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Elemental stoichiometry of Fungi and Bacteria strains from grassland leaf litter

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ABSTRACT

In most terrestrial environments, our knowledge of the elemental composition and stoichiometry of microorganisms stems from indirect whole community analyses. In contrast, we have little direct knowledge of the elemental composition of specific microorganisms and the variation between and within Fungi and Bacteria. To address this issue, we isolated and identified the elemental content of 87 strains of Fungi and Bacteria isolated from grassland leaf litter. The isolated strains were affiliated with a broad range of diversity including Ascomycota and Basidiomycota for Fungi, and Proteobacteria, Bacteroidetes, and Actinobacteria for Bacteria. The C:P and C:N but not N:P ratios were significantly higher in Fungi than in Bacteria. Extensive strain variation in elemental composition was partly linked to phylogeny and growth rate. Across all strains, the geometric mean C:N:P was 88:15:1. This overall ratio was significantly higher than reported for other leaf litter and terrestrial whole communities but closer to the canonical Redfield ratio characterizing marine microorganisms. This result warrants further investigation into the discrepancy between whole community and isolated strain elemental ratios.

1. Introduction

The ratios of carbon (C), nitrogen (N), and phosphorus (P) in the environment and within organisms link the biogeochemical cycles of these important elements (Sterner and Elser, 2002). Despite the importance of stoichiometric ratios, less is known about their magnitude and variation in microorganisms in terrestrial environments. In the most extensive comparison of different soil microbial communities, Cleveland and Liptzin (2007) found an average microbial C:N:P molar ratios of 60:7:1. On leaf litter, microbial communities also show low C:N:P ratios of 16:4:1 (Van Meeteren et al., 2008). These ratios are starkly lower than the averages observed in marine environments as described by the Redfield ratio (106:16:1). Such values suggest that terrestrial microorganisms generally are depleted in C and N – or enriched in P – compared to marine microorganisms.

There are several possible biological mechanisms for variations in elemental composition among communities (Sterner and Elser, 2002). First, differences in environmental conditions like temperature or nutrient availability can influence elemental composition (Woods et al., 2003; Scott et al., 2012; Xu et al., 2013). Second, the growth rate and associated allocation to growth vs. resource acquisition machinery may vary. Specifically, the growth rate hypothesis postulates negative relationships between C:P and N:P ratios and growth rate (Sterner and Elser, 2002). Third, specific lineages may have a unique elemental composition and thus changes in microbial community composition can lead to differences in elemental ratios (Quigg et al., 2003; Zimmerman et al., 2014). Some studies have suggested that Fungi in comparison to Bacteria have a higher C:N (Strickland and Rousk, 2010) but otherwise little is known about phylogenetically related differences in the elemental ratios among soil or leaf litter microorganisms.

To directly investigate the C:N:P ratios of microorganisms in leaf litter, we analyzed the elemental ratio of 87 phylogenetically-diverse fungal and bacterial strains isolated from a semi-arid Mediterranean grassland ecosystem. We asked if the average C:N:P ratios of Fungi and heterotrophic Bacteria from this habitat differed from each other, from the ratio observed in whole communities in terrestrial ecosystems (60:7:1), or from the Redfield ratio. We next

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asked if differences between microorganisms were due to phylogenetic constraints or growth rate differences. Finally, to make a direct comparison with marine microorganisms, we compared the stoichiometry of these leaf litter strains to a suite of previously analyzed marine heterotrophic Bacteria (Zimmerman et al., 2014). We hypothesized that average stoichiometric ratios of terrestrial Fungi and Bacteria would be statistically indistinguishable from Cleveland and Liptzin's ratio and significantly lower than the Redfield ratio and the average ratios of marine isolates. Furthermore, we expected that stoichiometric variation among isolates would in part be due to growth rate differences and phylogenetic history.

2. Material and methods

2.1. Fungi isolation and growth

45 fungal isolates were isolated from leaf litter samples from a Mediterranean grassland ecosystem at Loma Ridge, CA (33.4°N, 117.4°W, elevation 365 m). The site climate is characterized by hot dry summers, a mean annual temperature of 17 °C, and mean annual precipitation of 325 mm (Potts et al., 2012). Nearly all precipitation falls between October and April. Soils are a fine-loamy, mixed, thermic Typic Palexeralfs sandy loam (California Soil Resource Lab, http://casoilresource.lawr.ucdavis.edu) with a pH of 6.8. The litter layer consists of senesced plant material from primarily exotic annual grasses of the genera *Avena*, *Bromus*, and *Lolium* as well as annual forbs such as *Erodium*, *Lupinus*, and *Vicia*. Litter microbial communities are dominated by bacterial biomass, though Fungi represent up to ~20% of microbial biomass in summer (Alster et al., 2013). Fungal cultures were obtained from leaf litter using a dilution to extinction method. Briefly, leaf litter was homogenized in a blade grinder, suspended in sterile water, rinsed, and passed through a series of filters to obtain a size fraction between 106 and 212 μm. Filtrate was suspended in 30 ml of 0.6% carboxy-methyl-cellulose solution. 10 μl of filtrate was added to 500 μl solid malt extract agar (MEA) medium (agar 20 g/l, malt extract 5 g/l, yeast extract 5 g/l, C:N:P ratio w/o agar = 290:35:1) augmented with kanamycin and ampicillin (50 mg/l). Tubes were examined weekly for growth. Fungal cultures were transferred to liquid MEA medium and incubated overnight with shaking at room temperature and maintained at (22 °C). Before harvesting for elemental analysis, petri dishes containing MEA media with a pre-combusted (500 °C, 4 h) 47 mm GF/F filter (Whatman, Florham Park, NJ) were inoculated. Each isolate was propagated aseptically on a filter using a sterile cotton applicator wetted with 0.9% NaCl and placed on the top of MEA media during incubation. As a blank treatment, seven filters were humidified with NaCl. Petri dishes were incubated at room temperature for up to two months until sufficient biomass had appeared.

