

## ORIGINAL ARTICLE

# Changes in nutrient stoichiometry, elemental homeostasis and growth rate of aquatic litter-associated fungi in response to inorganic nutrient supply

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**Aquatic fungi mediate important energy and nutrient transfers in freshwater ecosystems, a role potentially altered by widespread eutrophication. We studied the effects of dissolved nitrogen (N) and phosphorus (P) concentrations and ratios on fungal stoichiometry, elemental homeostasis, nutrient uptake and growth rate in two experiments that used (1) liquid media and a relatively recalcitrant carbon (C) source and (2) fungi grown on leaf litter in microcosms. Two monospecific fungal cultures and a multi-species assemblage were assessed in each experiment. Combining a radioactive tracer to estimate fungal production (C accrual) with N and P uptake measurements provided an ecologically relevant estimate of mean fungal C:N:P of 107:9:1 in litter-associated fungi, similar to the 92:9:1 obtained from liquid cultures. Aquatic fungi were found to be relatively homeostatic with respect to their C:N ratio (~11:1), but non-homeostatic with respect to C:P and N:P. Dissolved N greatly affected fungal growth rate and production, with little effect on C:nutrient stoichiometry. Conversely, dissolved P did not affect fungal growth and production but controlled biomass C:P and N:P, probably via luxury P uptake and storage. The ability of fungi to immobilize and store excess P may alter nutrient flow through aquatic food webs and affect ecosystem functioning.**

*The ISME Journal* (2017) 11, 2729–2739; doi:10.1038/ismej.2017.123; published online 21 July 2017

## Introduction

Relative and absolute availability of nitrogen (N) and phosphorus (P) in ecosystems has changed as a result of human activity (Millennium Ecosystem Assessment, 2005). Little is known about the responses of heterotrophic microorganisms, especially aquatic fungi, to these shifts in nutrient availability. Apart from limiting the metabolic activity of microorganisms, N and P can also affect nutrient content of microbial biomass. Demand from microorganisms for energy and nutrients links their production to the cycling of C, N and P within ecosystems (Sinsabaugh and Follstad Shah, 2012; Sinsabaugh *et al.*, 2015). Thus, C, N and P cycling by microorganisms is partly controlled by the elemental composition of their biomass, making knowledge of the effects of increased exogenous nutrient availability on biomass stoichiometry, its variability and

on microbial activity (for example, growth rate) essential for understanding the role of microbes in nutrient cycles. Unfortunately, data on elemental stoichiometry and the degree of homeostasis in aquatic litter-associated microorganisms are scarce (Newell and Statzell-Tallman, 1982; Sanzone *et al.*, 2001; Findlay *et al.*, 2002). Virtually nothing is known about the stoichiometry, elemental homeostasis and responses to nutrients in aquatic fungi, despite their key role in litter processing (Gessner *et al.*, 2007; Danger *et al.*, 2016; Kuehn, 2016). Fungal biomass accrual and associated immobilization of N and P lead to strong control by fungi of C:N and C:P stoichiometry of decomposing plant litter (Tant *et al.*, 2013; Cornut *et al.*, 2015; Manning *et al.*, 2015). These increases in fungal biomass and nutrient content of detritus improve resource quality for detritivorous invertebrates, facilitating C and nutrient flow to higher trophic levels (Cross *et al.*, 2006, 2007).

Recent studies of freshwater bacteria suggest that both individual strains and natural assemblages can be stoichiometrically highly flexible, especially with respect to their C:P and N:P ratios (Cotner *et al.*,

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Received 20 September 2016; revised 16 May 2017; accepted 14 June 2017; published online 21 July 2017

2010; Scott *et al.*, 2012; Godwin and Cotner, 2014, 2015). Fanin *et al.* (2013) observed non-homeostasis in C:P and N:P, but not C:N, ratios in natural microbial communities within a terrestrial plant litter-microbe system that had roughly comparable contributions of fungi and bacteria. A recent study using laboratory microcosms showed that pure cultures of aquatic hyphomycetes are non-homeostatic (Danger and Chauvet, 2013). However, the use of a highly labile C source (glucose) and unrealistically high levels of dissolved N and P may limit generalization of this experimental finding.

Initial stoichiometric ratios of leaf litter entering streams (C:N > 50, C:P > 500; Enriquez *et al.*, 1993; Manning *et al.*, 2015) are considerably higher than C: nutrient ratios of microbial biomass (Stern and Elser, 2002). Thus, from the perspective of stoichiometric imbalance, and because carbon use efficiency of leaf litter-associated fungi is often quite high (averaging around 33%, but reaching 60% at high N and P availability; Suberkropp, 1991; Gulis and Suberkropp, 2003c), these microorganisms must either retain N and P from organic substrates more efficiently than C, or immobilize inorganic nutrients from stream water. Fungi, such as aquatic hyphomycetes, that dominate microbial communities on decaying plant litter in streams (Gessner *et al.*, 2007; Kuehn, 2016) are known to obtain N and P from both their substrate and the water column (Suberkropp, 1995; Cheever *et al.*, 2013). Mining N and P from organic substrates requires considerable energy and resource expenditure to produce extracellular enzymes to attack recalcitrant molecules and cleave amino or phosphate groups (Sinsabaugh *et al.*, 2014). Thus, fungi should preferentially use dissolved inorganic nutrients when available. Indeed, experiments in laboratory microcosms and whole-stream nutrient additions have shown that elevated dissolved nutrient concentrations stimulate fungal activity (that is, growth and sporulation rates, maximum fungal biomass, cumulative production, respiration) and litter decomposition (Gulis and Suberkropp, 2003a,b; Ferreira *et al.*, 2006; Gulis *et al.*, 2008; Suberkropp *et al.*, 2010; Rosemond *et al.*, 2015). However, in most of these experiments, both N and P were added, often at single levels of concentration. Thus, the possible differential responses of biomass nutrient stoichiometry vs activity (for example, growth rate) of aquatic fungi to gradients of dissolved N and P are poorly understood.

Here, we present results from two experiments designed to estimate elemental stoichiometry of aquatic fungi (C:N, C:P and N:P ratios), as well as the degree of elemental homeostasis under realistic conditions of N and P enrichment. In the second experiment, we also contrasted the effects of N and P on fungal stoichiometry with their effects on fungal growth and production. Both experiments included monocultures and multi-species fungal assemblages. In the first experiment, aquatic fungi were grown in

liquid media with varying concentrations and ratios of dissolved inorganic N and P and a relatively recalcitrant soluble C source (carboxymethylcellulose). Fungal biomass C, N and P contents were determined directly. The second experiment involved culturing fungi on a natural substrate (leaf litter) in microcosms simulating stream conditions at realistic levels of dissolved N and P availability. Nutrient stoichiometry of biomass was estimated from fungal C production ( $^{14}\text{C}$ -labeling) and dissolved N and P uptake over short intervals; fungal growth rates and production were also related to nutrient availability.

