


LETTER

Soil fungal mycelia have unexpectedly flexible stoichiometric C:N and C:P ratios

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Abstract

Soil ecological stoichiometry provides powerful theories to integrate the complex interplay of element cycling and microbial communities into biogeochemical models. One essential assumption is that microbes maintain stable C:N:P (carbon:nitrogen:phosphorus) ratios independent of resource supply, although such homeostatic regulations have rarely been assessed in individual microorganisms. Here, we report an unexpected high flexibility in C:N and C:P values of saprobic fungi along nutrient supply gradients, overall ranging between 7–126 and 20–1488, respectively, questioning microbial homeostasis. Fungal N:P varied comparatively less due to simultaneous reductions in mycelial N and P contents. As a mechanism, internal recycling processes during mycelial growth and an overall reduced N and P uptake appear more relevant than element storage. The relationships among fungal stoichiometry and growth disappeared in more complex media. These findings affect our interpretation of stoichiometric imbalances among microbes and soils and are highly relevant for developing microbial soil organic carbon and nitrogen models.

Keywords

C:N:P ratios, element homeostasis, fungal nutrient retranslocation, microbial carbon sequestration, mycelial growth, nutrient limitations, saprobic fungi, soil ecological stoichiometry, soil nutrient cycling.

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INTRODUCTION

Ecological stoichiometry represents an important field originally developed in aquatic ecosystems, with its principles being now also applied to terrestrial soil systems (Sturner and Elser, 2002). Models use stoichiometric theory for predicting soil nutrient cycles and carbon storage (Sardans *et al.*, 2012). Based on the general observation that C:N:P (carbon:nitrogen:phosphorus) ratios of soil microbial communities are more narrow than resource C:N:P, critical ratios of C:N and C:P are defined to predict nutrient demands of soil microbes, mineralisation versus immobilisation patterns and C sequestration versus respiration (Manzoni *et al.*, 2012; Zechmeister-Boltenstern *et al.*, 2015). Thus, ecological stoichiometry facilitates the incorporation of extremely complex processes into global models and predictions, especially in the context of global change (Hall *et al.*, 2011a).

These models make crucial assumptions that are critical for their validity (Mooshammer *et al.*, 2014; Spohn, 2016), one of them that heterotrophic soil microbes are homeostatic, i.e. maintain stable C:N:P ratios independently of the soil nutrient status (Persson *et al.*, 2010). Even though some models allow microbial C:N:P to vary slightly (McGill *et al.*, 1981; Nicolardot *et al.*, 2001), homeostatic flexibility is currently only attributed to microbial community shifts, not an actual stoichiometric flexibility in individuals (Buchkowski *et al.*, 2019). Fixed microbial stoichiometric ratios are interpreted as an indicator of

nutrient demands: If microbes need to maintain their narrow C:N ratios, N will be in limiting supply in substrates characterised by wider C:N ratios (Manzoni *et al.*, 2010). These assumptions of soil microbial homeostasis are supported by analyses of entire soil communities, which indeed show relatively little variation in microbial C:N:P ratios compared to soil resource variability (Cleveland and Liptzin, 2007; Hartman and Richardson, 2013). By contrast, we know astonishingly little about the stoichiometry of individual soil microbial groups.

Indeed, flexibility of C:N:P ratios in individual microbial species has rarely been analysed, and the few studies available mainly use aquatic isolates and partly result in contradictory results depending on the methods applied (Danger *et al.*, 2016). Surprisingly, some of these studies suggest that microbial C:N:P ratios may be less homeostatic than assumed for heterotrophic organisms. For aquatic bacteria, Scott *et al.* (2012) and Godwin and Cotner (2018) demonstrated high variation in C:P ratios for some but not all isolates (see also Makino *et al.*, 2003; Danger *et al.*, 2008). In case of saprobic fungi, aquatic hyphomycetes responded to varying element supply with non-homeostatic adjustments in C:P ratios, while C:N remained stable (Danger and Chauvet, 2013; Gulis *et al.*, 2017). In soils there are also indications that fungal C:N:P may exceed common textbook assumptions of C:N *c.* 10–20 and C:P *c.* 100–300 (Jennings, 1995; Strickland and Rousk, 2010). A recent meta-analysis demonstrated wide C:N and C:P ratios in few fungal samples (Zhang and Elser, 2017), as

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also reported for C:N in wood decomposing fungi under low N conditions (Levi and Cowling, 1969), while other studies indicate again only little flexibility (Heck, 1928; Egli and Quayle, 1986).

Physiological mechanisms causing non-homeostasis in microbes are still unknown. Most authors assume P storage, i.e. element uptake in excess as the exclusive mechanism (Scott *et al.*, 2012; Danger and Chauvet, 2013; Mooshammer *et al.*, 2014), although this only explains stoichiometric shifts in response to high element supply. The significance of such stoichiometric shifts for fungal growth and activity also remain unresolved. Available data show that N or P uptake can be independent of fungal growth, potentially related to storage mechanisms or primary C limitations (Levi and Cowling, 1969; Gulis *et al.*, 2017).

In order to understand stoichiometric adjustments in soil saprotrophic fungi in detail, we analysed fungal mycelial element concentrations in response to varying N, P and C supply in different growth media specifically developed for this question (varying from highly controlled to natural substrates). Derived from common assumptions in soil ecological stoichiometry, we tested the hypothesis that saprotrophic fungi are homeostatic, especially regarding their C:N ratios. Our results not only further question the general assumption of microbial homeostasis but also provide insights into the physiological mechanisms of element allocation in mycelia, and the significance of stoichiometric shifts for fungal growth and activity under varying conditions.

MATERIALS AND METHODS

Fungal material

Saprotrophic fungi isolated from grassland soils covering Mucoromycota, Basidiomycota and Ascomycota were used (see Supporting Information S1 and Table S1). Before the experiment, each isolate was divided into three repetitions and cultured separately on potato-dextrose agar with antibiotics to eliminate potential contaminants. Subsequently, repetitions were transferred to water agar (1.5%), in order to reduce nutrient storage in fungal tissues. Hereafter, repetitions were analysed separately for each fungal isolate and treatment.

