. 2004; Moore et al. 2011; Mooshammer et al. 2014; Zechmeister-Boltenstern et al. 2015; Liu et al. 2016; Tipping et al. 2016). The reason for the decrease is that C loss via respiration is much larger than net N mineralization and N loss via leaching, as shown by the ratio of C:N net mineralization that amounted to an average of 140 across all horizons.

C:P ratios of organic matter usually decrease from the Oi to the Oa horizon (Saggar et al. 1998; Spohn and Chodak 2015; Zechmeister-Boltenstern et al. 2015; Tipping et al. 2016). However, in the present study, the C:P ratios of organic matter did not decrease from the Oi to the Oa horizons as expected. Further, the N:P ratios of organic matter increased from the Oi to the Oa horizons (Fig. 1). This suggests that P is lost from the organic layer either due to leaching or due to plant uptake (Donald et al. 1993; Cortina et al. 1995). The low N:P ratios of net mineralization in the Oi and Oe horizons (Fig. 4) reveal that a disproportionally high amount of P, as compared to the N:P ratios of organic matter, was lost from the horizon in an inorganic form.

Conclusions

We conclude that C, N and P net mineralization depended on organic matter stoichiometry because we found significant correlations of mineralization rates and organic matter C:N:P ratios, and net N and P mineralization occurred below threshold C:N, C:P and N:P ratios as hypothesized. The threshold element ratio, at which net N and P mineralization started, differed between organic horizons, indicating that organic matter quality affected the threshold ratios. C mineralization was decoupled from net N and P mineralization as expected, while net N and P mineralization were closely correlated. Hence, we surmise that net N and P mineralization rates cannot be derived from C mineralization but require individual investigations. Chemical organic matter characteristics closely related to C mineralization were unrelated with net N and P mineralization in a principal component analysis and therefore likely less suitable for the prediction of the latter. Overall, the detailed analysis of C, N and P net mineralization rates and organic matter stoichiometry contributes to a better understanding of the interrelations between the C, N and P cycles in organic layers of forest ecosystems.

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Study III

2.3 Effects of long-term nitrogen addition on phosphorus cycling in organic soil horizons of temperate forests

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Effects of long-term nitrogen addition on phosphorus cycling in organic soil horizons of temperate forests

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Abstract High atmospheric nitrogen (N) deposition is expected to impair phosphorus (P) nutrition of temperate forest ecosystems. We examined N and P cycling in organic soil horizons of temperate forests exposed to long-term N addition in the northeastern USA and Scandinavia. We determined N and P concentrations, enzyme activities and net N and P mineralization rates in organic soil horizons of two deciduous (Harvard Forest, Bear Brook) and two coniferous (Klosterhede, Gårdsjön) forests which had received experimental inorganic N addition between 25 and 150 kg N ha^{-1} $year^{-1}$ for more than 25 years. Long-term N addition increased the activity of phosphatase (+ 180%) and the activity of carbon (C)- and N-acquiring enzymes (cellobiohydrolase: + 70%, chitinase: + 25%). Soil N enrichment increased the N:P ratio of organic soil horizons by up to 150%. In coniferous organic soil horizons, net N and P mineralization were small and unaffected by N addition. In deciduous organic soil horizons, net N and P mineralization rates were significantly higher than at the coniferous sites, and N addition increased net N mineralization by up to 290%. High phosphatase activities concomitant with a 40% decline in P stocks of deciduous organic soil horizons indicate increased plant P demand. In summary, projected future global

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increases in atmospheric N deposition may induce P limitation in deciduous forests, impairing temperate forest growth.

Keywords Chronic nitrogen deposition · Exoenzymes · Long-term experiments · Nutrient mineralization · Phosphatase · Soil stoichiometry

Introduction

Future increases in atmospheric nitrogen (N) deposition are expected to alter not only N, but also phosphorus (P) cycling in temperate forest ecosystems. This trend is already apparent in decreased foliar P concentrations observed in many temperate forests over the last several decades (Houdijk and Roelofs 1993; Flückiger and Braun 1998; Duquesnay et al. 2000; Ilg et al. 2009; Jonard et al. 2015; Talkner et al. 2015). Although the implementation of air pollution control measures has reduced N emissions in Europe and the USA (Xing et al. 2013; Vet et al. 2014; Li et al. 2016b), the amount of N deposition is still well above pre-industrial levels in these regions (Galloway et al. 2004; Simpson et al. 2014) and global rates of deposition are projected to double by 2050 (Galloway et al. 2004; 2008). Temperate and boreal forests, which are often N-limited, are particularly sensitive to high N inputs (Aber et al. 1989; Vitousek and Howarth 1991). High N inputs might cause P limitation in temperate forests (Mohren et al. 1986; Tessier and Raynal 2003; Gress et al. 2007; Talkner et al. 2015) because N-induced forest growth can increase plant P demand (Gradowski and Thomas 2006, 2008; Li et al. 2016a). For example, P limitation of trees has recently been reported in hardwood forests of the northeastern USA (Goswami et al. 2018).

Net mineralization of N and P, defined as the difference between gross mineralization and net immobilization, makes these nutrients available for plant uptake (Schimel and Bennett 2004; Bünemann et al. 2007). The mineralization of organic P is catalyzed by phosphatases, which are exoenzymes released by plants and soil microorganisms. Nitrogen addition has been shown to elevate soil phosphatase activity, likely as a result of increased P demand by plants and microorganisms (Treseder and Vitousek 2001; Wang et al. 2007; Marklein and Houlton 2012; Deng et al. 2017). The production of the N-rich phosphatases might be facilitated by high N availability (Treseder and Vitousek 2001; Wang et al. 2007; Marklein and Houlton 2012; Deng et al. 2017).

The activity of other enzymes besides phosphatases may also change in response to N availability. For example, the activities of the cellulose and the chitin degrading enzymes cellobiohydrolase and chitinase (β-1,4-*N*-acetylglucosaminidase) have been reported to increase (Weand et al. 2010a), whereas lignin degrading phenol oxidases are often inhibited by high N concentrations (Frey et al. 2004; Gallo et al. 2004; Waldrop and Zak 2006; Jian et al. 2016). Comparison of the activities of C-, N- and P-acquiring enzymes and their ratios can be used to identify the nutrient cycling processes in which organisms preferentially invest energy (Sinsabaugh et al. 2008, 2009; Sinsabaugh and Follstad Shah 2012; Herold et al. 2014). Such ratios might indicate, for example, whether microbial investment into P acquisition is increased compared to investment into N or C acquisition in response to long-term N addition.