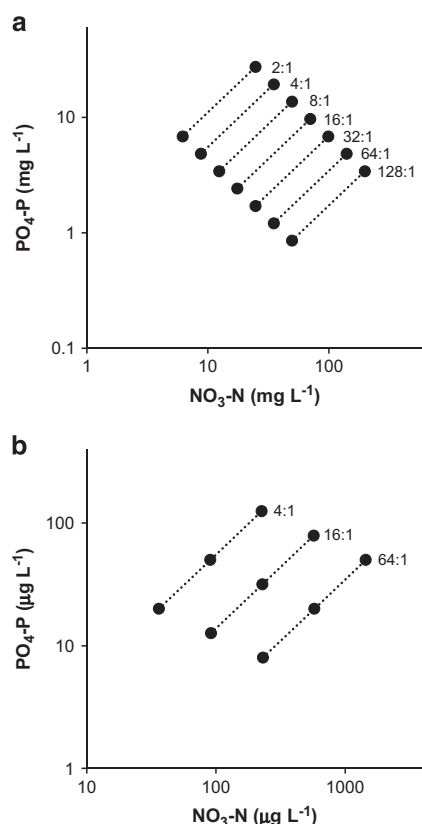
## Materials and methods

### *Dissolved nutrient treatments*

In Experiment 1, physiological responses of aquatic hyphomycetes to different concentrations and ratios of dissolved inorganic N and P were followed in liquid media with a soluble C source (Figure 1, Supplementary Table S1). Fourteen nutrient treatments included seven N:P ratios (2:1 to 128:1) at low and high nutrient concentration levels. Since these were batch cultures, initial concentrations were set relatively high. However, calculations based on biomass yield and reasonable average C:N:P expectations (Redfield ratio), as well as preliminary experiments, indicated that after 3-week incubations in some treatments all N or P would be incorporated into fungal biomass. In Experiment 2, aquatic fungi were grown on leaf litter in microcosms simulating stream conditions. Fungi were incubated at nine levels of dissolved nutrient availability (plus control, no added nutrients; Figure 1, Supplementary Table S2). Nutrient solutions spanned realistic N:P ratios (4:1 to 64:1) at three levels of nutrient concentrations ( $\text{NO}_3\text{-N}$ , 36–1445  $\mu\text{g l}^{-1}$ ;  $\text{PO}_4\text{-P}$ , 8–125  $\mu\text{g l}^{-1}$ ) found in pristine to polluted streams (Allan, 1995; Dodds and Welch, 2000). In both experiments, N and P were added as sterile solutions of  $\text{NaNO}_3$  and  $\text{KH}_2\text{PO}_4$ , respectively. Note, however, that in Experiment 1, initially high nutrient concentrations decreased during 3-week incubations due to fungal uptake, while in Experiment 2, solutions were replaced every 2 days to maintain low nutrient concentrations naturally found in streams.

### *Fungal inocula*

In both experiments, three fungal treatments were used: two monocultures (*Tricladium chaetocladium* 27-1, TC; *Heliscus lugdunensis* 62-1, HL) and a 6- or 7-species fungal assemblage (TC, HL, *Anguillospora filiformis* 23-4, *Articulospora tetracladia* 24-4, *Dimorphospora foliicola* 62-4, *Flagellospora sp.* 31-4, *Tetrachaetum elegans* 24-1; the last species was omitted in Experiment 2). All pure cultures were isolated from single conidia from decomposing leaf litter collected from streams at the Coweeta Long



**Figure 1** Concentrations of dissolved inorganic nitrogen (NO<sub>3</sub>-N) and phosphorus (PO<sub>4</sub>-P) and N:P ratios (molar) used in (a) Experiment 1 with fungi grown in liquid media and a soluble C source and (b) Experiment 2 with fungi grown on leaf litter in microcosms simulating natural stream conditions.

Term Ecological Research site (Otto, NC, USA). Experimental microcosms were inoculated with suspensions of conidia obtained by inducing sporulation from pure cultures. Growing colonies were kept at 15 °C in Petri dishes containing 1% malt extract and 2% agar (Difco, Sparks, MD, USA) for 3 weeks, at which point production of conidia was induced by agitation of agar slivers from fungal colonies in 25 ml of sterile deionized water on an orbital shaker (100 rpm) (Gulis and Suberkropp, 2003b). After 3 days, appropriate aliquots of conidia suspensions were used as inocula at ~600 conidia per microcosm.

#### Experiment 1. Aquatic fungi grown in liquid media

The base liquid medium used in this experiment contained carboxymethylcellulose (sodium salt) 8.38 g, CaCl<sub>2</sub> 0.1 g, MgSO<sub>4</sub> × 7 H<sub>2</sub>O 0.1 g, MnSO<sub>4</sub> × 7 H<sub>2</sub>O 0.004 g, FeCl<sub>3</sub> × 6 H<sub>2</sub>O 0.01 g, thiamine 0.01 mg (per 1 l). MgSO<sub>4</sub> and CaCl<sub>2</sub> were autoclaved separately, while micronutrients and thiamine were filter-sterilized. Inorganic nutrients (N and P) were added as sterile solutions of NaNO<sub>3</sub> and KH<sub>2</sub>PO<sub>4</sub>, respectively, at 14 combinations of concentrations and ratios (Figure 1, Supplementary Table S1). Liquid media were aseptically dispensed (25 ml)

into 75-ml tissue culture flasks with vented caps and inoculated with fungal spores. The flasks were placed on an orbital shaker in an inverted position to prevent the attachment of fungal colonies and incubated at 125 r.p.m. at 20 °C (16/8 h light/dark cycle) in an environmental chamber for 24 days.