Experimental design of nutrient manipulations

Nutrient availability was manipulated in growth media differing in complexity and C sources, namely, defined glucose medium, defined medium with cellulose as C source and soil-extract agar (SEA) manipulated by N, P, glucose and cellulose additions. Eight fungal isolates covering all three phyla were used (RLCS10, RLCS01, RLCS16, RLCS12, RLCS27, RLCS17, RLCS28, RLCS09; Table S1); in SEA medium, RLCS10 was replaced by RLCS22, since its growth in SEA was too limited for further analyses. For additional details and rationale of media preparation, see also Supporting Information S1.

Glucose medium

N and P availability were manipulated in defined medium with known growth conditions and nutrient limitation

patterns. This medium was designed in accordance with the law of the minimum, providing all elements and conditions in non-limiting supply to ensure N or P limitation of fungal growth only (Camenzind *et al.*, 2020). The resulting medium contained 5 g L⁻¹ glucose, 0.5 g L⁻¹ MgSO₄, 0.5 g L⁻¹ KCl, 0.1 g L⁻¹ CaCl₂, 0.1 g L⁻¹ NaFeEDTA, 5 mg L⁻¹ ZnSO₄, 0.05 mg L⁻¹ Na₂Mo₄, 0.05 mg L⁻¹ MnSO₄, 0.05 mg L⁻¹ H₃BO₄, 0.01 mg L⁻¹ CuSO₄, 1 mg L⁻¹ Thiamine HCl, 0.05 mg L⁻¹ biotin and 20 g L⁻¹ agar. N supply was manipulated by adding NH₄NO₃ testing five levels of N supply (molar C:N = 5, 20, 40, 80 and 200 (C:P = 100)). P availability was manipulated similarly by adding NaH₂PO₄ (molar C:P = 20, 100, 5000, 1000, 3000 (C:N = 20)).

Cellulose medium

For this experimental approach, glucose was replaced by cellulose (SigmaCell – highly purified fibres, Sigma-Aldrich, St. Louis, US) maintaining molar C contents. In a separate pre-test, we confirmed that no other elements or conditions were limiting and fungi were able to use cellulose as a C source, although to varying degrees (Table S2). N was manipulated as described above.

Nutrient manipulation in SEA

In order to obtain a more natural fungal growth substrate, we prepared an additional growth medium based on soil extract. Soil sampled at the original grassland site was used. Samples were autoclaved, mixed thoroughly with demineralised H₂O 1:3 (v:v) and sieved through 20 µm. The resulting liquid was supplemented with 20 g L⁻¹ agar. For nutrient manipulation, only the addition of N and P is possible in uncontrolled organic substrate. However, by concomitantly increased C availability by adding glucose (Glu) or cellulose (Cel), respectively, nutrient limitation was induced experimentally. Consequently, treatments comprised a control (Ctr; non-manipulated SEA), +N, +P, +Glu, +Glu+N, +Glu+P, +Cel, +Cel+N and +Cel+P. Glucose and Cellulose were added in equal molar C quantities, mimicking conditions in controlled media – 5 g L⁻¹ glucose and 4.5 g L⁻¹ cellulose. For N addition, 1.28 g L⁻¹ NH₄NO₃ was added, lowering soil C:N ratios of 15 experimentally to 5. P was added as 0.27 g L⁻¹ NaH₂PO₄ to shift soil C:P ratios of 1500 to 100 (based on original soil sample analyses). The parallel addition of C and N or C and P resulted in C:N ratios of *c.* 5.2 and C:P ratios of *c.* 75, respectively.

In glucose and cellulose media, fungi were grown in petri dishes in the dark for 12 days at 20°C (Ø 9mm; three repetitions of each isolate), only the fast-growing isolate RLCS01 was kept for 7 days in glucose media, while RLCS28 was grown for 27 days to obtain sufficient biomass. In SEA, all fungal strains (two repetitions each) were cultured for 26 days to ensure sufficient biomass formation for analyses.

Additional experiments evaluating N and P allocation in mycelia

Stoichiometric data derived from nutrient gradient tests suggested a differential allocation of N and P in fungal mycelia during growth. To test such patterns of element allocation, data of fungal strains growing on SEA and glucose media were analysed in this context.

Twelve fungal strains (RLCS01, RLCS16, RLCS27, RLCS15, RLCS13, RLCS28, RLCS22, RLCS17, RLCS09, RLCS18, RLCS12, RLCS11; $n = 3$) were grown on SEA, analysing N contents after 12 and 26 days. Additionally, the N content of inner and outer mycelium of four strains (RLCS12, RLCS16, RLCS22, RLCS28; $n = 3(2)$) was analysed after 26 days of growth. Therefore, the radius of fungal mycelia at four positions was determined, and partitioned by 2/3 (inner) and 1/3 (outer). Mycelia were cut and divided into inner and outer parts according to this separation, and element contents analysed separately.

Similarly, in an additional nutrient manipulation experiment, the inner and outer mycelium of fungi grown in glucose media with high N (C:N 20) and low N content (C:N 200) were harvested separately using the same mycelial partitioning. Four fungal strains were selected based on large variation of $1/H_{CN}$ values (see below), also using fungal strains previously characterised in another study (RLCS06, RLCS18, RLCS09, RLCS13; $n = 3(2)$; unpublished data).