Numerous studies have evaluated the effects of simulated N deposition in N addition experiments in forests of the temperate zones in North America and Europe (Dise and Wright 1992; Aber et al. 1993; Norton et al. 1999). Nitrogen addition has been shown to elevate N concentrations in foliage and soil organic horizons (e.g. Aber et al. 1993; Magill et al. 2004; Kjønaas and Stuanes 2008; Elvir et al. 2010), increase plant biomass (e.g. Aber et al. 1993; Magill et al. 1997; Lovett et al. 2013), stimulate nitrate leaching (e.g. Gundersen 1998; Jefts et al. 2004; Moldan et al. 2006; Lovett et al. 2013), and reduce soil cation concentrations (Currie et al. 1999; Moldan and Wright 2011). Further, net N mineralization often increases following N addition (Aber et al. 1993, 1995; Kjønaas et al. 1998; Jefts et al. 2004; Fatemi et al. 2016; Carrara et al. 2018). Despite this long history of research, the influence of chronic N deposition on P cycling is not well studied and the response of N:P ratios of vegetation and soils to long-term N addition is seldom reported (e.g. in Kjønaas et al. 1998; Kjønaas and Stuanes 2008; Weand et al. 2010a; Crowley et al. 2012). This underlines the need for further research of the influence of high N deposition on P cycling in temperate forests. Special consideration should be given to how element cycling processes in the organic horizon change upon increased N inputs because

organic horizons represent an important pool of P in forest soils that is rapidly mineralized by microorganisms and is very important for forest P nutrition (Ponge 2003; Huang and Spohn 2015; Spohn et al. 2018).

Our objective was to assess changes in N and P cycling and microbial nutrient acquisition in response to long-term N addition to temperate forests soils. Our hypotheses were that (i) N addition would increase N:P ratios of organic soil horizons, (ii) phosphatase activity would increase more strongly in comparison to the activities of C- and N-acquiring enzymes due to N addition and (iii) increased phosphatase activity would lead to increased net P mineralization. To test these hypotheses, we analyzed organic soil horizons of two deciduous and two coniferous long-term N addition experiments in the USA and Europe.

Materials and methods

Study sites and sampling

Two North American hardwood forest sites (Harvard Forest, Bear Brook) and two Scandinavian spruce forests (Klosterhede, Gårdsjön) exposed to long-term, experimental N addition were sampled. Harvard Forest (Massachusetts, USA, N42°32′, W72°10′) is located 330 m a.s.l., with mean annual precipitation and temperature being 1240 mm and 8.5 °C, respectively. The vegetation is dominated by black oak (*Quercus velutina*) and red oak (*Q. borealis*) mixed with other hardwood species including black birch (*Betula lenta*), red maple (*Acer rubrum*) and American beech (*Fagus grandifolia*). The soils are mainly Cambisols (Inceptisols; USDA Taxonomy), developed from sandy tills of the Gloucester series. The organic horizon has an average thickness of 5 cm and a bulk density ranging from 0.17 (N150) to 0.24 cm^{-3} (control), depending on N treatment. There are three N addition treatments with application rates (since 1988) of 0 (N0), 50 (N50) and 150 (N150) kg N ha^{-1} $year^{-1}$ in the form of ammonium nitrate (NH_4NO_3). Each treatment plot has a size of 30 × 30 m. Ambient N deposition at the site is currently 8–10 kg N ha^{-1} $year^{-1}$ (Aber et al. 1995; Magill et al. 2004; Schwede and Lear 2014).

Bear Brook is located in Maine, USA (N44°52′, W68°06′) and has a mean annual precipitation of 1400 mm and a mean annual temperature of 4.9 °C. Its lower elevations, which were sampled in this study, are dominated by American beech (*Fagus grandifolia*), sugar maple (*Acer saccharum*) and red maple (*Acer rubrum*). The soils are mainly Podzols (Spodosols) developed from quartzite and gneiss covered by an organic horizon with an average thickness of 4 cm (bulk density: 0.09 g cm^{-3}, both control and N addition plots). The Bear Brook experiment consists of two adjacent watersheds. The 11 ha watershed "West Bear" has received 25 kg N ha^{-1} $year^{-1}$ in the form of ammonium sulfate ($(NH_4)_2SO_2$) since 1989 and the 10 ha watershed "East Bear" has served as a control. Ambient deposition is 3 kg N ha^{-1} $year^{-1}$ (Norton et al. 1999; Fernandez et al. 2010; SanClements et al. 2010).

Klosterhede (Denmark, N56°29′, E008°24′) is situated at 27 m a.s.l., with a mean annual precipitation of 860 mm and mean annual temperature of 9 °C. A 108 year-old Norway spruce (*Picea abies*) plantation grows on Podzols (Spodosols) developed from glacial outwash sands. The organic horizon is on average 11 cm thick and has bulk densities of 0.10 (control) and 0.13 g cm^{-3} (N addition). The experiment was part of the "NITREX project" (Wright and van Breemen 1995) and consists of a 500 m^2 N-addition plot surrounded by three control plots. The N-addition plot has received 35 kg N ha^{-1} $year^{-1}$ in the form of NH_4NO_3 in monthly doses since 1992; ambient N deposition is approximately 25 kg N ha^{-1} $year^{-1}$ (Dise and Wright 1992; Gundersen and Rasmussen 1995; Gundersen 1998). Samples were taken from 15 × 15 m subplots.

The Gårdsjön experiment (Sweden, N58°04′, E12°03′), which was also part of the "NITREX project", is situated at 135-145 m a.s.l. with mean annual precipitation of 1100 mm and mean annual temperature of 6.4 °C. The dominant tree species is Norway spruce (*Picea abies*) with Scots pine (*Pinus sylvestris*) growing in drier areas. The main soil types are Orthic Humic Podzols and Gleyed Humoferric Podzols (Spodosols) developed from glacial till covered by an 8 cm thick organic horizon with a mean bulk density of 0.18 g cm^{-3} (both control and N addition). We sampled the catchment G2 (0.5 ha), which has received 40 kg N ha^{-1} $year^{-1}$ in the form of NH_4NO_3 in small doses (128 mg N l^{-1}) along with each rain event since 1991, and the control catchment F1 (3.7 ha). Ambient N deposition at the site is 12 kg N ha^{-1} $year^{-1}$ (Dise and Wright 1992;

Andersson et al. 1998; Moldan et al. 2006; Seftigen et al. 2013).