After the experiment was terminated, the contents of flasks were filtered through pre-combusted pre-weighed glass-fiber filters (Whatman 934-AH) to capture fungal mycelia and spores. Two filters per flask (for N and P analyses) were prepared and rinsed 4 × with saline solution. Filters were dried at 60 °C for at least 24 h. The filters were then weighed to determine fungal biomass and analyzed for N or P. N content of fungal biomass was determined with a CHN elemental analyzer at the Odum School of Ecology, University of Georgia, USA. P content of fungal biomass was determined using a modification of the combustion and hot HCl extraction procedure of Andersen (1976), followed by spectrophotometry using the ascorbic acid molybdenum blue method (APHA, 1995). National Institute of Standards and Technology standards for both N and P were used as a reference.

#### Experiment 2. Aquatic fungi grown on leaf litter in microcosms simulating stream conditions

**Laboratory microcosms.** Laboratory microcosms specifically designed and shown to simulate stream conditions (with respect to turbulence and aeration necessary to ensure growth of aquatic hyphomycetes on leaf litter; Suberkropp, 1991) were used to obtain sufficient leaf-associated fungal biomass. Microcosms (glass cylinder, 33 mm in diameter × 120 mm long with aeration and drain tubes attached and covered by a glass cap) were combusted overnight (500 °C) and acid-washed before the experiments. Each chamber was stocked with pre-weighed leaf disks (11.2 mm in diameter) obtained from freshly abscised, air-dried red maple (*Acer rubrum* L.) leaves. Before the experiment, leaf disks were leached in deionized water overnight to remove excess nutrients, dried (60 °C) and weighed in groups of 30. Chambers stocked with dry leaf disks were autoclaved for 20 min at 121 °C. Microcosm solutions (40 ml) were aseptically pipetted into chambers and the leaf disks were allowed to soak until saturated. Solutions contained inorganic N and P added as sterile stocks of NaNO<sub>3</sub> and KH<sub>2</sub>PO<sub>4</sub>, respectively, at nine combinations of concentrations and ratios (Figure 1, Supplementary Table S2), as well as 0.25 g l<sup>-1</sup> of 3-(N-morpholino) propanesulfonic acid to maintain pH of 7.0 throughout the experiment. Microcosms inoculated with appropriate fungal treatment (see above) were incubated at 15 °C with aeration (100 ml sterile air per min, regulated with individual flow meters) to simulate stream conditions (Suberkropp, 1991). Every 2 days, nutrient solutions were aseptically drained from each chamber and replaced with fresh sterile



solutions in order to maintain nutrient concentrations and ratios. After 15 days, leaf disks were aseptically removed for fungal production and nutrient uptake assays.

**Fungal growth rate and production.** Fungal growth rate and production were estimated from rates of  $^{14}\text{C}$ -acetate incorporation into ergosterol (Suberkropp and Weyers, 1996; Suberkropp and Gessner, 2005). Leaf disks (8) with associated fungal biomass underwent short-term (5 h) incubations with sodium [ $^{14}\text{C}$ ]acetate (2.387 MBq per sample; ViTrax, Placentia, CA, USA) in 15-ml centrifuge tubes with nine nutrient solutions (Figure 1, Supplementary Table S2; 10 ml total volume, 5 mM acetate, in triplicate). Three sets of uninoculated disks in 3-(N-morpholino) propanesulfonic acid-buffered solution with no nutrients added, as well as formalin-killed controls, were also run. Tubes were incubated flat on a shaker at 15 °C. After incubation, tubes were placed on ice, centrifuged (5 min at 1000 g, 4 °C), the supernatant decanted into separate tubes for nutrient analyses, and then frozen. Leaf disks were later lyophilized, weighed and used for quantification of radiolabeled ergosterol.

Fungal biomass associated with leaf disks was estimated by ergosterol extraction and quantification by high-performance liquid chromatography. Ergosterol was extracted using the solid-phase extraction technique (modified from Gessner and Schmitt, 1996) using Oasis HLB 3cc cartridges (Waters, Milford, MA, USA; Gessner, 2005). Final elution of ergosterol was achieved with four portions (300–400  $\mu\text{l}$  each) of isopropanol and the volume in each vial was then calculated based on weight and specific density ( $\delta_{\text{isopropanol}} = 0.786 \text{ g cm}^{-3}$  at 25 °C). Ergosterol content was determined via high-performance liquid chromatography (Shimadzu 10VP equipped with a Whatman Partisphere C18 reverse-phase column set at 40 °C). The UV detector was set at 282 nm and the flow rate of 100% methanol at 1 ml min $^{-1}$ . Two 100- $\mu\text{l}$  injections per sample were used. A standard curve based on external ergosterol standards (Acros Organics, Morris Plains, NJ, USA) was used to convert high-performance liquid chromatography areas to ergosterol concentrations. Ergosterol content per sample was calculated using the volume of lipid extract. A conversion factor of 4.5 mg ergosterol per g fungal dry mass was used to estimate fungal biomass of *H. lugdunensis* (Raviraja *et al.*, 2004) and 5.5 mg g $^{-1}$  for all other species (Gessner and Chauvet, 1993).

For each sample, ergosterol fractions from two injections were collected (Advantec SF-3120), pooled in a 20-ml scintillation vial, mixed with Ecolume scintillation cocktail (ICN Biomedicals, Cleveland, OH, USA) and assayed for radioactivity using a scintillation counter (Beckman LS-6500, Fullerton, CA, USA) that corrected for quenching. Instantaneous growth rates and fungal production were calculated assuming the exponential growth

model (Gessner and Chauvet, 1997). This procedure also allowed to estimate the amount of fungal C produced during our short 5-h incubations. Data on fungal C production together with fungal dissolved N and P uptake data (see below) were used to calculate fungal elemental ratios (C:N:P).