Fungal growth and stoichiometric analyses

At the end of experiments, agar media were melted in a microwave and mycelium collected on a 20 μm mesh. To remove superficial element traces, mycelia were washed with 1 L of $> 90^\circ\text{C}$ demineralised H_2O , and cleaned with 0.1 M HCl for 1 min. This washing method was inevitable to ensure complete removal of agar medium from the mycelium (Maynard *et al.*, 2017), but we showed in a separate experiment that it did not alter element concentrations or treatment effects (Fig. S1). Fungal material was freeze dried and kept at -20°C . Fungal dry biomass was determined, as well as fungal density [mg cm^{-2} mycelium] based on the actual size of the mycelium, since mycelial density as a trait proved to be a good estimate of fungal fitness and indicator of nutrient limitations (Camenzind *et al.*, 2020). Fungal enzymatic activity was assessed as an additional important response trait (for details see Supporting Information S1). For element analyses, fungi were milled and C and N contents determined with an Elemental Analyzer (EuroEA, HekaTech, Germany). P content was analysed after aqua regia digestion (1:4 HCl: HNO_3) by ICP-OES analyses (Optima 2100 DV, Perkin Elmer, Germany). In cellulose media, P contents were only analysed for four isolates (RLCS16, RLCS17, RLCS27 and RLCS28) in media with C:N of 5 and 200 respectively. Likewise, in SEA media, P contents were only analysed for four isolates (RLCS12, RLCS22, RLCS27 and RLCS28).

Statistical analyses

All statistical analyses were done in R version 3.6.1 (R Core Team, 2019).

The homeostatic coefficient $1/H$ was calculated to analyse the response of fungal stoichiometric ratios to varying nutrient supply in glucose and cellulose media (Persson *et al.*, 2010). For example, $1/H_{CN}$ represents the regression slope of fungal \log_{10} (C:N) correlated with \log_{10} (C:N) in media, and takes values between 0 (strictly homeostatic) and 1 (non-homeostatic) (for more details see also Sterner and Elser

(2002)). The same was calculated for $1/H_{CP}$ and $1/H_{NP}$ values, for the latter using the whole gradient of N and P manipulation in glucose media. Differences in isolate-specific $1/H$ values among different element gradients were calculated by paired t-tests. Variations in fungal stoichiometric ratios in fertilised SEA media were analysed by linear mixed-effects models, taking isolate as random effect into account (`lme()`; package *nlme* (Pinheiro *et al.*, 2020)). Here, in case of non-normality, data were log-transformed.

Details of the results and analyses of fungal growth responses to varying nutrient supply in glucose, cellulose and SEA medium are shown in Figs S2, S3 and S4, respectively.

Total fungal element masses [mg] were correlated with fungal biomass [mg] in each medium type, in order to understand the stability of different elements in fungal mycelia under varying resource conditions. In this case, data were normalised within isolates for each element and medium type, respectively, by standardisations between 0 and 1. Model outputs are based on linear random slope models, using isolate as random factor. Explanatory power of biomass versus nutrient manipulation on total fungal element masses was assessed by the comparison of sums of squares, evaluated by type III analyses of variances of model outputs (package *lmerTest* (Kuznetsova *et al.*, 2017)).

To assess simultaneous decreases in fungal N and P concentrations [%] in both, N and P manipulations, linear correlations were done by linear-mixed effects models with isolate as random factor for each medium and nutrient manipulation respectively. Relative deviations in element concentrations [% deviation from maximum values of each isolate] were used to compare effect sizes of element reductions among isolates. In SEA media, added carbon sources were included as fixed effect, since element concentrations were significantly affected by cellulose and glucose additions. Marginal R^2 values were calculated using function `r.squaredGLMM()` (package *MuMIn* (Barton, 2019)).

Differences in fungal C:N values of inner versus outer mycelium in SEA medium, as well as of fungi growing 12 or 26 days, respectively, were determined by paired t-tests (one sided). To evaluate the effects of treatment (low vs. high N), position (in vs. outer mycelium) and isolate on fungal C:N and C:P values in low and high N glucose media, three-way type III analyses of variances were applied.

The predictive power of stoichiometric ratios for fungal growth and activity was determined by linear correlations of growth responses (viz. fungal biomass, density, enzymatic activity and the ratio of leucine-aminopeptidase and phosphatase activity) with stoichiometric ratios, applying linear mixed-effects models (`lmer()`; package *lme4* (Bates *et al.* 2012)). Marginal (variance explained by fixed effects only) and conditional (variance explained by fixed effects and random effects due to varying intercepts among isolates) R^2 values were calculated with function `r.squaredGLMM()`.

RESULTS

Fungal C:N:P ratios in response to varying nutrient supply

Fungal C:N and C:P ratios were highly flexible for most isolates in all media types and nutrient manipulations, compared

to more stable N:P ratios. Along a defined gradient of N and P supply using glucose as a C source, fungal C:nutrient ratios positively correlated with C:nutrient supply in the medium, with $1/H_{CN}$ and $1/H_{CP}$ values of isolates ranging from 0.16 to 0.65 (Fig. 1a and b). At high N supply (medium C:N = 5), fungal C:N only varied within 8 to 18, while reaching a maximum of 84 at low N supply (C:N = 200). Likewise, fungal C:P ranged from 23 to 199 at high P (C:P = 20), compared to values up to 1114 at low P supply (C:P = 3000) (Fig. 1). By contrast, shifts in N:P ratios resulted in lower $1/H_{NP}$ than $1/H_{CN}$ and $1/H_{CP}$ ($P < 0.01$), with most isolates remaining relatively homeostatic (Fig. 1c).

In similarly controlled N and P manipulations with cellulose as a more costly C source, the C:N ratio was likewise non-homeostatic in some isolates, overall even reaching higher C:N values up to 126 (Fig. S5a), while again N:P remained relatively stable ($1/H_{NP}$ 0.02–0.13; Fig. S5b). Also in complex soil-extract agar (SEA) varying N, P and C supply strongly affected fungal C:N:P ratios. In non-manipulated SEA fungal C:N:P was on average 103:7:1. N and P additions alone did not lower ratios significantly, whereas the addition of cellulose and glucose significantly increased C:N and C:P ratios four- to fivefold, respectively (Fig. 2). Increased C availability consistently led to higher fungal biomass (in parallel with C uptake) (Fig. S4), while N and P concentrations remained low. The supplementary addition of N and P again reduced C:N and C:P ratios to levels of the control, without affecting biomass production (Fig. 2, Fig. S4). Increased availability of N and P both affected C:P and C:N ratios to a similar extent. Consequently, N:P remained more stable than C:nutrient ratios, only varying up to twofold (Fig. 2c).