The organic horizon at Harvard Forest and Bear Brook was sampled in July 2016. Samples (20 × 20 cm) were collected from six replicate plots and divided into leaf litter (Oi horizon) and organic soil (Oe + Oa horizons). Samples were collected from Klosterhede and Gårdsjön in March–April 2017 following the same methods. Samples were stored at 4 °C and shipped immediately on ice to the University of Bayreuth, Germany. All Oe + Oa horizons were sieved to remove roots and stones and stored moist at 4 °C. They were characterized for total C, total and available N and P concentrations as well as microbial C, N and P concentrations, N and P stocks, pH, exoenzyme activity and net N and P mineralization rates. Leaf litters were homogenized by hand, dried at 60 °C, and subsequently analyzed for total C, N and P concentrations.

Soil characteristics

Gravimetric water content and maximum water holding capacity of the soils was determined following Naeth et al. (1991). Plastic tubes closed with a fine cloth at the bottom were filled with 1 cm of field-moist soil for two replicates from each site, weighed, and water saturated for 48 h. Samples were then drained for 48 h on a water saturated sand bath at 5 °C and weighed again. During draining, the tops of the tubes were covered with a wet cloth to prevent water loss via evaporation. Sample dry weights were determined after 48 h drying at 60 °C. The bulk density was calculated for the organic soil horizons of both N addition and control plots in six replicates.

Soil pH was measured with a gel electrode (WTW) in a suspension of moist soil and deionized water at a ratio of 1:5 (w/v). Total C and N concentrations were measured on dried (60 °C) and finely ground subsamples using a CN analyzer (Vario MAX, Elementar). Total soil P concentrations were measured with an inductively coupled plasma-optical emission spectroscopy (ICP-OES, Vista-Pro radial, Varian) after pressure digestion in concentrated nitric acid. Nitrogen and P stocks were calculated from total N concentrations (total P concentrations) and soil bulk density measurements.

Net mineralization

Net N and P mineralization were determined by incubating soil (ca. 50 g), adjusted to 60% water holding capacity, at 15 °C for 11 weeks. Net N mineralization was determined based on the increase in ammonium (NH_4^+) and nitrate (NO_3^-) concentrations extracted with cold water from 2.5 g dry-weight equivalents of moist soil in weekly (1st month) to biweekly intervals with a ratio of 1:20 (w/v). NH_4^+ and NO_3^- concentrations were measured with flow-injection analysis (FIA-Lab, MLE). The NH_4^+–N + NO_3^-–N concentrations determined at the first measurement were defined as the available N concentration. In the same incubation, net P mineralization was determined based on the increase in phosphate (PO_4^-) concentrations in Bray-1 extracts (0.03 M NH_4F + 0.025 M HCl). For this purpose, 5 g dry-weight equivalent of moist soil were regularly extracted in a ratio of 1:10 (w/v). PO_4^- concentrations in the extracts were measured spectrophotometrically using a microplate reader (M200 pro, Tecan) according to the method of Murphy and Riley (1962). To prevent interference with the color formation of the assay, fluoride ions were neutralized with 0.1 M boric acid before addition of the molybdate blue reagent. The PO_4^-–P concentration determined at the first measurement was termed available P. Net N and P mineralization rates were calculated as the increase in NH_4^+–N plus NO_3^-–N and PO_4^-–P concentrations, respectively, over time.

Soil microbial biomass

Soil microbial biomass C, N and P were determined by chloroform-fumigation extraction (Brookes et al. 1984, 1985; Vance et al. 1987) of subsamples (C and N: 5 g, P: 2.5 g) from the incubation experiment described above. Samples were fumigated for 24 h at room temperature. For microbial C and N, controls and fumigated samples were extracted in 0.5 M K_2SO_4 with a ratio (w/v) of 1:5 (Joergensen et al. 1995). The C and N concentrations in the extracts were determined with a CN analyzer (multi N/C 2100, Analytik Jena). For microbial P, controls and fumigated samples were extracted in Bray-1 solution (0.03 M NH_4F + 0.025 M HCl, Bray and Kurtz (1945)) in a ratio of 1:10. PO_4 concentrations in the extracts were measured spectrophotometrically with a

microplate reader (M200 pro, Tecan) using the method of Murphy and Riley (1962) modified as above. The Bray-1 solution has been shown to be a very efficient extractant for microbial P (Khan and Joergensen 2012). Soil microbial biomass C, N and P were calculated as the difference of C, N and P concentrations in fumigated and control samples, and corrected by a factor of 2.22 for C and N and by 2.5 for P, respectively (Jenkinson et al. 2004).

Exoenzyme activity

The activities of cellobiohydrolase, chitinase and phosphatase were determined using the fluorogenic substrates 4-methylumbelliferyl-β-D-cellobioside, 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide and 4-methylumbelliferyl-phosphate according to Marx et al. (2001) and German et al. (2011). Briefly, 1 g moist soil was dispersed in 50 ml deionized water with ultrasound and 50 µl of this soil slurry were transferred to black 96-well microplates in four analytical replicates. These samples were diluted with 50 µl sterile deionized water and amended with 100 µl of a 1 mM substrate solution. The plates were incubated at 30 °C for 210 min and fluorescence was measured after 30, 60, 90, 150 and 210 min. Fluorescence values were corrected for quenching of the soil, as well as for the fluorescence of substrate, and soil enzyme activity calculations were based on end-point measurements (German et al. 2011). The ratios of cellobiohydrolase-to-chitinase, cellobiohydrolase-to-phosphatase and chitinase-to-phosphatase were determined. For this calculation, the natural logarithms of the specific enzyme activities in µmol g organic C^{-1} h^{-1} were used (Sinsabaugh et al. 2008; Herold et al. 2014).