2.2. Fungi biomass isolation using freeze-drying

Filters with Fungi biomass were removed from the agar plates and collected into 5 ml vials. The vials were flash frozen in liquid nitrogen and stored at −80 °C until further processing. The frozen filters were freeze-dried overnight with a pressure below 30 Pa at −25 °C in a freeze-drying system (Freezone 4.5, Labconco, Kansas City, MO) and homogenized with a disperser (Ultra-Turrax T8, IKA Wilmington, NJ) until obtaining a fine powder. The culture powder was stored at −80 °C in 5 ml pre-combusted (500 °C, 4 h) glass vials until further analysis.

2.3. Bacteria isolation and growth

5–10 g of leaf litter were weighed and washed down through a sieve column of 106, 212, and 2000 μm pores with 750 ml–1000 ml of autoclaved, distilled water to separate leaf litter particles. Leaf litter particles were then collected from the 106–212 μm fraction in a sterile Falcon tube and rinsed with 5–10 ml of distilled water to create a litter suspension. The suspension was then poured over a Millipore Sterifil 47 mm Aseptic Vacuum Filter System suspension chamber set up with a sterile, nylon Millipore Sterifil 47 mm Filter O-ring with 100 μm pores. 200 ml of distilled water was poured down the suspension chamber and collected as the first wash water. The first wash was then inoculated onto LB media plates and incubated at room temperature. 42 individual colonies were picked and re-transferred onto LB plates. This process was repeated three times to ensure clonal isolation.

Each culture was then incubated in pre-filtered (0.22 μm) LB media (C:N:P ratio = 189:49:1) at 25 °C with shaking (225 rpm) and growth was monitored at OD_{600}. We first identified the full growth curve and estimated the growth rate in the logarithmic phase. On a following run, we then sampled replicate cultures in mid-log phase for particulate elemental content and flow cytometry. Specifically, technical replicate samples of cells from 250 μl samples were captured on pre-combusted (500 °C, 4 h) GF/F filter (Whatman, Florham Park, NJ). Replicate 500 μl samples were fixed at final concentration of 0.1% glutaraldehyde and stored in liquid nitrogen. Once defrosted, samples for flow cytometry were incubated in the dark for 15 min with 2 μl 10,000× diluted SybrGreen (Life Technologies, Grand Island, NY). Samples were then diluted with autoclaved and 0.2 μm filtered media and counted on an Accuri C6 Flow Cytometer (BD Biosciences, San Jose, CA).

2.4. Determination of particulate organic material

To quantify particulate organic carbon (POC) and nitrogen (PON), filter samples were thawed and allowed to dry overnight at 65 °C. We weighed three replicates of each sample (isolates and blanks) with a microbalance and then packed the material into a 30 mm tin capsule (CE Elantech, Lakewood, NJ) and analyzed C and N content on a FlashEA 1112 nitrogen and carbon analyzer (Thermo Scientific, Waltham, MA), following the protocol of Sharp (1974). POC and PON concentrations were calibrated using known quantities of atropine and peach leaves at each run. The amount of particulate organic phosphorus (POP) was determined in each sample (three replicates per isolate and blank) using a modified ash-hydrolysis method (Lomas et al., 2010). We also directly determined the media composition (without agar) for both Fungi and Bacteria using the POC, PON, and POP protocols.

2.5. PCR and phylogenetic analysis

Genomic DNA was extracted from morphologically distinct cultures using 10 μl each of the extraction and dilution solutions from the Extract-N-amp Plant kit (Sigma–Aldrich, St. Louis, MO) and incubated according to the manufacturer's directions. To identify fungal strains, we PCR amplified the ITS region as well as the adjacent ~600 bp of 28S rRNA using the ITS1F and TW13 primers (Gardes and Bruns, 1993). Approximately 0.1 μl of each DNA extract was added to a PCR cocktail containing 1.2U Taq polymerase, 1× PCR buffer containing 1.5 mM $MgCl_2$, 200 μM of each dNTP, and 0.5 μM of each primer in a final volume of 25 μl. For Bacteria, we PCR amplified the full 16S rRNA gene using the pA (5′-AGAGTTTGATCCTGGCTCAG-3′) and pH (5′-AAGGAGGTGATCCAGCCGCA-3′) primers as designed by Edwards et al. (1989). Approximately 5 μl of each DNA extract was added to a PCR cocktail containing 0.3 μl of Taq polymerase (5 units per μl), 15.75 μl of Premix F (Epicentre, Madison, WI), and 50 μM of each primer in a final volume of 26.5 μl. Sequences were generated with Sanger paired-end sequencing and were deposited in GenBank under the

Table 1
C:N:P molar ratios (±std) of cultures of Fungi and Bacteria.