Quantification and correlation of fungal C, N and P contents in nutrient manipulation experiments

In homeostatic fungal growth, not only would the ratio of elements but also the relative concentration of each element in mycelia have to remain constant. In this scenario, fungal size would be the only determinant of respective total element masses in mycelia, irrespective of nutrient supply, i.e. the larger a fungus grows the more elements it accumulates. This was the case for the total mass of fungal C, which showed a strong linear correlation with biomass (Fig. 3a). Comparing model sums of squares (see pie charts Fig. 3), biomass was the primary determinant for total C mass (Fig. 3d), except for a weak treatment effect of glucose fertilisation in SEA (Fig. 3a, Fig. S7I). Total N and P masses, however, were less strongly coupled to fungal biomass production. The correlation with fungal biomass was also positive, but R^2 and slope values were much lower (Fig. 3b and c). Mostly, nutrient

manipulations exerted stronger impacts on total fungal N and P masses than biomass production (see pie charts Figs 3e and f). Thus, contrary to the homeostatic assumption that C, N

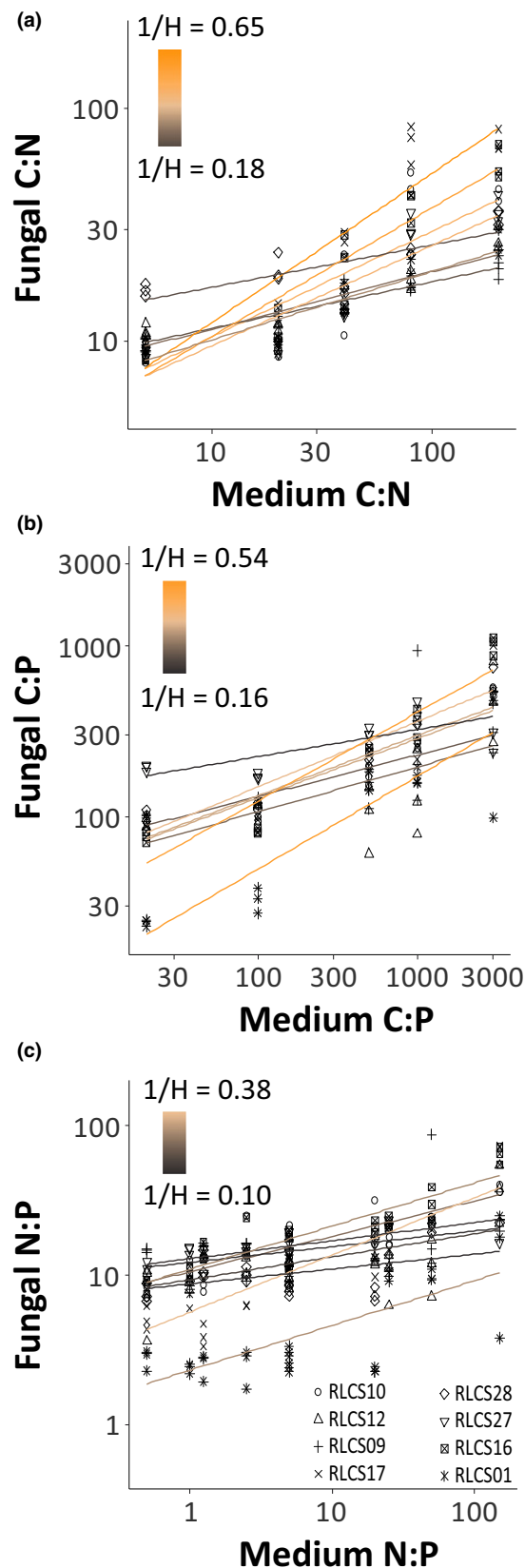


Figure 1 Stoichiometric responses in fungal C:N:P ratios to varying N and P supply in defined glucose media. (a, b and c) depict shifts in fungal C:N, C:P and N:P ratios in response to varying media supply ratios (axes are \log_{10} transformed). Lines represent the linear response of fungal stoichiometry, symbols data points of individual isolates (see legend). Line colors depict $1/H$ values calculated for each fungal isolate, with maximum and minimum values shown in the respective color legend.

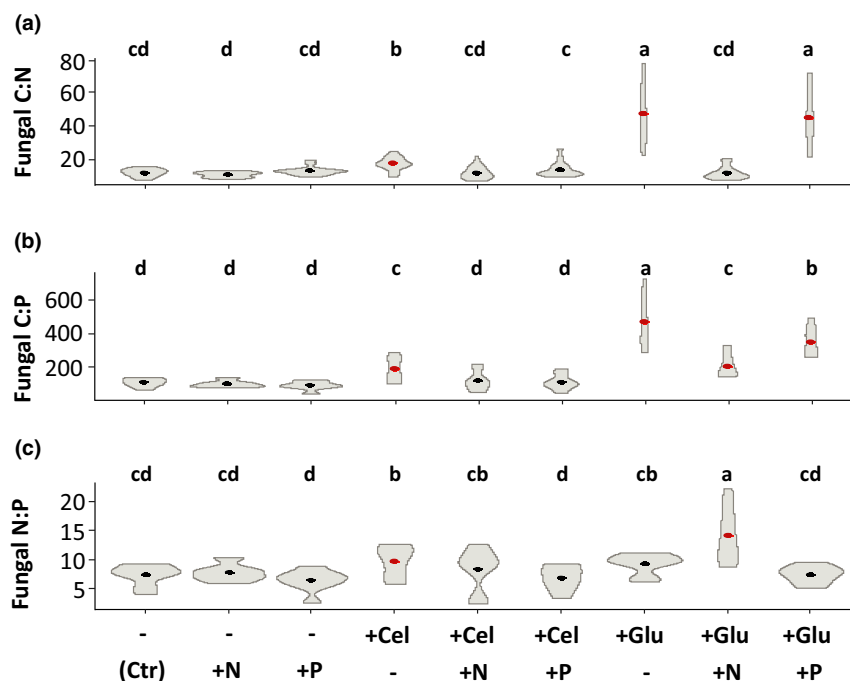


Figure 2 Fungal stoichiometric responses to nitrogen (N), phosphorus (P), cellulose (Cel) and glucose (Glu) manipulations in soil extract agar. Fungal C:N (a), C:P (b) and N:P (c) values are illustrated as violin plots, a mirrored density plot showing overall data distribution, with average values illustrated by dots (8 (a) or 4 (b and c) isolates, 2 repetitions each). Letters indicate deviations among treatments (linear mixed-effects model), red dots deviations from control values ($P < 0.05$). Large variations are mainly driven by distinct differences among isolates (see Fig. S6).