Statistical analyses

Data were tested for significant site-specific differences between N addition treatments and controls. The normality of the data was examined visually by QQ-plots and analytically by Shapiro–Wilk tests, and the homogeneity of variances were checked with Levene's tests. In all cases in which normality assumptions were met, analyses of variance (ANOVA) followed by Tukey's multiple comparisons tests were used for Harvard Forest, which had three treatments, and t-tests were used for Bear Brook, Klosterhede and Gårdsjön, which each had two treatments. Where assumptions of normality were violated, Kruskal–Wallis tests followed by multiple comparisons tests according to Dunn (Pohlert 2014) were calculated for Harvard Forest, and Wilcoxon rank sum tests were used for the other sites. The relationships between cellobiohydrolase, chitinase and phosphatase activity as well as net N and P mineralization and different fractions of soil C, N and P were analyzed with Spearman rank correlations. Where linear regressions were used, all residuals were checked for normal distribution and homoscedasticity. If assumptions for least-squares regression were not met, robust linear regression (Yohai 1987; Koller and Stahel 2011) was used with the R package "robustbase" (Rousseeuw et al. 2015). The influence of control and N addition treatments on regression analyses were examined with analyses of covariance (ANCOVA). All statistical analyses were performed in R version 3.2.2 (R Core Team 2015).

Results

N and P of leaf litters and organic horizons

The total N concentration of the leaf litter increased significantly at all sites due to N addition, on average by 17% (Table 1). The total N concentration of the organic soil horizon also increased at two of the four sites, on average by 42% (Table 2). The total N stock of the organic soil horizon increased significantly at Klosterhede (+ 15%), but decreased at Bear Brook (− 27%) in response to N addition (Table 2). The available N concentration in the organic horizon was significantly elevated at all sites upon N addition (Table 2).

The total P concentration of the leaf litter declined significantly at Gårdsjön, but increased at Bear Brook (Table 1). The total P concentration of the organic horizon decreased at Harvard Forest by 29%, but increased at Klosterhede by 20%. Total P concentrations of leaf litter and the organic soil horizon were significantly lower in the coniferous than in the deciduous forests ($P < 0.001$, Tables 1, 2). The total P stocks of the coniferous organic soil horizons were not affected by N addition. In contrast, P stocks were significantly reduced in the deciduous organic soil horizons due to N addition by 55 and 35% at Harvard

Table 1 Total C, N and P concentrations and C:N:P ratios of the leaf litter from control and N-addition plots at Harvard Forest, Bear Brook, Klosterhede and Gårdsjön

Site	Treatment	Total C (mg g^{-1})	Total N (mg g^{-1})	Total P (mg g^{-1})	C:N ratio	C:P ratio
Harvard Forest	Control	475 ± 04	16.7 ± 1.3	1.16 ± 0.04	32.9 ± 2.8	1039 ± 053
	+ 50 kg N	481 ± 03	**18.9 ± 1.3***	1.24 ± 0.04	29.8 ± 2.1	1001 ± 037
	+ 150 kg N	**493 ± 07*****	17.8 ± 1.1	0.97 ± 0.10	32.3 ± 2.1	**1324 ± 139***
Bear Brook	Control	464 ± 02	16.0 ± 1.1	0.84 ± 0.04	33.8 ± 2.2	1422 ± 056
	+ 25 kg N	469 ± 06	**19.5 ± 1.3*****	**0.95 ± 0.04****	**27.2 ± 2.1*****	**1265 ± 034****
Klosterhede	Control	477 ± 16	17.7 ± 0.5	0.82 ± 0.04	31.4 ± 1.2	1501 ± 060
	+ 35 kg N	**496 ± 05***	**20.0 ± 0.3*****	0.76 ± 0.05	**28.9 ± 0.4****	**1642 ± 049****
Gårdsjön	Control	498 ± 03	13.5 ± 1.3	0.78 ± 0.11	43.2 ± 3.8	1665 ± 226
	+ 40 kg N	**510 ± 09****	**17.5 ± 1.6*****	**0.62 ± 0.13***	**34.3 ± 3.8****	2220 ± 590

All ratios were calculated on a molar basis and all values are given as means and standard deviations. Asterisks mark the level of significance for all significant differences between the N addition treatment and the respective control (bold)

Levels of significance: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$

Forest and Bear Brook, respectively (Table 2). The available P concentrations of the organic soil horizon was elevated at Harvard forest by 67% due to N addition.

N:P ratios were increased in all leaf litters and organic soil horizons due to N addition, and the increases were significant for all sites except for Bear Brook (leaf litter) and Gårdsjön (organic soil horizon, Fig. 1). Overall, the N:P ratios of leaf litter and organic soil horizons were lower in the deciduous forests than in the coniferous forests (leaf litter: $P < 0.05$, organic soil horizons: $P < 0.001$).

Total C, pH and microbial biomass

Total C concentrations of leaf litter and organic soil horizons increased significantly under N addition at Harvard Forest, Klosterhede and Gårdsjön (Tables 1, 2). Nitrogen addition did not affect total C stocks of the organic soil horizons, with the exception of a significant increase at Gårdsjön (by 9%). Organic horizon soil pH was significantly reduced at Harvard Forest and Bear Brook in response to N addition. Microbial biomass C declined in response to N addition at Harvard Forest in the N150 treatment, but was not affected at the other sites (Table 2). Microbial biomass N and P concentrations and microbial biomass C:N, C:P, and N:P ratios were also unaffected by long-term N addition (Table 3).

Exoenzyme activities in organic soil horizons

Phosphatase activity in the organic horizon increased significantly in response to N addition at all sites, except for Klosterhede (Fig. 2c). Cellobiohydrolase activity increased significantly at Bear Brook and Gårdsjön, and chitinase activity increased significantly at Klosterhede and Gårdsjön upon N addition (Fig. 2a, b). On average, phosphatase activity increased more strongly due to N addition (260%) than chitinase (80%) and cellobiohydrolase activity (150%); however, ratios of phosphatase-to-cellobiohydrolase or chitinase activity were only significantly increased in response to N addition at Harvard Forest (Fig. 2).

Phosphatase activity was negatively correlated with total P concentrations ($R^2 = 0.46$, $P < 0.001$) and positively with total N concentrations ($R^2 = 0.26$, $P < 0.001$) and N:P ratios ($R^2 = 0.65$, $P < 0.001$; Fig. 3). An exponential model best described the negative relationship between phosphatase activity and P concentrations in the organic soil horizons. Phosphatase activity was also strongly positively correlated with N:P and C:P ratios and total C and N concentrations (Online Resource 1). Cellobiohydrolase exhibited similar correlations with these variables, while chitinase activity was only weakly positively correlated with them (Online Resource 1).