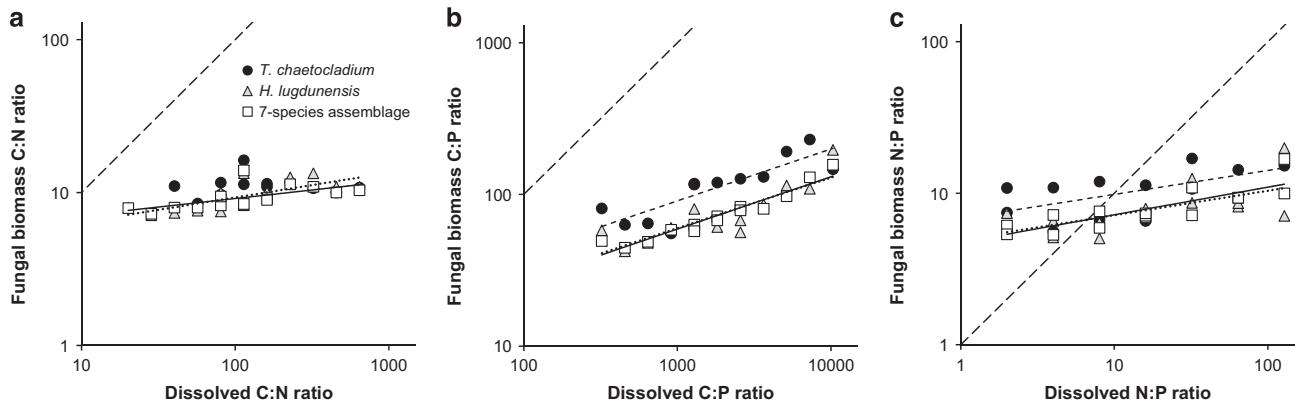
**Nutrient uptake rates.** Fungal nutrient uptake per sample (or per unit of newly produced fungal C, see above) was estimated from the difference between the initial and final (after 5-h incubation) nutrient concentrations for each sample (10 ml solution in a tube). Owing to the radioactivity in our samples, a Hach kit 2429800 and a modification of the Hach method 8192 (scaled down 10-fold to run smaller samples) was used according to the manufacturer's suggestions to determine nitrate concentrations (Hach, Loveland, CO, USA). The method is based on nitrate Cd reduction followed by spectrophotometric determination of the colored product at 507 nm. Soluble reactive phosphorus (SRP) was estimated by spectrophotometry using the ascorbic acid molybdenum blue method (APHA, 1995). For both nitrate-N and SRP, final absorbance values were corrected for color that leached from leaf litter during sample handling, and nutrient concentrations were then calculated based on standard curves.

#### Data analyses

Molar nutrient ratios of fungal biomass were estimated directly in Experiment 1 from C, N, P content of biomass captured on filters. Filter blanks were processed along with the samples and values were corrected. In Experiment 2, stoichiometric ratios of fungal biomass were calculated based on C production and simultaneous estimates of dissolved inorganic N and P uptake over the 5-h incubations. Fungal nutrient uptake rates were corrected using values from control samples incubated in solutions with no added nutrients. Since dissolved nutrient concentrations or their levels (low, medium and high) could not be the same among nutrient ratio treatments (4:1, 16:1 and 64:1) in our experimental design (Figure 1), we could use only a regression approach (not ANOVA) for the full data sets. Specifically, the effects of dissolved nutrient ratios or concentrations ( $\text{NO}_3\text{-N}$  or SRP) on fungal biomass stoichiometry were analyzed by linear regression of  $\log_{10}$ -transformed data. For each data point, three replicate samples originating from three flasks or microcosms were used. The assessment of the degree of elemental homeostasis was based on the homeostatic coefficient,  $H$ :

$$H = \frac{\log_{10}(x)}{\log_{10}(y) - \log_{10}(c)}$$

where  $x$  is the resource (medium) nutrient stoichiometry (for example, C:P, C:N or N:P ratio),  $y$  is the fungal nutrient stoichiometry and  $c$  is a constant (Sternner and Elser, 2002; Persson *et al.*, 2010). We



**Figure 2** Relationships between nutrient ratios (molar) in liquid media (Experiment 1) and (a) C:N, (b) C:P and (c) N:P stoichiometry of fungal biomass. Slopes ( $1/H$  values, see Table 1) between dissolved C:N ratio and fungal biomass C:N were either non-significant or less steep (linear regressions, TC  $R^2 = 0.001$ ,  $P = 0.92$ ; HL  $R^2 = 0.53$ ,  $P = 0.003$ ; assemblage  $R^2 = 0.41$ ,  $P = 0.013$ ) when compared to observed slopes between dissolved C:P ratio and fungal biomass C:P (TC and HL  $R^2 = 0.72$ ,  $P < 0.001$ ; assemblage  $R^2 = 0.92$ ,  $P < 10^{-7}$ ) or between dissolved N:P ratio and fungal biomass N:P (TC  $R^2 = 0.35$ ,  $P = 0.043$ ; HL  $R^2 = 0.42$ ,  $P = 0.012$ ; assemblage  $R^2 = 0.70$ ,  $P < 0.001$ ). Means of three replicates are shown. Dashed lines represent 1:1 ratio.

**Table 1** Summary of biomass nutrient stoichiometry and degree of elemental homeostasis in aquatic fungi grown in Experiment 1 (liquid media) and Experiment 2 (leaf litter substrate)

	C:N ratio			C:P ratio			N:P ratio		
	range	mean	1/H	range	mean	1/H	range	mean	1/H
<i>Experiment 1 (liquid media)</i>									
<i>T. chaetocladium</i>	8.2–16.5	11.2	0.01 <sup>a</sup>	50–408	122	0.34	5.3–28.9	10.9	0.16
<i>H. lugdunensis</i>	7.0–14.4	9.7	0.16	37–205	80	0.33	3.4–21.5	8.3	0.16
Seven-species assemblage	6.9–14.2	9.4	0.11	36–166	78	0.34	4.0–19.1	8.2	0.18
<i>Experiment 2 (leaf litter)</i>									
<i>T. chaetocladium</i>	8.0–23.5	14.6	—	26–466	151	—	2.2–34.0	10.2	0.53
<i>H. lugdunensis</i>	3.1–9.0	5.9	—	31–196	76	—	4.0–31.8	13.3	0.50
Six-species assemblage	6.7–27.2	13.3	—	21–228	86	—	1.5–14.2	6.8	0.31

$1/H$  values of 0 correspond to strict homeostasis, while values approaching 1 would indicate non-homeostasis or stoichiometric plasticity.

<sup>a</sup>Not significant.

calculated  $1/H$ , which is the slope of the regression between  $\log_{10}(x)$  and  $\log_{10}(y)$  and varies between 0 and 1. Microorganisms with  $1/H$  approaching 0 are considered strictly homeostatic and those with  $1/H$  approaching 1 are highly plastic with respect to their biomass elemental ratios (Persson *et al.*, 2010). Regression slopes ( $1/H$ ) with respect to C:nutrient ratios were calculated separately for *T. chaetocladium*, *H. lugdunensis* and multispecies fungal assemblages.