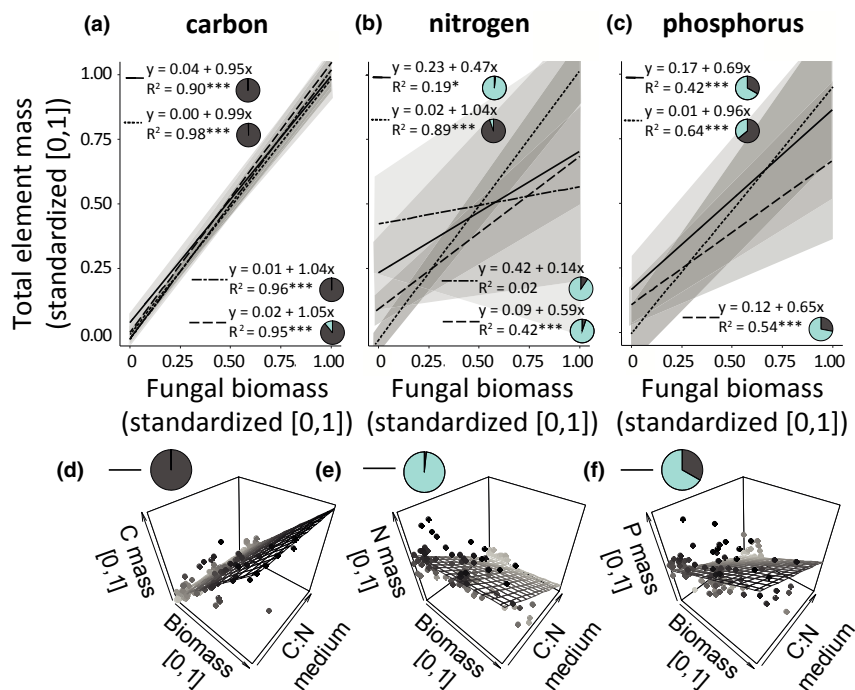


Figure 3 Correlations of total fungal (a) carbon (C), (b) nitrogen (N) and (c) phosphorus (P) mass [mg] with fungal biomass [mg] in different nutrient manipulation experiments (both standardised [0,1] for comparisons among elements and fungal isolates). Lines show predicted model outputs from linear mixed effects models, with grey areas illustrating 95% confidence intervals. Line types indicate respective nutrient manipulation experiments (solid: N manipulation in glucose medium; dashed: P manipulation in glucose medium; dotted: N manipulation in cellulose medium and longdashed: fertilisation experiment in soil extract agar). For correlations conducted in each medium, respective formulas of regression lines as well as R^2 values are given. Pie charts illustrate the relative variation in element masses determined by fungal biomass (dark grey) versus the respective nutrient manipulation treatments applied (light blue). 3D correlation graphs (d and e) further illustrate the relative contribution of fungal biomass versus nutrient manipulation to total element mass, exemplarily shown for N manipulations in defined glucose media.

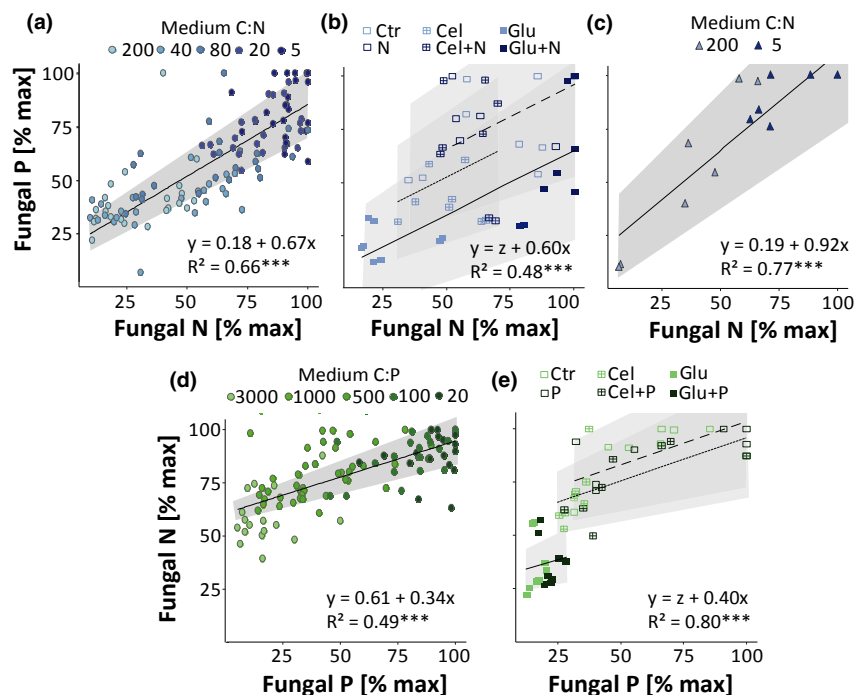


Figure 4 Positive correlations of relative fungal nitrogen (N) and phosphorus (P) concentrations [% deviation from maximum values] in defined glucose media (a and d), soil-extract agar (b and e) and defined cellulose medium (c). The relative deviation of fungal concentrations of the manipulated element is depicted on the x-axis, while the relative concentrations of the respective other element (unchanged in media) is plotted on the y-axis. The chosen relative values allow to assess effect sizes in element reductions, and reduce isolate-specific variability by data standardisation. Colors and symbols depict respective treatments applied (see legends), black lines predicted model outputs from linear mixed effects models, with grey areas illustrating 95% confidence intervals (*** $P < 0.001$).

and P concentrations remain constant within fungal mycelia independent of environmental conditions, fungal mycelia must have physiological mechanisms to reduce or increase P and N concentrations, while C remains relatively constant (Fig. S7).