Table 2 Characteristics of organic soil horizons of control and N-addition plots at Harvard Forest, Bear Brook, Klosterhede and Gårdsjön

Site	Treatment	Total C (mg g^{-1})	Total N (mg g^{-1})	Total P (mg g^{-1})	Total C stock (kg m^{-2})	Total N stock (g m^{-2})	Total P stock (g m^{-2})
Harvard Forest	Control	168 ± 29	08.3 ± 1.1	1.14 ± 0.10	1.63 ± 0.35	076.1 ± 08.3	11.93 ± 1.33
	+ 50 kg N	215 ± 58	11.5 ± 2.7	**0.89 ± 0.04*****	1.79 ± 0.30	096.7 ± 19.7	**07.83 ± 2.38****
	+ 150 kg N	**245 ± 60***	**13.2 ± 3.2***	**0.74 ± 0.08*****	2.11 ± 0.88	111.6 ± 41.1	**05.39 ± 0.88*****
Bear Brook	Control	278 ± 25	14.8 ± 1.5	0.92 ± 0.18	1.10 ± 0.20	058.6 ± 12.1	03.20 ± 0.45
	+ 25 kg N	297 ± 15	16.7 ± 2.8	0.80 ± 0.10	0.83 ± 0.27	**042.7 ± 13.2***	**02.08 ± 0.74***
Klosterhede	Control	377 ± 26	12.1 ± 1.1	0.42 ± 0.05	4.99 ± 0.35	160.7 ± 14.3	05.56 ± 0.65
	+ 35 kg N	**441 ± 24****	**15.2 ± 0.3*****	**0.50 ± 0.02***	5.38 ± 0.30	**185.3 ± 03.2****	06.08 ± 0.23
Gårdsjön	Control	450 ± 24	14.8 ± 2.6	0.58 ± 0.06	6.30 ± 0.34	207.3 ± 36.6	08.20 ± 0.89
	+ 40 kg N	**491 ± 08****	17.4 ± 1.8	0.56 ± 0.04	**6.87 ± 0.11****	243.8 ± 24.7	07.88 ± 0.53

C:N ratio	C:P ratio	Microbial C (mg g^{-1})	Microbial N (mg g^{-1})	Microbial P (mg g^{-1})	Available N (μg g^{-1})	Available P (μg g^{-1})	pH
23.6 ± 2.0	0380 ± 051	1.20 ± 0.11	0.20 ± 0.02	0.18 ± 0.04	066 ± 012	77.8 ± 29.8	4.7
21.7 ± 1.0	**0629 ± 185***	1.18 ± 0.17	0.21 ± 0.05	0.20 ± 0.03	068 ± 012	**37.9 ± 17.4****	4.4
21.8 ± 1.6	**0877 ± 289****	**0.94 ± 0.17***	0.15 ± 0.01	0.13 ± 0.04	**097 ± 167****	**12.8 ± 04.4*****	**3.5*****
22.0 ± 1.8	0807 ± 167	1.63 ± 0.47	0.32 ± 0.10	0.34 ± 0.09	095 ± 021	39.0 ± 42.1	4.9
22.8 ± 2.8	**1042 ± 100****	1.23 ± 0.19	0.23 ± 0.02	**0.18 ± 0.07****	**188 ± 081***	42.4 ± 20.4	**4.0** **
36.3 ± 1.6	2326 ± 141	1.18 ± 0.19	0.18 ± 0.05	0.13 ± 0.02	006 ± 002	04.8 ± 01.0	4.2
35.0 ± 1.5	2369 ± 139	1.23 ± 0.18	0.18 ± 0.04	0.14 ± 0.03	**015 ± 005****	05.5 ± 01.0	4.1
38.3 ± 3.2	2011 ± 327	2.07 ± 0.29	0.41 ± 0.03	0.24 ± 0.06	003 ± 003	05.3 ± 01.7	4.4
32.2 ± 2.5**	2189 ± 139	1.85 ± 0.35	**0.35 ± 0.05***	0.20 ± 0.07	**054 ± 040****	04.1 ± 02.1	4.3

The stoichiometric ratios were calculated on a molar basis and all values are given as means with standard deviations. Asterisks mark the level of significance for all significant differences between the N-addition treatment and the respective control (bold)

Levels of significance: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

Net N and P mineralization rates

Net N and P mineralization was substantially higher in deciduous forests than in coniferous forests ($P < 0.001$). Net N mineralization ranged between 0.09 and 0.56 μmol N g^{-1} day^{-1} at the deciduous forest sites (Harvard Forest, Bear Brook) and between 0.02 and 0.05 μmol N g^{-1} day^{-1} at coniferous forests (Klosterhede, Gårdsjön) across all treatments (Fig. 4). Net P mineralization was between 2.6 and 12.7 nmol P g^{-1} day^{-1} in deciduous forests and 0.0 and 0.4 nmol P g^{-1} day^{-1} in coniferous forests. Net N and P mineralization were only affected by N addition treatments in deciduous organic soil horizons. Net N mineralization increased significantly in the Harvard Forest N150 treatment (+ 290%) and at Bear Brook (+ 210%) compared to the control. Net P mineralization increased significantly in response to N addition at Bear Brook (+ 400%), whereas it decreased significantly in the N50 treatment at Harvard Forest (+ 60%).

Net N mineralization was strongly correlated with several fractions of C, N and P and organic soil horizon C:N:P stoichiometry across all sites and treatments, whereas net P mineralization was only related to P fractions (Online Resource 1). Net N mineralization correlated strongly positively with dissolved organic N (r = 0.89), the N:P and C:P ratios (r = 0.77 and 0.78, respectively), and total N concentrations of the organic horizons (r = 0.59). In addition, net N mineralization was strongly negatively correlated with total P concentrations (r = − 0.74, Online Resource 1).