## Results

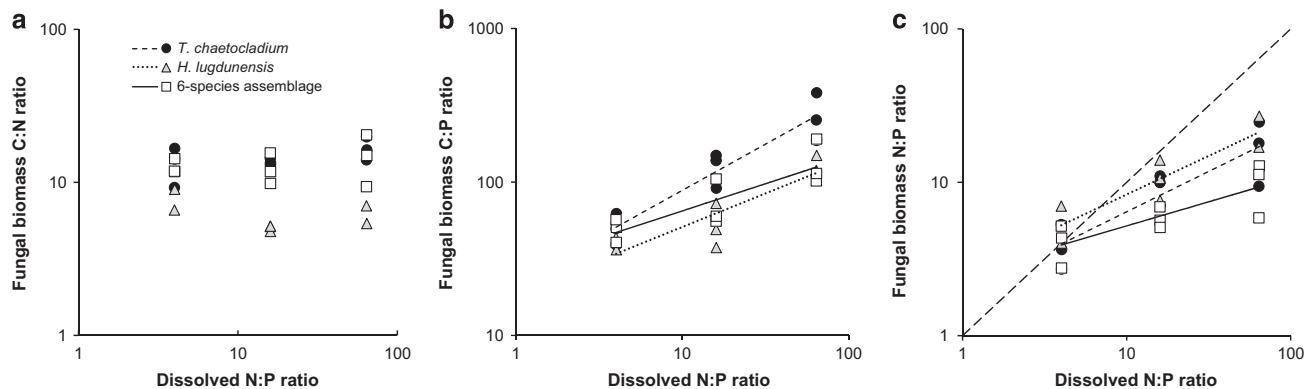
### Experiment 1. Aquatic fungi grown in liquid media

Fungal biomass C:N ratios ranged from 6.9 to 16.5 in different treatments (Figure 2a, Table 1). However, dissolved C:N ratio had no or only small effect on C:N of fungal biomass (linear regressions, TC  $R^2 = 0.001$ ,  $P = 0.92$ ; HL  $R^2 = 0.53$ ,  $P = 0.003$ ; assemblage  $R^2 = 0.41$ ,  $P = 0.013$ ), with all  $1/H$  values being  $< 0.16$ , indicating relatively strict homeostasis. In contrast, fungal C:P ratios increased sharply at lower

P availability or higher C:P ratio of the medium (TC and HL  $R^2 = 0.72$ ,  $P < 0.001$ ; assemblage  $R^2 = 0.92$ ,  $P < 10^{-7}$ ), with all  $1/H$  coefficients  $> 0.33$ , indicating higher plasticity (Figure 2b, Table 1). Fungal C:P ratios ranged from 36 to 408 depending on P availability and fungal species. The N:P ratio of the medium also affected N:P ratios of fungal biomass (TC  $R^2 = 0.35$ ,  $P = 0.043$ ; HL  $R^2 = 0.42$ ,  $P = 0.012$ ; assemblage  $R^2 = 0.70$ ,  $P < 0.001$ ). However, the  $1/H$  values (range 0.16–0.18) were considerably lower than those for fungal C:P. Overall, under the varying N:P ratios and levels of nutrient concentrations in the medium, fungal biomass N:P ratios ranged from 3.4 to 28.9 (Figure 2c, Table 1).

### Experiment 2. Aquatic fungi grown on leaf litter in microcosms simulating stream conditions

Fungal biomass stoichiometry was estimated as the ratio of fungal C produced to the amount of nutrient taken up during the same time period. Overall, fungal biomass C:N ratios (molar) ranged from 3.1 to 27.2 (Table 1, Figure 3a, Supplementary Figure S1),



**Figure 3** Effects of dissolved N:P ratios (molar) on elemental stoichiometry of fungal biomass associated with decaying leaf litter (Experiment 2). Dissolved N:P ratios did not affect (a) fungal biomass C:N (linear regressions,  $R^2 = 0.05\text{--}0.25$ ,  $P = 0.17\text{--}0.57$ ), but positively affected (b) fungal biomass C:P (TC  $R^2 = 0.89$ ,  $P < 0.001$ ; HL  $R^2 = 0.76$ ,  $P = 0.011$ ; assemblage  $R^2 = 0.72$ ,  $P = 0.004$ ) and (c) fungal biomass N:P (TC  $R^2 = 0.79$ ,  $P = 0.001$ ; HL  $R^2 = 0.81$ ,  $P = 0.006$ ; assemblage  $R^2 = 0.65$ ,  $P = 0.009$ ) indicating that fungi were not homeostatic with respect to their C:P and N:P ratios. Means of three replicates are shown. Dashed line represents 1:1 ratio.

depending on the fungal species (or assemblage) and dissolved nutrient availability. *H. lugdunensis* had the lowest mycelial C:N ratio, that is, its biomass was the most N-rich, while *T. chaetocladium* and the 6-species fungal assemblage had C:N ratios generally above 10. However, there was no significant effect of dissolved N:P ratio or  $\text{NO}_3\text{--N}$  concentration on fungal C:N (Figure 3; linear regressions,  $P > 0.05$ ). Thus, litter-associated fungi were also relatively homeostatic with respect to their C:N ratio.

Fungal C:P ranged from 21 to 466 (Table 1, Figure 3b, Supplementary Figure S1) depending on fungal species (or assemblage) and nutrient availability. Both dissolved N:P ratios (Figure 3; linear regression, TC  $R^2 = 0.89$ ,  $P < 0.001$ ; HL  $R^2 = 0.76$ ,  $P = 0.011$ ; assemblage  $R^2 = 0.72$ ,  $P = 0.004$ ) and SRP concentrations (all treatments combined,  $R^2 = 0.34$ ,  $P = 0.002$ ) affected fungal C:P ratios. Thus, litter-associated fungi were not homeostatic with respect to their C:P ratio.

Fungal N:P ranged from 1.5 to 34 (Table 1, Figure 3, Supplementary Figure S1), being lowest on average for the six-species assemblage. Dissolved N:P ratios had a significant effect on fungal biomass N:P (TC  $R^2 = 0.79$ ,  $P = 0.001$ ; HL  $R^2 = 0.81$ ,  $P = 0.006$ ; assemblage  $R^2 = 0.65$ ,  $P = 0.009$ ). Nitrate–N concentration did not affect fungal N:P ratio, while higher SRP resulted in lower fungal N:P (all treatments combined,  $R^2 = 0.31$ ,  $P = 0.004$ ). The overall pattern was somewhat similar to the relationship between dissolved nutrients and fungal C:P, suggesting that P rather than N drove fungal N:P ratios. In all treatments, fungi were not homeostatic with respect to their N:P ratios, as the  $1/H$  coefficient varied from 0.31 to 0.53.