	ID	Organism	C:N	C:P	N:P
Ascomycota	f1	*Alternaria* sp.	11.1 ± 1.63	243 ± 67.8	22.4 ± 7.53
	f2	*Hypocrea koningii*	17.4 ± 4.56	106 ± 4.80	6.39 ± 1.60
	f3	*Hypocreales*	5.22 ± 0.07	76. ±1.995	14.7 ± 0.27
	f5	*Hypocreales*	13.5 ± 3.22	215 ± 65.5	16.4 ± 4.93
	f10	*Davidiella* sp.	9.42 ± 1.65	256 ± 37.3	27.3 ± 2.19
	f63	*Davidiella* sp.	14.0 ± 2.72	223 ± 62.9	16.3 ± 5.02
	f13	*Pleosporales*	5.29 ± 0.18	81.5 ± 3.55	15.4 ± 0.93
	f26	*Cercophora* sp.	6.32 ± 0.81	45.7 ± 4.06	7.26 ± 0.40
	f33	*Alternaria* sp.	12.2 ± 0.42	50.7 ± 13.8	4.15 ± 0.99
	f37	*Fusarium* sp.	28.2 ± 7.32	64.0 ± 12.6	1.87 ± 1.30
	f64	*Capronia brabeji*	9.72 ± 0.06	87.6 ± 12.5	9.01 ± 1.25
	f69	*Sordariomycetes*	12.5 ± 1.15	168 ± 9.76	13.5 ± 1.37
	f81	*Gibberella* sp.	10.6 ± 1.29	89.3 ± 16.0	8.66 ± 2.74
	f101	*Tetracladium* sp.	10.1 ± 1.24	69.5 ± 8.67	6.94 ± 1.24
	F205	*Lewia* sp.	5.98 ± 0.31	54.5 ± 0.74	9.13 ± 0.59
	F210	*Neofusicoccium* sp.	20.8 ± 12.7	101 ± 38.9	5.60 ± 1.95
	f220	*Dothideomycetes*	4.93 ± 0.03	102 ± 7.65	20.7 ± 1.51
	f274	*Dothideomycetes*	8.66 ± 0.93	185 ± 12.5	22.0 ± 1.68
	f222	*Helotiales*	9.81 ± 1.02	124 ± 33.5	12.5 ± 2.29
	f223	*Dothidea* sp.	15.2 ± 1.86	316 ± 119	21.2 ± 8.65
	f226	*Phaeomoniella* sp.	10.2 ± 0.63	225 ± 47.4	22.0 ± 3.48
	f233	*Kabatiella bupleuri*	10.3 ± 3.54	180 ± 29.3	19.5 ± 8.74
	f242	*Pleosporales*	4.92 ± 0.10	102 ± 8.25	20.8 ± 2.06
	f245	*Pleosporales*	5.52 ± 0.67	69.4 ± 8.45	12.6 ± 1.21
	f272	*Exophiala* sp.	4.88 ± 0.41	74.1 ± 3.09	15.2 ± 0.65
	f276	*Pleurophoma* sp.	6.72 ± 0.05	80.9 ± 11.7	12.0 ± 1.71
	f280	*Phaeosphaeria* sp.	6.48 ± 7.74	64.8 ± 38.2	16.9 ± 8.81
	f285	*Helotiales*	5.30 ± 0.21	103 ± 12.5	19.4 ± 2.64
	f288	*Epicoccum nigrum*	6.31 ± 0.00	76.7 ± 7.45	12.2 ± 1.19
Basidiomycota	f53	*Cryptococcus* sp.	4.57 ± 0.10	90.0 ± 3.71	19.7 ± 1.15
	f61	*Cryptococcus* sp.	9.69 ± 1.12	188 ± 20.8	19.5 ± 2.83
	f65	*Rhodotorula. benthica*	4.55 ± 0.05	169 ± 49.0	37.1 ± 10.4
	f67	*Rhodotorula* sp.	4.63 ± 0.02	92.2 ± 6.75	19.9 ± 1.43
	f80	*Cryptococcus* sp.	12.6 ± 2.08	178 ± 24.8	21.3 ± 9.88
	f98	*Cryptococcus* sp.	6.30 ± 0.51	41.6 ± 0.94	6.62 ± 0.38
	f99	*Cryptococcus* sp.	5.89 ± 0.17	69.9 ± 2.41	11.9 ± 0.76
	f230	*Cryptococcus* sp.	11.3 ± 0.57	227 ± 3.71	31.1 ± 20.0
	f261	*Cryptococcus* sp.	4.68 ± 0.19	75.2 ± 2.88	16.1 ± 0.27
Zygo	f102	*Mucor flavus*	6.99 ± 0.49	128 ± 7.00	18.4 ± 0.33
	f104	*Mucor racemosus*	8.07 ± 0.05	75.9 ± 15.8	9.40 ± 1.97
	f109	*Mucor racemosus*	8.92 ± 0.98	78.8 ± 13.6	9.01 ± 2.51
No sequence	f68		14.0 ± 0.76	158 ± 38.0	11.4 ± 2.86
	f235		9.13 ± 2.14	200 ± 52.0	21.8 ± 0.60
	f284		4.65 ± 0.54	64.3 ± 23.5	14.0 ± 5.44
	f287		8.14 ± 0.55	51.9 ± 9.86	6.40 ± 1.39
Fungi (geometric mean ± 95%)			8.30 (7.25–9.51)	106 (90.9–125)	13.3 (11.2–15.8)
	ID	Organism	C:N	C:P	N:P
Actinobacteria	b109	*Curtobacterium* spp.	4.40 ± 0.84	121 ± 9.49	28.2 ± 7.55
	b115	*Curtobacterium* spp.	4.58 ± 0.93	70.9 ± 17.5	16.2 ± 7.12
	b123	Nocardioidaceae	5.78 ± 0.22	98.9 ± 22.8	17.1 ± 3.30
	b126	*Cryocola* spp.	4.03 ± 0.42	53.2 ± 5.17	13.2 ± 0.09
	b127	*N. bacterium*	4.03 ± 0.94	87.7 ± 3.06	22.5 ± 5.99
	b136	*C. flaccumfaciens*	5.39 ± 0.63	69.5 ± 0.15	13.0 ± 1.48
	b145	*C. flaccumfaciens*	4.13 ± 0.63	76.9 ± 2.17	18.8 ± 2.33
	b163	*C. flaccumfaciens*	4.68 ± 0.42	128 ± 40.6	27.7 ± 11.1
	b171	*C. flaccumfaciens*	5.24 ± 1.15	109 ± 21.7	21.7 ± 8.90
	b173	*Arthrobacter* spp.	6.99 ± 0.38	72.0 ± 23.1	10.2 ± 2.75
	b174	*Cryocola* spp.	5.24 ± 0.64	52.1 ± 4.98	10.1 ± 2.17
	b177	*S. luteola*	10.9 ± 0.79	175 ± 22.9	16.1 ± 0.94
	b186	*Frigoribacterium* spp.	5.10 ± 0.56	75.8 ± 39.0	15.4 ± 9.33
	b201	*C. michiganensis*	4.43 ± 1.16	115 ± 8.10	27.0 ± 8.88
	b212	*P. cousiniae*	3.95 ± 0.54	66.9 ± 3.08	17.2 ± 3.11
	b213	*Curtobacterium* spp.	8.49 ± 2.56	92.5 ± 11.6	11.2 ± 2.01
	b219	*R. cercidiphylli*	2.38 ± 0.30	89.2 ± 5.27	37.9 ± 7.03
	b221	*Rhodococcus* spp.	2.83 ± 0.31	81.0 ± 35.5	29.5 ± 15.8
	b222	*Sanguibacter* spp.	4.26 ± 0.01	61.2 ± 5.30	14.4 ± 1.28
	b223	*Microbacterium* spp.	4.17 ± 0.77	66.2 ± 4.91	16.3 ± 4.16
Bacteroidetes	b29	*Chryseobacterium* spp.	4.61 ± 0.48	64.9 ± 0.72	14.2 ± 1.31
	b31	*Dyadobacter* spp.	4.67 ± 0.23	69.1 ± 7.44	14.8 ± 0.86
	b41	*Flavobacterium* spp.	2.90 ± 0.04	106 ± 24.6	36.7 ± 9.02
	b134	*P. borealis*	5.55 ± 0.30	53.0 ± 6.31	9.53 ± 0.62
	b209	*Dyadobacter* spp.	4.88 ± 0.09	154 ± 58.7	31.7 ± 12.6
Proteobacteria	b2	*E. billingiae*	3.79 ± 0.40	91.6 ± 25.0	24.7 ± 9.20