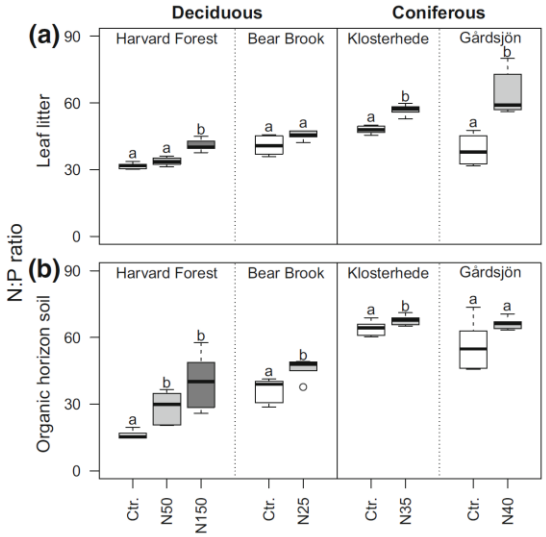

Fig. 1 N:P ratios of **a** leaf litter and **b** organic soil horizons of control and N addition treatments at Harvard Forest, Bear Brook, Klosterhede and Gårdsjön. Different lowercase letters indicate significant site-specific differences ($P < 0.05$)

Discussion

Long-term N addition significantly increased the N:P ratio and phosphatase activity in the organic horizon at three of the four forest sites. This response was particularly obvious in the deciduous forests where the P stock of the organic horizon was also significantly reduced in response to N addition. In the coniferous organic horizons, net N and P mineralization rates were negligible and were not affected by N addition, indicating efficient microbial uptake of these nutrients. In the deciduous organic soil horizons, net N and P mineralization rates were significantly higher than in the coniferous forests and net N mineralization at these sites increased due to high chronic N inputs.

Exoenzyme activity

Phosphatase activity increased strongly in response to long-term N addition (Fig. 2c). This observation, in combination with the positive linear relationship between phosphatase activity and the total N concentrations of the organic soil horizons (Fig. 3), confirms previous findings (Naples and Fisk 2010; Weand et al. 2010b; Marklein and Houlton 2012). The observed relationship between total N concentrations and phosphatase activity may be due to an increased P demand of plants and potentially of microorganisms exposed to long-term N addition (Clarholm 1993; Olander and Vitousek 2000). The high N supply is likely beneficial for the synthesis of N-rich enzymes, including phosphatase (Allison and Vitousek 2005). Phosphatase activity was negatively correlated with total P concentrations and positively correlated with C:P and N:P ratios of the organic soil horizons (Fig. 3, Online Resource 1). This finding is in agreement with previous studies showing that the soil P concentration regulates phosphatase activity (Spiers and McGill 1979; Marklein and Houlton 2012), with declining activity where P is abundant (Juma and Tabatabai

Table 3 Molar microbial biomass C:N, C:P and N:P ratios in the organic soil horizons of long-term N addition experiments at the deciduous forest sites Harvard Forest and Bear Brook and the coniferous forest sites Klosterhede and Gårdsjön

Site	Treatment	C:N ratio	C:P ratio	N:P ratio
Harvard Forest	Control	7.3 ± 1.1	16.8 ± 2.2	2.3 ± 0.3
	N50	6.2 ± 0.4	15.1 ± 1.9	2.3 ± 0.5
	N150	6.6 ± 0.6	19.5 ± 5.9	2.9 ± 0.8
Bear Brook	Control	6.0 ± 0.4	12.6 ± 2.3	2.1 ± 0.5
	N	6.0 ± 0.5	15.7 ± 1.3	2.8 ± 0.4
Klosterhede	Control	8.0 ± 1.7	25.6 ± 2.7	3.3 ± 1.0
	N	7.9 ± 0.8	22.9 ± 3.1	2.9 ± 0.4
Gårdsjön	Control	5.9 ± 0.6	24.4 ± 5.2	4.2 ± 1.2
	N	6.2 ± 0.8	20.9 ± 2.7	3.4 ± 0.5

Values are given as mean with standard deviation (n = 6)

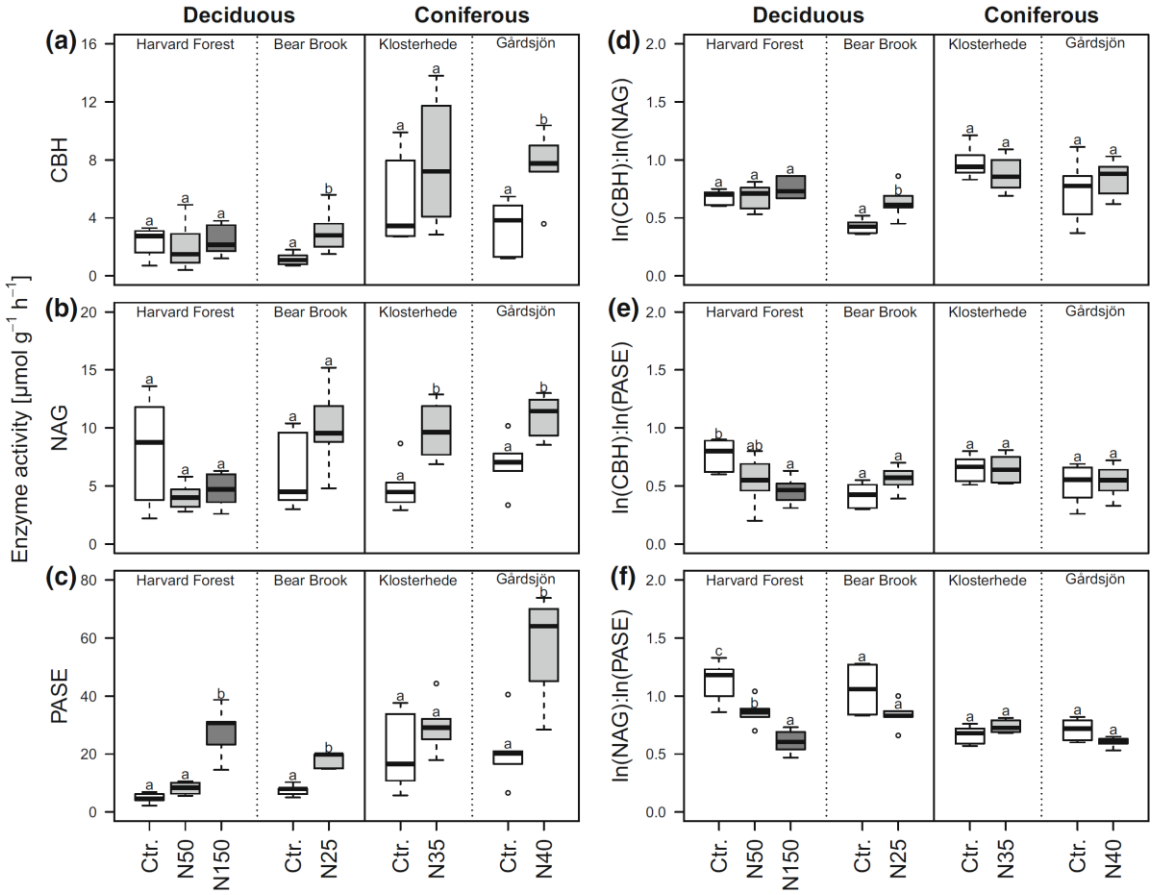

Fig. 2 Activities of the enzymes **a** cellobiohydrolase (CBH), **b** chitinase (NAG), **c** phosphatase (PASE) and ratios of the natural logarithms of specific enzyme activities, **d** CBH:NAG, **e** CBH:PASE, **f** NAG:PASE in the organic soil horizons of control and N addition treatments at Harvard Forest, Bear Brook, Klosterhede and Gårdsjön. Different lowercase letters indicate significant site-specific differences ($P < 0.05$)

1977, 1978; Olander and Vitousek 2000; Moscatelli et al. 2005; Marklein and Houlton 2012).