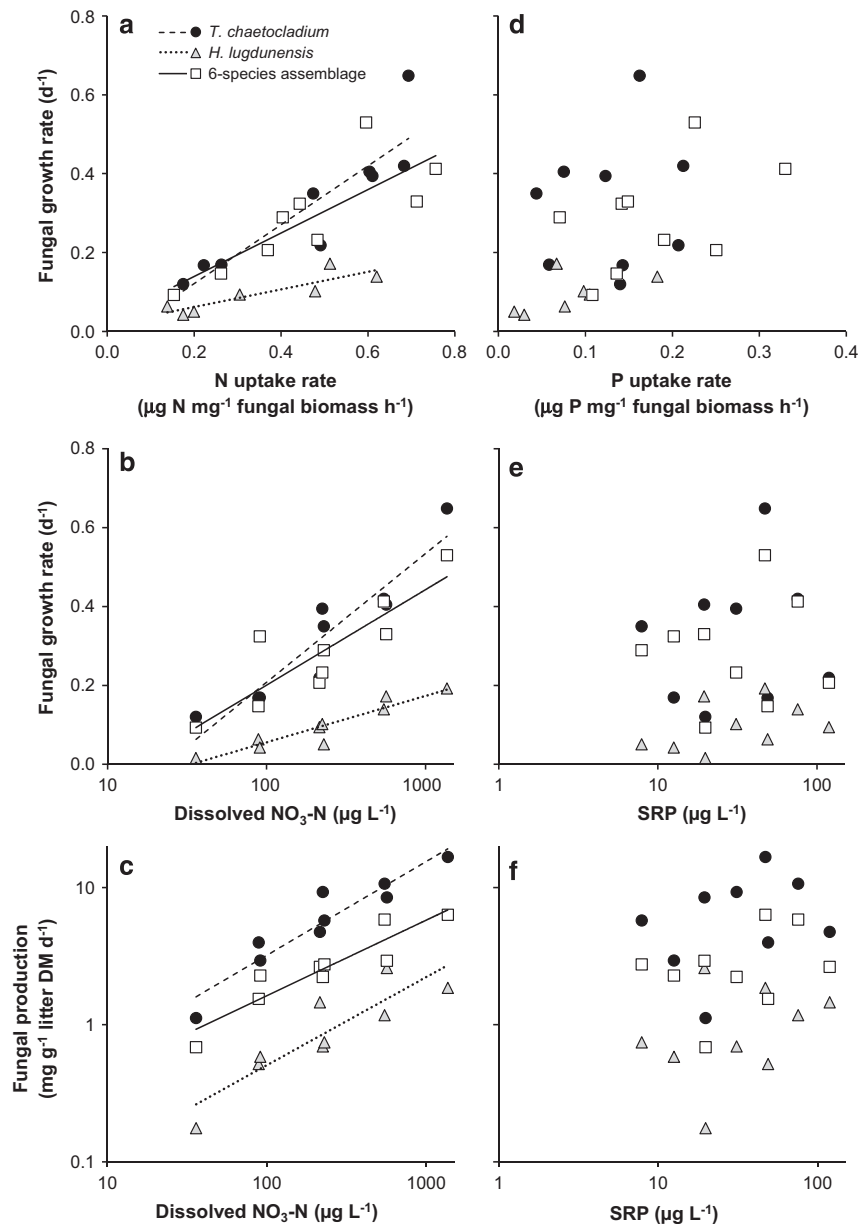
Fungal growth rates and production were stimulated by dissolved  $\text{NO}_3\text{--N}$  availability (Figures 4b and c, TC  $R^2 = 0.88$  and  $0.89$ ; HL  $R^2 = 0.88$  and  $0.79$ ; assemblage  $R^2 = 0.76$  and  $0.85$ , respectively,  $P \leq 0.002$  in all cases). Fungal growth rates were also strongly correlated with fungal nitrate–N uptake

rates (Figure 4a, TC  $R^2 = 0.79$ ,  $P = 0.001$ ; HL  $R^2 = 0.78$ ,  $P = 0.008$ ; assemblage  $R^2 = 0.66$ ,  $P = 0.008$ ). No effects of inorganic P uptake rate or SRP concentration on fungal growth rate or production were found (Figures 4d–f).

We found a significant positive relationship between fungal growth rate and biomass C:N when all data were analyzed together (Figure 5a,  $R^2 = 0.48$ ,  $p < 0.001$ ), but not for individual taxa or the six-species assemblage, again suggesting that C:N ratios are not flexible within taxa. We also found no relationship between fungal growth rates for individual species or multi-species assemblages and their C:P ratios or P content (Figure 5b).

## Discussion

Aquatic fungi are intimately associated with plant litter, making physical separation of fungal biomass from its natural substrate impossible. Aquatic fungi also obtain N and P from both their substrate and the water column (Suberkropp, 1995; Gulis et al., 2006), complicating estimation of their biomass nutrient ratios and assessment of elemental homeostasis (Cherif and Loreau, 2007; Fanin et al., 2013). Using a radioactive tracer (Experiment 2) allowed us to measure fungal production (C accrual), providing the first estimates of nutrient stoichiometry and homeostasis in fungi grown on natural substrates under realistic nutrient concentrations. Both N and P were kept at concentrations commonly found in streams, and our corresponding fungal biomass accrual on leaf litter was comparable to values from natural plant litter in streams (Gessner et al., 2007), suggesting that our results are ecologically meaningful. Moreover, our results from Experiment 1 using a soluble, relatively recalcitrant C source (carboxymethylcellulose) were surprisingly similar to those obtained with the radioactive tracer, despite different approaches.