Since fungal N:P ratios were more stable than C:nutrient ratios in all media types, fungal N and P must have responded to nutrient manipulations in a concerted way, even though only the availability of one element changed (Figs 1 and 2). Correlating the relative reductions in N and P concentrations in fungal mycelia within the different applied nutrient gradients supported this conclusion (Fig. 4). For example, along a gradient of C:N manipulated in defined glucose medium, under conditions of low N supply, the proportion of N in fungal mycelia decreased on average to 31 % of its maximum values. In parallel, even though the P supply in the medium was not experimentally manipulated, the mycelium P concentration was reduced to an average of 41 % of its highest value, resulting in a correlation with an R^2 of 0.66 and a slope as high as 0.67 between fungal N and P concentrations (Fig. 4a). In case of N manipulations, the parallel shift in P concentration was comparably strong for all media tested (Fig. 4a, c and e, Fig. S7), whereas in P manipulated media the parallel shift in N was slightly weaker, indicated by lower slope values (Fig. 4b and d). Still, correlations were also very clear. None of the elements positively correlated with C concentrations, which slightly increased in N- and P-limited

conditions in some isolates in glucose media (Fig. S7c and f) and raised from 35.5 ± 1.9 % (mean \pm SE) in SEA to 44.3 ± 2.2 % in SEA + Glu ($P < 0.001$) (Fig. S7l).

Element allocation in fungal mycelia

The ability to reduce or increase N and P concentrations (in parallel) within the fungal mycelium may be related to spatial patterns (e.g. retranslocation/recycling) or a general decline throughout the mycelium. In fungal mycelia grown on SEA, we indeed observed different C:N ratios in inner vs. outer regions of mycelia (Fig. 5a) due to twofold increases in relative N concentrations in outer parts (Fig. S8). Since high N concentrations were only present in the outer, more recently produced and likely more active mycelium portion, the relative amount of N decreased during fungal growth causing also temporal shifts in C:N ratios (Fig. 5b, Fig. S8). We tested whether this simple element allocation pattern also explained non-homeostatic shifts in C:N ratios due to limiting N supply (Fig. 1), namely, a simple spatial reduction in the outer N-rich zone (Fig. 6). Again, all fungal isolates had lower C:N (and also C:P) ratios in outer mycelial parts, however, this differential allocation was not affected by N supply rates (Fig. 5c, Fig. S9). Shifts in fungal C:N ratios occurred equally in inner and outer mycelial parts (Fig. S9).

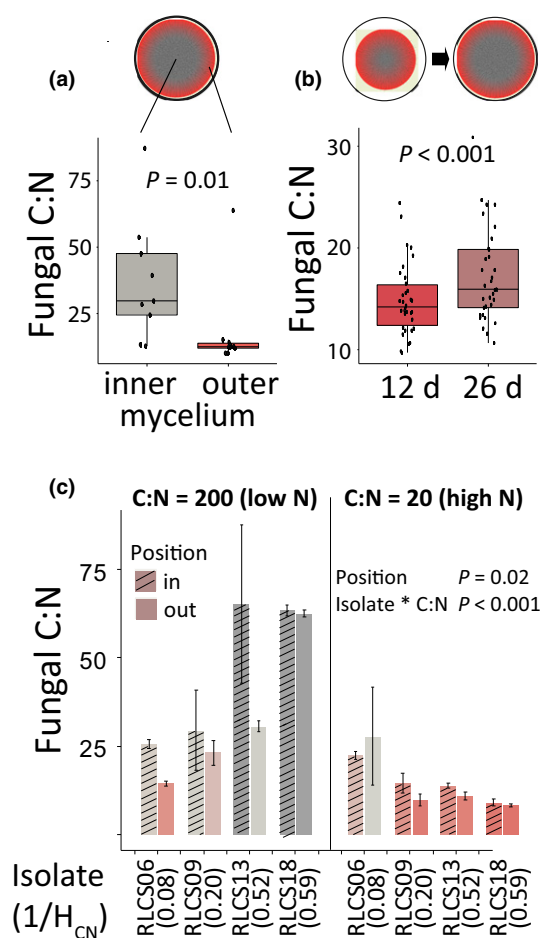


Figure 5 Spatial and temporal shifts in fungal C:N ratios are depicted, with colours illustrating fungal nitrogen concentrations (red: high, grey: low). Stoichiometry was compared in (a) inner vs. outer parts of the mycelium (4 fungal isolates, 3 (or 2) repetitions each), and in fungi grown for 12 or 26 days (b) on soil-extract agar (12 fungal isolates, 3 repetitions each), as well as in (c) inner vs. outer parts of mycelium grown in glucose media with low (C:N = 200) and high (C:N = 20) N supply (4 fungal isolates, 3 repetitions each). (a and b) Dots represent data points underlain by box-and-whisker plots. *P*-values are based on paired *t*-tests. Illustrations depict potential element distributions in fungal mycelia (see Fig. 6). (c) Bars illustrate mean values of fungal C:N ratios measured in inner (hatched bars) and outer (filled bars) parts of the fungal mycelium. Tested fungal isolates (3 or 2 repetitions each) differ in $1/H_{CN}$ values, previously determined along a larger gradient of C:N (see Fig. 1). Given *P*-values are based on three-way type III analyses of variances.

Relation of stoichiometric shifts with fungal growth and activity

In a defined glucose medium characterised by N or P limitations alone, varying fungal C:N:P values were good linear predictors of fungal growth and activity (Fig. S2, Table S4 and S6). In parallel to shifts in element concentrations, biomass production, fungal density and enzymatic activity were positively affected by higher N supply, as well as biomass and density by increasing P supply (Fig. S2). Thus, models examining the explanatory power of fungal C:N and C:P ratios distinguished them as good predictors of fungal growth, activity and the ratio of phosphatase and leucine-aminopeptidase production (Table S4 and S5).