Table 1 (*continued*)

	ID	Organism	C:N	C:P	N:P
	b4	*Pseudomonas* spp.	3.90 ± 0.21	71.2 ± 0.95	18.3 ± 0.74
	b7	*Pseudomonas* spp.	3.45 ± 0.60	47.2 ± 2.75	13.8 ± 1.60
	b9	*P. synxantha*	3.32 ± 0.23	52.3 ± 11.5	15.9 ± 4.57
	b12	*Pseudomonas* spp.	4.01 ± 0.17	67.2 ± 3.95	16.8 ± 0.29
	b17	*Pseudomonas* spp.	3.86 ± 0.12	62.2 ± 21.4	16.1 ± 5.05
	b21	*Pseudomonas* spp.	3.50 ± 0.32	61.2 ± 8.49	17.5 ± 0.84
	b27	*Erwinia* spp.	3.34 ± 1.03	55.7 ± 0.93	17.5 ± 5.13
	b38	*D. zoogloeoides*	4.92 ± 0.29	61.0 ± 3.45	12.4 ± 0.02
	b47	*D. zoogloeoides*	4.71 ± 0.62	65.8 ± 0.88	14.1 ± 1.68
	b49	*V. paradoxus*	4.61 ± 0.09	40.1 ± 3.37	8.71 ± 0.89
	b114	*P. poae*	5.40 ± 0.44	43.6 ± 7.86	8.04 ± 0.81
	b117	*Pseudomonas* spp.	3.80 ± 0.00	58.7 ± 0.85	15.5 ± 0.24
	b122	*P. fluorescens*	4.32 ± 0.23	43.5 ± 9.40	10.0 ± 1.65
	b124	*P. fluorescens*	3.93 ± 0.38	59.2 ± 8.94	15.0 ± 0.81
	b132	*D. zoogloeoides*	5.14 ± 0.10	59.3 ± 10.0	11.6 ± 2.17
	b160	*E. billingiae*	3.44 ± 0.08	52.9 ± 4.16	15.4 ± 0.87
Bacteria (geometric mean − CI 95%)			4.59 (4.06–4.81)	71.8 (64.6–79.7)	16.4 (14.6–18.5)
Total (geometric mean − CI 95%)			C:N	C:P	N:P
			6.12 (5.52–6.80)	88.1 (79.4–97.7)	14.7 (13.3–16.4)

accession numbers KF733300–340 for Bacteria and KF733341–381 for Fungi, respectively. For a few strains, we could not identify the SSU rRNA sequence and these strains were excluded from the phylogenetic analysis.