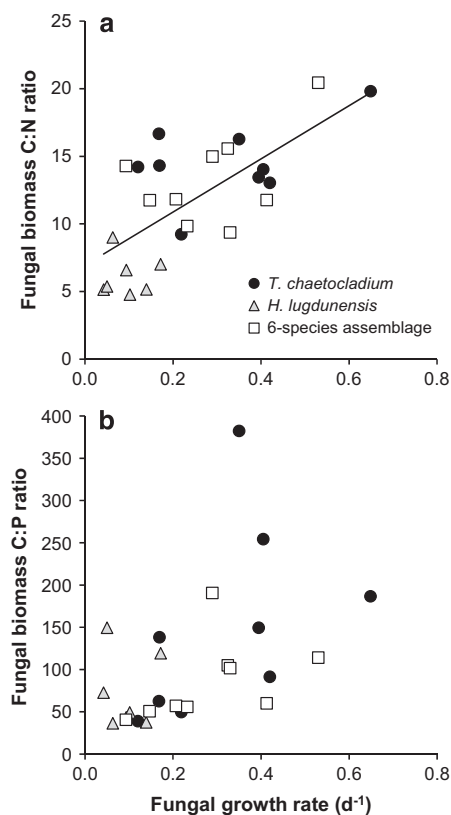


**Figure 4** Relationships between dissolved inorganic N and P availability or uptake rates and fungal growth rates or production associated with decaying leaf litter. Elevated  $\text{NO}_3\text{-N}$  uptake rates (**a**) led to faster fungal growth rates (linear regression, TC  $R^2 = 0.79$ ,  $P = 0.001$ ; HL  $R^2 = 0.78$ ,  $P = 0.008$ ; assemblage  $R^2 = 0.66$ ,  $P = 0.008$ ). Similar positive relationships were found for dissolved  $\text{NO}_3\text{-N}$  concentrations and fungal growth rates (**b**) (TC  $R^2 = 0.88$ ,  $P < 0.001$ ; HL  $R^2 = 0.88$ ,  $P < 0.001$ ; assemblage  $R^2 = 0.76$ ,  $P = 0.002$ ) and production (**c**) (TC  $R^2 = 0.89$ ; HL  $R^2 = 0.79$ ; assemblage  $R^2 = 0.85$ ,  $P \leq 0.001$  in all cases). No effects of inorganic P uptake rate or SRP concentration on fungal growth rates or production were found (**d–f**,  $P \geq 0.17$  in all cases).

The range of fungal C:N ratios in our experiments (3.1–27.2; Table 1) was broader than that reported in physiological experiments using rich liquid media with labile C sources (12.4–30.6; Danger and Chauvet, 2013; Grimmer et al., 2013) and was affected by species identity (we found very low C:N ratios of *H. lugdunensis* in Experiment 2). Fungal C:P ratios ranged from 21 to 466 and were generally lower than values reported by Danger and Chauvet (2013) and Grimmer et al. (2013) (76–1499), but broadly comparable to those of terrestrial litter-associated fungi (Mouginot et al., 2014). We also

found generally lower fungal N:P ratios (1.5–34.0) than those published previously. In our experiments, N:P ratios of multi-species fungal assemblages appeared somewhat less variable than those observed for single species, however, we found no significant differences in the degree of N:P and C:P non-homeostasis among the multi-species assemblages and monocultures (Figures 2b,c and 3c, Table 1, comparisons of slopes,  $P > 0.59$ ). Published indirect estimates of fungal C:N ratios from aquatic ecosystems or for aquatic microbial communities dominated by fungi have yielded a relatively narrow





**Figure 5** Relationships between growth rates and biomass elemental stoichiometry of fungi associated with decaying leaf litter. Higher biomass C:N ratios (a) corresponded to faster growth rates (linear regression, all fungal treatments combined,  $R^2 = 0.48$ ,  $P < 0.001$ ), while no relationships between fungal growth rates and C:P ratios (b) were found.

C:N range of 6–13 (Newell and Statzell-Tallman, 1982; Sanzone *et al.*, 2001; Findlay *et al.*, 2002). No estimates of fungal C:P from aquatic systems exist, while in terrestrial ecosystems, C:P estimates for ectomycorrhizal fungi range from 45–179 (Olsson *et al.*, 2008) to as high as 116–3900 (Wallander *et al.*, 2002).

Mean C:N:P of aquatic hyphomycetes was estimated at 92:9:1 and 107:9:1 in Experiment 1 and 2, respectively, which differs somewhat from the canonical Redfield ratio for marine plankton (106:16:1; Redfield, 1958), with a seemingly higher C:N ratio (~11:1) in fungi. Our ratios also suggest that aquatic fungi are more P-rich than algae, but less 'nutrient-dense' than bacteria (Sternner and Elser, 2002; Makino and Cotner, 2004). However, recent studies suggest that natural assemblages of heterotrophic lake bacteria may show great flexibility in their P content and nutrient stoichiometry, with extremely high C:P ratios under severe P limitation (Godwin and Cotner, 2015).

The C:N and C:P ratios of fungal biomass from Experiment 2 are likely slight overestimates. We assumed that all N and P incorporated into fungal biomass during our short radiotracer incubations was derived from dissolved inorganic N and P.

However, fungi can also acquire N and P from their organic substrates (such as leaf litter), and these fluxes into fungal biomass were not accounted for in our calculations. However, mining N and P from plant litter requires considerable energy expenditures to produce enzymes to attack complex organic molecules and cleave amino or phosphate groups (Sinsabaugh and Follstad Shah, 2012; Sinsabaugh *et al.*, 2014). Thus from an energetic standpoint, aquatic fungi are expected to rely heavily on dissolved inorganic N and P, if they are available. Indeed, recent experimental estimates of the importance of dissolved vs litter-derived N (Cheever *et al.*, 2013) suggest that the dissolved inorganic pool is by far the major N source for aquatic fungi. Moreover, N and P mineralization at early to mid-stages of decomposition (Experiment 2) was likely very low, and it is now well established that dissolved N and P in nutrient-addition experiments stimulate fungal activity, leading to considerable net nutrient immobilization (Gulis and Suberkropp, 2003a; Ferreira *et al.*, 2006; Gulis *et al.*, 2006; Cornut *et al.*, 2015). Small uncertainties in our estimates of fungal biomass (and C) due to interspecific variability in ergosterol content may have also affected our estimates of fungal C:nutrient ratios. To minimize this effect, we used a specific conversion factor of 4.5 mg ergosterol per g of fungal biomass for *H. lugdunensis*, while an average conversion factor (5.5 mg per g) was used for the other treatments, including our six-species assemblage (Gessner and Chauvet, 1993; Raviraja *et al.*, 2004).

While aquatic fungi maintained relatively constant C:N ratios regardless of changes in resource stoichiometry or dissolved N concentration (that is, homeostasis), fungal C:P and, hence, N:P ratios varied significantly depending on dissolved C:P and N:P ratios and absolute P availability, suggesting plasticity of P content. Danger and Chauvet (2013) also reported non-homeostasis of C:P and N:P in aquatic hyphomycetes in high-P liquid cultures, with 1/*H* values slightly higher (0.55–0.75) than those reported here for the experiment using leaf litter. The non-homeostatic pattern in fungal C:P and N:P ratios suggests the possibility of P storage (for example, in the form of polyphosphates) when dissolved P is plentiful, as has been observed in stream periphyton (Rier *et al.*, 2016). Grimmer *et al.* (2013) reported no correlation between RNA and DNA content and P content in aquatic fungi, supporting the notion of P storage. In terrestrial fungi, P content may also be flexible, since different groups of fungi can retain and store excess, metabolically inactive P during periods of luxury supply (Beever and Burns, 1980; Bucking and Heyser, 1999).