By contrast, in complex media with more diverse C sources, observed strong shifts in C:N and C:P values did not allow to predict N or P limitations. Along varying N supply with cellulose as a C source, fungal traits did not respond positively to increasing N availability, showing no indication of N limitation despite high fungal C:N values (Fig. S3). Likewise, in soil extract agar, the addition of N and P alone did not affect biomass production or fungal density, nor did it show a significant interaction with the addition of cellulose or glucose (Fig. 2b and c). By contrast, cellulose and glucose additions shifted fungal biomass from 7.8 mg (± 2.7) to 19.9 mg (± 2.1) and 64.4 mg (± 10.6), respectively. Only enzymatic activity responded positively to N additions, while glucose strongly suppressed fungal enzymatic activity also in the presence of additional N (Fig. 2d). Consequently, stoichiometric ratios did not correlate with fungal growth or activity in cellulose and SEA media.

DISCUSSION

Contrary to the proposed hypothesis of element homeostasis, we observed high flexibility in fungal C:P and even C:N ratios, reaching values far beyond common estimates of microbial stoichiometry (Cleveland and Liptzin, 2007; Strickland and Rousk, 2010), with maxima of 1488 and 126, respectively. Induced N and P limitations under controlled conditions reduced the relative amount of fungal N and P concentrations on average by 69 and 81%, respectively, causing wide fungal C:nutrient ratios, while increasing C availability in more natural substrate (i.e. SEA) allowed fungi to build up on average eight times more biomass despite strongly widening C:N and C:P ratios. These results show that soil fungi can adjust C:nutrient ratios much more flexibly than expected (at least under the varying experimental conditions applied). Fungal N:P also showed variations, but the consistent parallel shift in N and P concentrations resulted in more homeostatic fungal N:P than C:nutrient ratios. This may be a general pattern in fungi: Gulis *et al.* (2017) also reported lower $1/H$ values for N:P than C:P in aquatic hyphomycetes, while Zhang and Elser (2017) realised that fungal N:P was closer to the canonical Redfield ratio than C:N and C:P average values (Redfield, 1958). However, potential underlying mechanisms and general stoichiometric patterns revealed here have not been described before.

Mycelial internal retranslocation and shifted nutrient uptake explain stoichiometric flexibility

Strict homeostasis is typically assumed for heterotrophs, for example, in metazoans with a determined body shape, whereas in addition to other mechanisms discussed below the modular indeterminate growth form of autotrophic plants allows more flexible adjustments (Güsewell, 2004; Persson *et al.*, 2010). In soil fungi, an indeterminate mycelial lifestyle may also permit stronger stoichiometric flexibility than expected. Fungi cannot only dynamically translocate elements within their mycelium depending on element demands (Watkinson *et al.*, 2006), but also internally recycle elements and cytoplasm. Mycelial tip growth is sustained by cytoplasm transport towards active hyphal tips (Moore *et al.*, 2011), and recycling mechanisms of

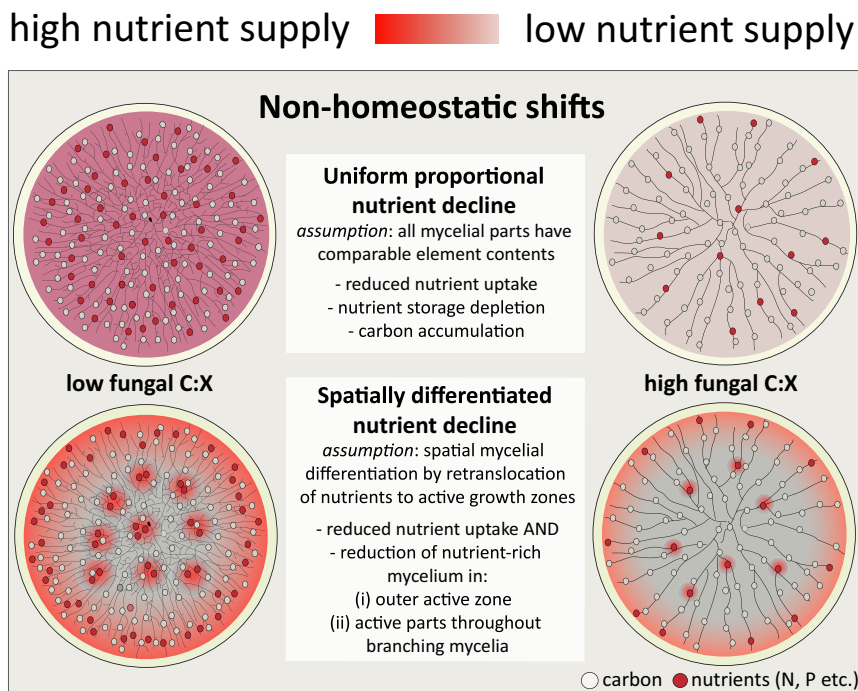


Figure 6 Illustration of potential mechanisms in fungal mycelia allowing flexible carbon:nutrient (C:X) adjustments to nutrient-limited conditions. Colours indicate element contents throughout different mycelial parts (red: low C:X, i.e. high nutrient contents; grey: wide C:X, i.e. low nutrient contents). Little grey dots illustrate the distribution of C throughout mycelia, red dots nutrients, e.g. nitrogen and phosphorus. Mycelial growth form depicts typical adaptations to reduced nutrient supply (decreased biomass, density and activity (Fig. S2; Camenzind *et al.*, 2020)).

hyphal autolysis by intracellular degrading enzymes allow efficient mycelial expansion (Reyes *et al.*, 1990; Lilly *et al.*, 1991; Pusztahelyi *et al.*, 2006). Given that N and P are more abundant in active cytoplasm, while C is also mainly bound in the hyphal wall structure, variable C:nutrient ratios in contrast to more stable N:P appear to be the logical outcome of this mycelial growth (only about 2 % of N is bound in cell walls compared to 60–70 % in proteins (Paustian and Schnürer, 1987a; Peter, 2005)) (Fig. 6). Not only the parallel shifts in fungal N and P but also the spatial and temporal variability of C:N and C:P ratios in fungal mycelia observed here lend strong support to this idea, since N and P accumulated in the outer “active growth” zone and proportionately decreased with progressing mycelial expansion. Previous reports of more active outer mycelia (Zheng, 2015) and temporal relative reductions in RNA and N and P concentrations (Levi and Cowling, 1969; Newell and Stutzell-Tallman, 1982; Grimmer *et al.*, 2013) also support this result. Whether the inner mycelium is dead or simply less active (and still involved in transport; Fricker *et al.*, 2017) is currently unclear.