Cellobiohydrolase and chitinase activity also increased with N addition, mainly in the coniferous forests (Fig. 2a, b), suggesting that N addition at these nutrient poor sites led to increased microbial C demand and facilitated enzyme synthesis of N-rich hydrolytic enzymes. However, the increase in phosphatase activity in response to N addition was much larger than the increase in cellobiohydrolase and chitinase activity, indicating that long-term N addition predominantly increased P demand.

Net N and P mineralization

Net N and P mineralization in the organic horizon were substantially higher in the deciduous forests than in the coniferous forests (Fig. 4). This is most likely because soil microorganisms experienced greater N and P scarcity in the coniferous forest soils compared to the deciduous forest soils, and therefore took up the available forms of the two nutrients almost completely. This is supported by the stoichiometry of the organic soil horizons. The C:N and N:P ratios in the coniferous forests were above the thresholds for net N mineralization in organic soils, which amount to 20–40 for the C:N ratio (Parton et al. 2007; Moore et al. 2011) and 60 for the N:P ratio (Heuck and Spohn

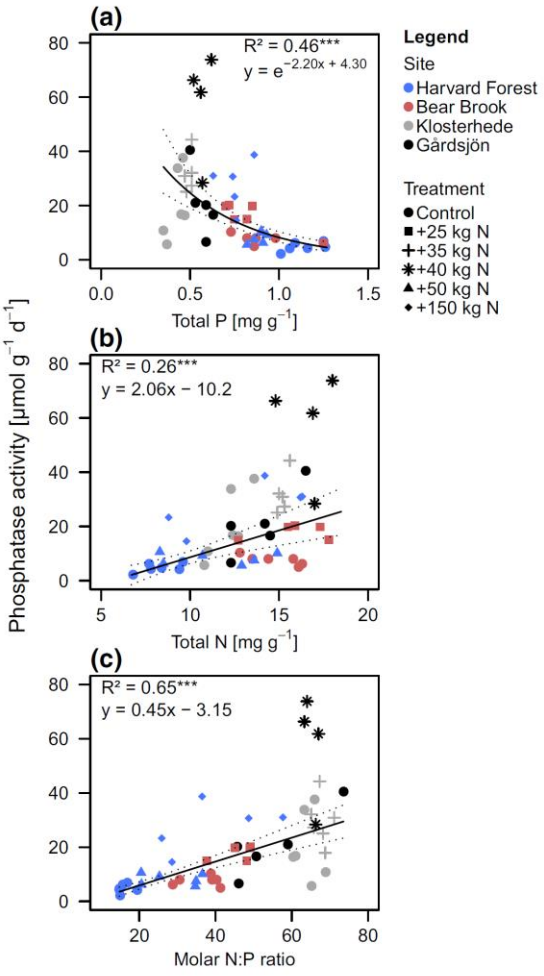

Fig. 3 Relationship between phosphatase activity and **a** total soil P concentration, **b** total soil N concentration and **c** molar N:P ratio of the organic soil horizons. Sites are distinguished by colors (blue: Harvard Forest, red: Bear Brook, grey: Klosterhede, black: Gårdsjön) and treatments are distinguished by symbols (control: circle, + 25 kg N: square, + 35 kg N: plus, + 40 kg N: star, + 50 kg N: triangle, +150 kg N: diamond). An exponential model was fitted for (**a**) and linear models for (**b**) and (**c**). Fitted lines are presented with 95%-confidence intervals (dotted lines), R^2 and the regression equations. Levels of significance are: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$

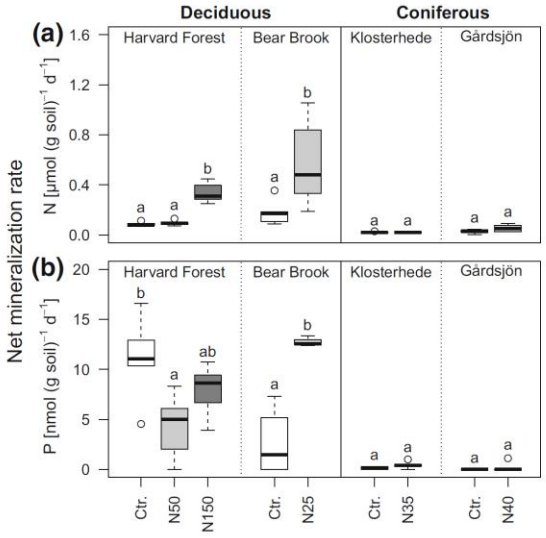

Fig. 4 Net mineralization rates of **a** N and **b** P in organic soil horizons of control and N addition treatments at Harvard Forest, Bear Brook, Klosterhede and Gårdsjön. Different lowercase letters indicate significant site-specific differences ($P < 0.05$)

2016). Above these thresholds, N availability is so low that no net release of inorganic N occurs, and microorganisms immobilize all N when decomposing organic matter (Parton et al. 2007; Moore et al. 2011; Heuck and Spohn 2016; Spohn 2016). Analogously, net P mineralization was not observed in the coniferous forests, presumably because the threshold C:P (300–1700, Blair 1988; Moore et al. 2011) and N:P ratios (40, Heuck and Spohn 2016) for net P mineralization were exceeded. In the deciduous organic soil horizons, N addition increased microbial net N mineralization. This may have occurred because available N concentrations exceeded microbial N demand due to N addition, and thus microbes released surplus N in inorganic forms during organic matter decomposition (Prescott et al. 1992). This likely allowed the microbes to maintain their biomass C:N:P stoichiometry (Table 3) despite changes in organic matter stoichiometry (Table 2), and is in agreement with the theory of the microbial biomass stoichiometry being homeostatic (Cleveland and Liptzin 2007; Xu et al. 2013; Spohn 2016). In addition, variation in the microbial community composition of deciduous and coniferous organic horizons may have contributed to the differences in net N and P mineralization rates because of higher fungal:bacterial ratios in soils of temperate coniferous forests than in deciduous forest soils (Fierer et al. 2009).