Isolate identity was tentatively assigned as the best identified match to a sequence within the GenBank nr/nt database inferred with a blastn alignment (Altschul et al., 1997). For phylogenetic analyses, the ITS portions of each sequence were removed and 558 bp of the 28S rRNA were aligned using the local pairwise alignment setting of MAFFT (Katoh et al., 2009) with the Chytrid *Spizellomyces punctatus* added as an outgroup. For Bacteria, we aligned the 16S rRNA sequences using the SINA aligner (www.arb-silva.de) (Pruesse et al., 2012). A maximum likelihood tree with 100 bootstrap replications using a transition/transversion ratio = 2, a constant base rate variation among sites, and empirical base frequencies was estimated using PHYLIP v. 3.68 (Felsenstein, 2006).

We tested for a significant phylogenetic signal in the variation in elemental ratios among the fungal strains. We used both a simple Mantel test as encoded in the R package 'ade4' (Dray and Dufour, 2007) and Pagel's λ in the R package 'phytools' (Pagel, 1999; Revell, 2012). The R package 'caper' was used for phylogenetic generalized least square regression to estimate the correlation between growth rates and elemental ratios while accounting for a phylogenetic autocorrelation (Orme et al., 2012). The input tree for all phylogenetic analyses was a maximum likelihood tree estimated from original alignment using the majority consensus tree of 100 bootstrap runs as topological guide. We determined that the lognormal transformed ratios could be approximated with a normal distribution. Thus, differences among geometric mean ratios were determined with one- or two-sample *t*-tests on log-transformed ratios. Geometric means and 95% confidence intervals were calculated by back-transforming the means and 95% confidence intervals of the log-transformed data.

3. Results

3.1. Elemental stoichiometry of cultures

The 45 fungal and 42 bacterial strains from grassland leaf litter represented a broad range of phylogenetic diversity based on SSU rRNA sequencing (Table 1). The fungal isolates were affiliated with the phyla Ascomycota, Basidiomycota, and Zygomycota and from a total of 25 genera. The bacterial isolates were affiliated with Proteobacteria, Bacteroidetes, and Actinobacteria from a total of 18 genera. The phylogenetic distribution of the strains fell within the phylogenetic community composition based on metagenomic sequencing of the total community. In a metagenomics survey of leaf litter at the same site, these three fungal phyla constituted more than 99% of the fungal sequences. For Bacteria, the isolated phyla represented more than 90% of the bacterial sequences in the metagenome.

We grew the cultures in a high carbon to nutrient media to reflect the availability of these elements in soil and leaf litter environments. Across all strains, the geometric mean C:P, N:P, and C:N molar ratios [±95% CI] were 88.1 [79.4,97.7], 14.7 [13.3,16.4], and 6.1 [5.5,6.8], respectively (Table 1). All three ratios were significantly different from the soil microbial biomass ratios reported previously (Fig. 1, two-sample *t*-test, $p < 0.05$). However, there was also an overlap in the distribution of elemental ratios between our strains and field communities (Fig. 1). The geometric mean C:P ratio was significantly lower than the Redfield C:P ratio (one-sample *t*-test, $p < 0.001$), but the C:N and N:P ratios were not significantly different from Redfield ratios. The fungal and bacterial strains also differed from one another (Fig. 2, Table 1). Both the C:P and C:N geometric mean ratios were significantly higher for Fungi compared to Bacteria (two-sample *t*-test, $p < 0.001$). The N:P ratios were more similar for the two domains, but slightly higher for Bacteria (two-sample *t*-test, $p = 0.046$).

3.2. Variation in stoichiometry within Fungi and Bacteria

Within domains, the strains exhibited extensive variation in elemental ratios despite identical growth conditions (Fig. 3). For Fungi, the ratios among the three phyla did not differ significantly (1-way Kruskal–Wallis test, $p > 0.05$), and the pairwise similarity of ratios between fungal strains was not correlated with phylogenetic distance (Table 2). We did, however, observe evidence of phylogenetic conservatism of the N:P ratio using Pagel's lambda metric, even though the ratios were highly variable among closely related fungal strains. For example, seven different strains of *Cryptococcus* had large differences in all three elemental ratios (Table 1).

In contrast to Fungi, Bacteria displayed a greater degree of phylogenetic structuring, particularly for the C:P and N:P ratios. The bacterial C:P ratio was significantly different among bacterial phyla (1-way Kruskal–Wallis test, $p < 0.05$). Specifically, strains affiliated with Proteobacteria had a lower ratio compared to Bacteroidetes