Non-homeostasis of C:P and N:P ratios in aquatic fungi parallels recent findings for aquatic heterotrophic bacteria. While some studies suggested relative homeostasis in aquatic bacteria (Makino *et al.*, 2003; Makino and Cotner, 2004), experiments with bacterial assemblages from different lakes



showed highly variable P content and marked non-homeostasis of C:P and N:P ratios (Tezuka, 1990; Cotner *et al.*, 2010; Godwin and Cotner, 2015). For example, Godwin and Cotner (2015) reported C:N:P of bacterial assemblages from lakes to range from 28:7:1 to as high as 8500:1200:1 under severe P limitation.

Responses of natural assemblages of heterotrophic microorganisms may deviate from homeostasis due to differences in optimal cellular N:P ratio and nutrient demand among microbial taxa, resulting in shifts in community structure across gradients of N and P availability (Tezuka, 1990; Chrzanowski *et al.*, 1996; Makino and Cotner, 2004; Godwin and Cotner, 2014). While such shifts have already been documented for aquatic bacteria, only circumstantial evidence exists for fungi. Changes in dominance patterns among aquatic hyphomycetes associated with leaf litter have been observed in response to whole-stream nutrient enrichment (N+P) (Gulis and Suberkropp, 2003a, 2004). Such changes in assemblage composition may drive shifts in both fungal and detrital stoichiometry. Fungal biomass can account for up to 18–23% of total detrital mass (Suberkropp, 1995), and it is nutrient-rich (low C:nutrient ratio) compared to uncolonized plant litter. Fungal biomass accrual and associated immobilization of dissolved nutrients as litter decomposes leads to decreases in C:N and C:P ratios of detritus and hence increased resource quality for detritivores (Cornut *et al.*, 2015). Since detritivorous invertebrates prefer to consume litter colonized by fungi, and even favor patches colonized by particular fungal species (Suberkropp, 1992), nutrient immobilization and variable fungal C:P and N:P may have strong effects on food quality and nutrient transfer to detritivores and higher trophic levels (Manning *et al.*, 2016).

We found no relationship between fungal growth rates on plant litter for individual fungal species or multi-species assemblages and their C:P ratios or P content. The Growth Rate Hypothesis (GRH; Sterner and Elser, 2002; Elser *et al.*, 2003) suggests that organisms with higher growth rates show higher P content (lower C:P ratios) due to faster rates of metabolism and protein synthesis that require higher concentrations of P-rich RNA. A plausible explanation for any lack of relationship between growth rate and fungal C:P may be (i) P storage and/or (ii) nutrient limitation. Hence, the GRH may not be valid under conditions of N limitation (Franklin *et al.*, 2011) or, in the case of aquatic fungi, when either dissolved N or P (or both) are limiting, so that fungi switch from relying on dissolved nutrients to mining N and P from plant litter. Grimmer *et al.* (2013) explicitly tested the GRH in several aquatic hyphomycetes in liquid cultures and also found no evidence to support it. Since a similar lack of support for the GRH has been reported for microalgae (Flynn *et al.*, 2010) and aquatic heterotrophic bacteria when P-limitation was relaxed (Makino and Cotner, 2004), the broad applicability of the

GRH to aquatic microbial communities may be limited. Interestingly, we saw a positive relationship between fungal growth rate and biomass C:N when all data were analyzed together, suggesting that the fastest growing taxa have the highest nutrient use efficiency (that is, can build biomass with the highest C:N ratios), but within taxa, C:N is not flexible.

Our findings of the positive effect of dissolved N, but not P, on fungal growth rates add to other data (Cheever *et al.*, 2012, 2013; Gulis *et al.*, unpublished) suggesting that fungal activity, but not C:N ratio, is driven by dissolved N availability. Conversely, fungal biomass C:P and N:P stoichiometry, but not activity, was driven by dissolved P availability, probably via luxury P uptake and storage. These findings have important implications for our understanding of organic matter processing and stream ecosystem functioning. Fungi may stabilize the stoichiometry of detritus and facilitate nutrient transfer to detritivorous invertebrates. N limitation (for example, low dissolved N:P ratios) would theoretically decrease fungal growth and litter decomposition rates. However, even when fungal biomass is relatively low, luxury P uptake by fungi would decrease litter C:P ratios and make detritus more palatable to detritivores. Conversely, P limitation (such as high dissolved N:P ratios common in many polluted streams) should not strongly constrain fungal growth and microbial litter decomposition rates. In this scenario, when fungal biomass is high but P-poor, detrital C:P ratio would still be decreased sufficiently to provide palatable resources to detritivores. Even though the effects of dissolved N and P may occur via different pathways (via changing litter associated biomass or its nutrient content), fungi-mediated shifts in detrital stoichiometry are a critical nexus for the effects of nutrients on litter breakdown and associated metazoans (Manning *et al.*, 2015).

Our results demonstrate that aquatic fungi have N and P content and C:N, C:P and N:P ratios intermediate to those reported for algae and bacteria, which can be explained by the overall metabolic activities or growth rates typical of these microbial groups (fastest in the case of bacteria and slowest for algae). However, while the aquatic fungi in our experiments were capable of maintaining nearly constant biomass C:N ratio, regardless of changes in resource stoichiometry or absolute concentrations of dissolved N, fungal C:P ratio varied depending on N:P supply ratio and P concentration. Thus, aquatic fungi are relatively homeostatic with respect to their biomass C:N ratio, but flexible with respect to their C:P and N:P ratios. The ability of aquatic fungi to immobilize and store P when it becomes available due to excessive nutrient loading may alter the flow of nutrients through aquatic food webs and affect ecosystem functioning, since many aquatic detritivores rely on litter-associated microorganisms as sources of C, N and P.

## Conflict of Interest

The authors declare no conflict of interest.

## Acknowledgements

We thank Rong Su for assistance with HPLC of radiolabeled ergosterol samples. Financial support from the National Science Foundation (DEB 0919054 to VG and DBI 0923063 to KAK) is gratefully acknowledged.

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