Despite a consistent increase in phosphatase activity in response to N addition, we did not find a consistent increase in net P mineralization. In deciduous forests, net P mineralization both increased (Bear Brook) and decreased (Harvard Forest), whereas in the coniferous forests, net P mineralization rates were

very small across all treatments. Elevated phosphatase activity only increases net P mineralization if substrate concentrations are sufficiently high, and the microbial P demand is satisfied. This seemed to be the case at Bear Brook, but not at Harvard Forest. Another possible explanation for these divergent responses of the two decideous sites is that different forms of N were added to soils at the two sites. Ammonium nitrate was added at Harvard Forest, whereas ammonium sulfate was used at Bear Brook. The added sulfate may have contributed to the additional P release, because sulfate might exchange with sorbed phosphate (Geelhoed et al. 1997).

Taken together, in deciduous organic soil horizons high rates of net N and P mineralization (Fig. 4) led to the formation of high concentrations of inorganic N and P (Table 2) that are potentially plant available. In contrast, in the coniferous organic soil horizons, net rates of N and P mineralization, and concentrations of plant available inorganic N and P were much lower (Table 2). Thus, in the deciduous but not in the coniferous forests, increased microbial net N and P mineralization may have facilitated increased plant nutrient uptake. The significant declines in P concentrations and P stocks in the deciduous organic soil horizons in response to long-term N addition (Table 2) most likely resulted from an increased P demand of the plants that led to increased P uptake. The increased P demand was likely caused by the high availability of N. This is supported by increased aboveground biomass and annual net primary productivity of trees in the N addition plots at Harvard Forest (Magill et al. 2004; Savage et al. 2013). Yet, aboveground biomass was unaffected by N addition at Bear Brook (Elvir et al. 2010), which might be due to the addition of sulfate together with N that may have led to desorption of adsorbed phosphate (see above). Substantial net P mineralization in the organic horizons of the deciduous forests likely allowed for transfer of inorganic P from the organic soil horizons into the vegetation by plant uptake or into the mineral soil by leaching. In the long-term, this may force plants to acquire P from the mineral soil, where P strongly adsorbs to minerals and therefore is more difficult to obtain.

Nutrient stocks of the organic horizon

The significant decrease in P stocks of the organic horizon in the two deciduous forests (Table 2) can most likely be attributed to increased plant P uptake caused by the high N availability (see above). The organic horizon is an important pool of P for forest nutrition (Ponge 2003; Huang and Spohn 2015; Spohn et al. 2018), and a significant decline in P stocks of this pool might lead to plant P limitation in the long-term if N inputs remain high. Foliar P concentrations of several deciduous tree species are currently decreasing in Europe (Duquesnay et al. 2000; Ilg et al. 2009; Talkner et al. 2015). The reason for this decrease is not known. However, our study indicates that one possible explanation is atmospheric N deposition.

The N:P ratio of leaf litter and the organic soil horizon increased at most sites due to long-term N addition (Fig. 1), which is in agreement with previous results for foliage and the organic soil horizon of the site Gårdsjön (Kjønaas et al. 1998; Kjønaas and Stuanes 2008). The increase in the N:P ratios resulted from the decreased P stocks as well as from the large N additions that increased organic horizon N stocks either directly due to incorporation of added N in the organic horizon (Magill et al. 1997; Gundersen 1998) or indirectly due to increased foliar N concentrations (Aber et al. 1993; Gundersen 1998; White et al. 1999; Magill et al. 2004; Kjønaas and Stuanes 2008; Fernandez and Norton 2010).

Conclusions

Long-term N addition consistently increased phosphatase activities and N:P ratios of the organic horizons in both coniferous and deciduous temperate forests, indicating increased plant P demand, which confirms our first two hypotheses. In the deciduous forests, net N and P mineralization rates were substantially higher than in the coniferous forests. In contrast to our third hypothesis, high phosphatase activities caused by N addition did not consistently translate into high net P mineralization rates. This can be attributed to the organic horizon stoichiometry that differed among deciduous and coniferous forests, and to a very efficient immobilization of P by the microbial biomass in the coniferous organic horizons. We observed a decline in the P stocks of the organic horizons due to N addition in the deciduous but not in the coniferous forests. The decline in P stocks of the deciduous organic horizons indicates that in the long-term, high N inputs might lead to decreased plant P

uptake and even to plant P limitation in deciduous forests.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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Contributions to the included manuscripts

Study I

Soil microbial biomass C:N:P stoichiometry and microbial use of organic phosphorus. Christine Heuck, Alfons Weig and Marie Spohn.
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C. Heuck:	65%	laboratory works, data analysis, manuscript preparation
A. Weig:	10%	laboratory works, manuscript preparation
M. Spohn:	25 %	research design, laboratory works, discussion of results, manuscript preparation

Study II

Carbon, nitrogen and phosphorus net mineralization in organic horizons of temperate forest: stoichiometry and relations to organic matter quality. Christine Heuck, Marie Spohn.
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C. Heuck:	70%	research design, laboratory works, data analysis, manuscript preparation
M. Spohn:	30%	research design, discussion of results, manuscript preparation

Study III

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C. Heuck:	50%	research design, laboratory works, data analysis, manuscript preparation
G. Smolka:	20%	laboratory works
E. Whalen:	5%	sampling, data provision
S. Frey:	5%	data provision, discussion of results
P. Gundersen:	5%	sampling, data provision
F. Moldan:	5%	sampling, data provision
M. Spohn:	10%	research design, discussion of results, manuscript preparation

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Publications

First author

Heuck, C., Weig, A., Spohn, M., 2015. Soil microbial biomass C:N:P stoichiometry and microbial use of organic phosphorus. *Soil Biology & Biogeochemistry* 85, 119-129. doi: 10.1016/j.soilbio.2015.02.029.

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(§ 8 Satz 2 Nr. 3 PromO Fakultät)
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