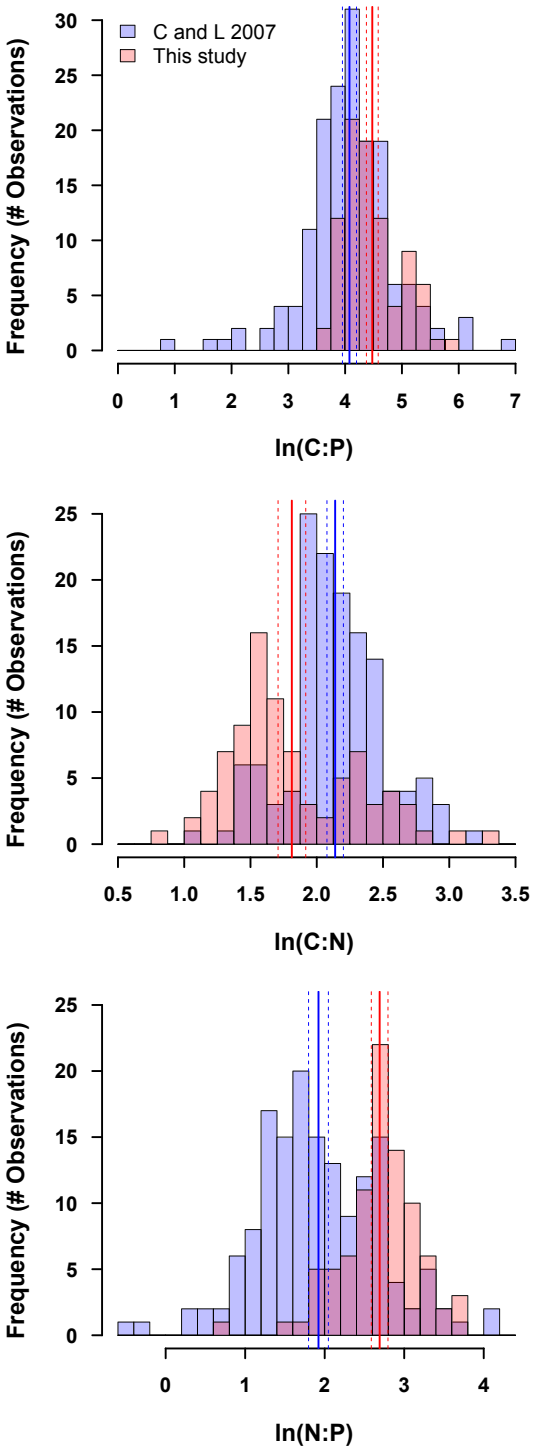

Fig. 1. Variation in the cellular C:N:P molar ratios for field communities (appendix A, Cleveland and Liptzin, 2007) as well as cultured strains of Fungi and Bacteria ($n = 87$). The solid lines represent the mean of log-transformed values and the dashed lines represent the 95% confidence interval of the mean.

and Actinobacteria (Tukey's posthoc test, $p < 0.05$). In contrast, the C:N and N:P ratios did not differ significantly among phyla. However, both the C:P and N:P ratios (but not C:N) were phylogenetically correlated (Table 2). This result was supported by Pagel's lambda, which was much higher for C:P and N:P compared to C:N (Table 2).

For the bacterial strains, we were also able to estimate the cell number and thus quantify the specific cellular content of C, N, and P. The average cell quota of the cultures was 4.4 pg C, 0.4 pg N, and 0.07 pg P. Furthermore, faster growing cells had significantly lower C:P and C:N but not N:P ratios (Fig. 4). To evaluate if this correlation was due to any phylogenetic structuring of the growth rate and elemental ratios, we then performed a phylogenetic generalized least square regression. Growth rate was still negatively related to C:P and C:N but not N:P ratios (Fig. 4), suggesting that, independent of phylogeny, differences in growth strategy influence the elemental ratios of these bacterial strains.

Finally, we compared the elemental stoichiometry of the leaf litter Bacteria to that of a suite of marine Bacteria (C:N:P = 77:17:1) previously analyzed (Zimmerman et al., 2014). Despite large differences in growth conditions between the two sets of strains, the average ratios did not differ (two-sample t-test, $p > 0.05$).

4. Discussion

In this study, we provide the first systematic analysis of elemental ratios across a wide phylogenetic range of Bacteria and Fungi strains from grassland leaf litter. We identify extensive variation in the elemental stoichiometry between strains but also some links to phylogenetic history and growth physiology. Our results are consistent with a high Fungi C:N ratio previously detected in direct elemental measurements of ectomycorrhizal mycelia and fruiting bodies in forest soil and cultures (Van Veen and Paul, 1979; Lodge, 1987; Wallander et al., 2003). A high Fungi C:P ratio has also been seen (Stark, 1972; Lodge, 1987). For Bacteria, the lower C:P and C:N ratios are similar to other studies of heterotrophic Bacteria including a range of marine and terrestrial heterotrophic cultures (Van Veen and Paul, 1979; Vrede et al., 2002; Makino et al., 2003; Cotner et al., 2010; Zimmerman et al., 2014). This difference suggests that Bacteria are generally depleted in carbon. Thus, a greater fungal abundance may lead to an increase in C:nutrient ratios in microbial communities. However, the specific chemical differences underlying such differences are currently unknown.

Past research has clearly identified that most microorganisms are not homeostatic, and their cellular elemental content is dependent on the specific environmental conditions (Sterner and Elser, 2002). In our study, higher observed C:nutrient ratios in Fungi than Bacteria could be due to the higher C:nutrient content of the fungal growth medium. However, this explanation is unlikely because the influence of resource supply ratios is usually only observed under resource limited growth (Klausmeier et al., 2004; Bonachela et al., 2013). We harvested the cultures during exponential growth when there was essentially unlimited access to resources. Thus, the gap in elemental ratios between Fungi and Bacteria is more likely driven by differences in elemental requirements under these growth conditions.