As a mechanism for non-homeostasis in microbial individuals, mainly P storage has been discussed so far (Fricker *et al.*, 2008; Scott *et al.*, 2012). In plants, by contrast, diverse additional processes are known, including shifted element uptake, nutrient resorption and investment in different tissues (Frost *et al.*, 2005). Our data as well as previous stoichiometric assessments in fungi also provide evidence for more complex mechanisms in fungi (Fig. 6; Levi and Cowling, 1969). Resorption and efficient use of N and P is supported by the spatial stoichiometric flexibility (here, retranslocation).

However, the response to low N conditions (after only 12 days) was not driven by a simple reduction in the outer active zone, but may rather relate to small-scale spatial processes throughout the young growing mycelium (Fig. 6; see also Klein and Paschke (2004)). Beside a spatial reduction in active cytoplasm, the parallel shift in fungal N and P concentrations may also relate to reduced uptake (as described for plants) and a decline in both elements throughout the mycelium. Here, homeostatic N:P ratios simply relate to a tight coupling of the synthesis of N-rich proteins with P-demanding ribosomal activity (Loladze and Elser, 2011).

Regarding element storage, the parallel reduction in N and P concentrations lend little support to N or P storage as the major mechanism of non-homeostasis. Only along the applied P gradient may polyphosphate stores have been depleted before reducing internal P (and N) concentrations below optima (Scott *et al.*, 2012), which may also explain the lack of enzymatic responses to P limitation (Fig. S2). On the other hand, C storage may enhance wide C:element ratios, for example, in glucose supplemented SEA media (Wilson *et al.*, 2010). Future studies need to apply detailed chemical and microscopic analyses to reveal the contribution of these physiological mechanisms to mycelial non-homeostasis (Hall *et al.*, 2011b).

In complex growth substrates, fungal C:N:P values do not indicate nutrient demands

Shifts in stoichiometric ratios can be useful to predict organismic nutrient demands, as applied for variable leaf N:P ratios in plants (Güsewell, 2004). However, the observed shifts in C:

N:P ratios only allowed to infer fungal N or P demands in controlled media with the respective element as the only limitation (Camenzind *et al.*, 2020). In complex growth substrates, i.e. more “costly” C sources and unknown limitation patterns, stoichiometric ratios were not related to responses in fungal growth or activity to N and P supply. In complex media, (co-) limitation by various elements may impede simple causalities (Kaspari and Powers, 2016). The parallel shifts in C:N and C:P ratios reported here, which were also observed for other elements (unpublished data), complicate the interpretation of stoichiometric shifts: Wide C:P ratios, for example, are indicative of both, limitations of N and P and potentially also other nutrients. Here, N:P may be explored as a preferential stoichiometric indicator of N vs. P limitations and species-specific demands (see also Hartman and Richardson, 2013; Mougint *et al.*, 2014). In addition, C limitation in soil appears to primarily control fungal biomass production – as shown in SEA media – most likely due to the investment of C into hyphal wall structures compared to the efficient (re)use of cytoplasmic N and P contents (Paustian and Schnürer, 1987b; Camenzind *et al.*, 2018).

Implications for soil carbon sequestration and nutrient cycling

What are the implications of these findings, and why do analyses of soil microbial communities primarily show homeostasis despite this apparent flexibility in individuals? Our results imply that most saprobic fungal species do adjust their stoichiometry and nutrient use efficiency to resource supplies, a finding not only giving interesting new insights into mycelial growth dynamics but also affecting our view of microbially driven element cycles. Since stoichiometric models in soil are based on whole microbial communities, our data on fungal individuals do not directly indicate flexible C:N:P ratios at the community level. Still, if many individuals in a microbial community were non-homeostatic, this would subsequently also allow for non-homeostatic adjustments in soil microbial communities. So far, occasionally observed C:N:P plasticity in soil microbes was only explained by community shifts (Fanin *et al.*, 2013; Mooshammer *et al.*, 2014). However, in case of the “true non-homeostasis” described here, interpretations of soil microbial C:N:P may be re-evaluated: the commonly observed low microbial C:N:P would not be indicative of high N and P demands, but rather of C limitation but sufficient (or co-limiting) nutrient supply. Differences in stoichiometric flexibility among bacteria and fungi may also result in homeostasis at the soil community level, since bacteria are assumed to be homeostatic (even though aquatic studies also start to challenge this view (Scott *et al.*, 2012; Godwin and Cotner, 2018)), but are still relevant for models starting to recognise different decomposer groups (Waring *et al.*, 2013; Riley *et al.*, 2014). Regarding biogeochemical cycles, not only the lack of homeostasis but also the suggested differential use of C vs. nutrients in fungi is relevant. High structural C demand for biomass build up compared to an efficient use of nutrients by internal recycling not only shape fungal nutrient use efficiency but especially subsequent C sequestration (Liang *et al.*, 2019). It is likely that fungal necromass is enriched in C, as indicated by our stoichiometric analyses; which suggests that C

sequestration by fungi may occur without large nutrient losses (van Groenigen *et al.*, 2017).

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AUTHORSHIP

TC developed the idea, the methods and experimental design, conducted and supervised the laboratory work, performed the statistical analyses and data interpretation and wrote the manuscript with input from all authors. KPG conducted parts of the experimental laboratory work and helped developing the methods. JL and MCR aided in interpreting the results and worked on the manuscript. MCR also gave support during the development and laboratory work of the project.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

We confirm that, should the manuscript be accepted, the data supporting the results will be archived in Dryad and the data DOI will be included at the end of the article (<https://doi.org/10.6084/m9.figshare.13048064>).

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