Consistent with past research of heterotrophic microorganisms (Zimmerman et al., 2014), our study also reveals extensive variation in the elemental ratios within both Bacteria and Fungi despite the same growth conditions. This variation is likely driven by differences in functional traits like growth optimum, nutrient acquisition, and resource requirements and indicates that the specific composition of leaf litter communities will have an impact on the elemental stoichiometry. For Fungi, there appears to be little phylogenetic structure to the elemental composition such that closely related strains can have very different ratios. For Bacteria, the phylum Proteobacteria displays a lower C:P ratio in comparison to Bacteroidetes and Actinobacteria. To our knowledge, this is the first time a difference in the elemental stoichiometry across bacterial phyla has been described – although this may be an artifact of our strain selection. Within these phyla, we also find extensive variation but these differences are correlated to phylogenetic distance. Thus, the C:N:P ratios of terrestrial Bacteria appear to be

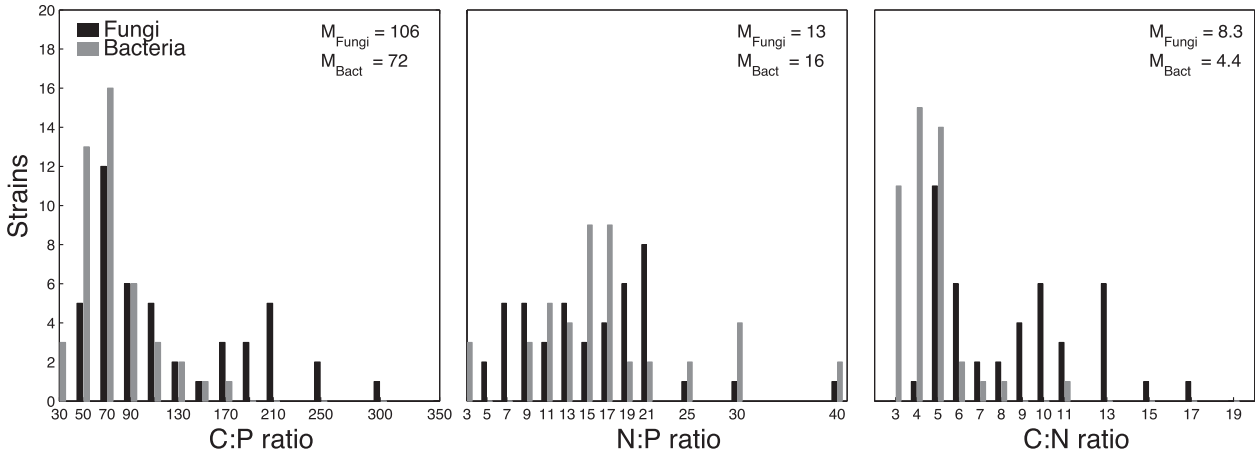

Fig. 2. Variation in the cellular C:N:P molar ratios of Fungi ($n = 45$) and Bacteria ($n = 42$) strains. M is the geometric mean ratio.

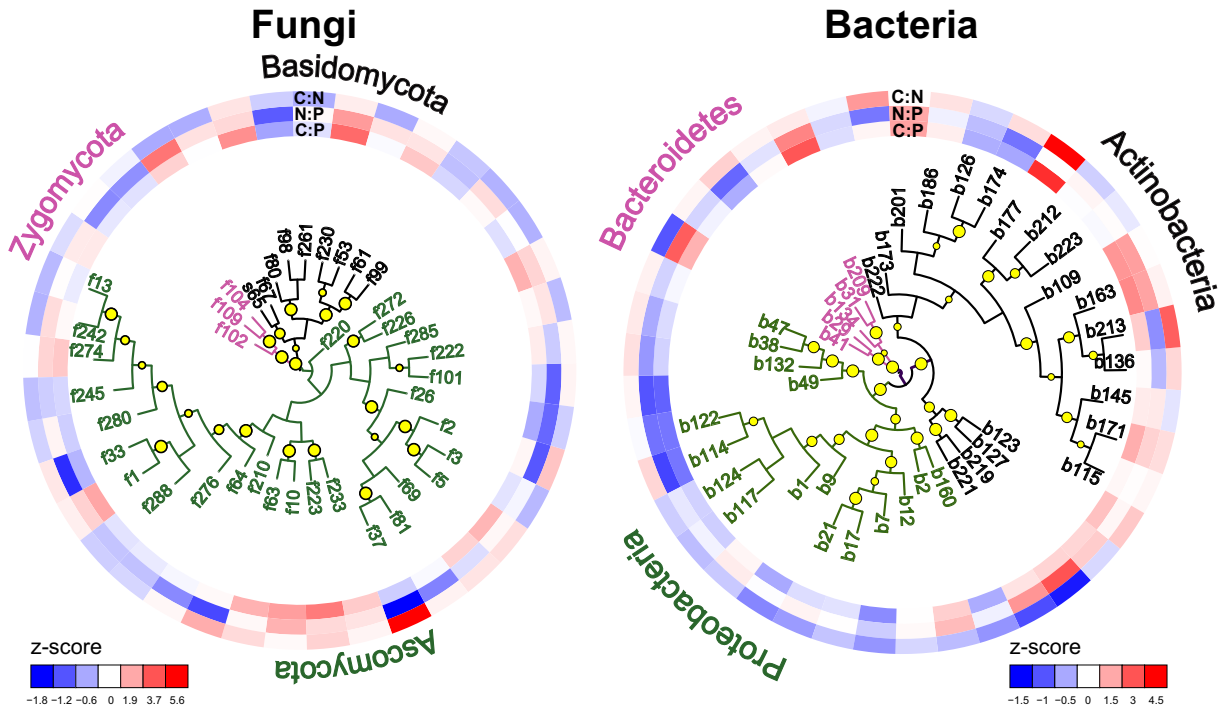

Fig. 3. Phylogenetic distribution of cellular elemental ratios in Fungi and Bacteria. Each phylogenetic maximum likelihood tree is a majority consensus of 100 bootstrap runs using Phylip and visualized in iTOL (Letunic and Bork, 2007). The yellow circles represent nodes with at least 50% support and the diameter represents the support level. The *z*-scores are based on the median to account for the non-normal probability density distributions of elemental ratios (Martiny et al., 2013b).

Table 2
Tests for phylogenetic signal of C:N:P ratios in Fungi and Bacteria.

	C:P	N:P	C:N
Fungi ($N = 40$)			
Mantel R	−0.01	−0.02	0.04
p	0.54	0.58	0.24
Pagel's λ	0.11	0.71	0.17
p	0.72	0.01	0.36
Bacteria ($N = 41$)			
Mantel R	0.15	0.22	−0.04
p	0.07	0.01	0.60
Pagel's λ	0.42	0.89	0.20
p	0.05	0.01	0.45

phylogenetically constrained, as has been seen for phytoplankton lineages (Quigg et al., 2003; Martiny et al., 2013a). We also observe variations in the elemental stoichiometry of Bacteria that can be linked to their growth physiology. In support of the growth rate hypothesis (Sterner and Elser, 2002), we find a significant negative correlation between C:nutrient ratios and growth rate in Bacteria. This connection has been elusive among marine microorganisms (Martiny et al., 2013b; Zimmerman et al., 2014), but it appears that leaf litter Bacteria respond physiologically according to this theory.

The mean elemental stoichiometry of our fungal and bacterial strains differ significantly from the mean whole community ratios observed in litter and soil (Cleveland and Liptzin, 2007; Van Meeteren et al., 2008). Especially the N:P ratio is much higher in cultures and match the ratios observed in marine communities.

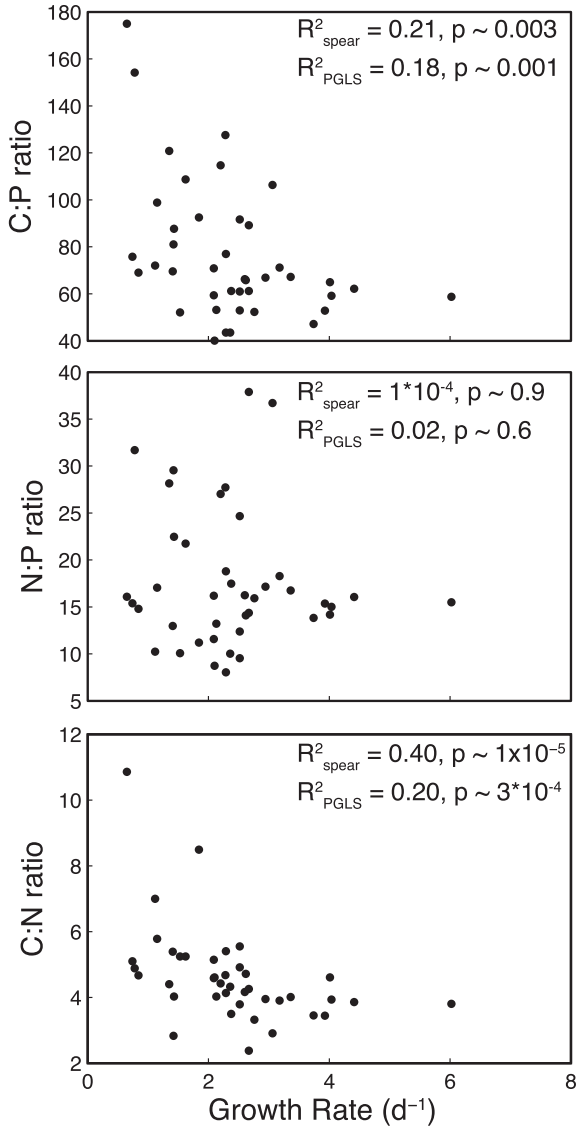

Fig. 4. Link between growth rate and cellular C:N:P molar ratios in Bacteria ($n = 42$). R and p values were either estimated using a Spearman rank correlation and phylogenetic generalized least square regression.

However, our mean ratios fall within the overall distributions reported in Cleveland and Liptzin (2007) and thus overlapping with at least some reported ratios for whole communities, even though our geometric mean ratios were statistically different. No method of measuring community stoichiometry is perfect, and methodological biases must be considered in the interpretation of our results. Culture-based methods do not fully sample the microbial community, so the observed elemental ratios may not be representative of the whole community. Our culture approach also means that growth conditions are not identical to field conditions and we chose a specific medium with lower C:nutrient ratios than plant material (McGroddy et al., 2004). However, the latter bias may lead to even higher C:nutrient ratios in microorganisms growing on plant material. Nevertheless, unless the isolated strains are perfectly homeostatic, the mean elemental ratios of Bacteria and Fungi in our study will likely be influenced by the growth conditions.

It is also worth considering that the fumigation technique used for whole community analysis can be biased. Previous studies have suggested that the approach may preferentially extract cytoplasmic material rich in nucleic acids and possibly co-extract non-cellular material, thereby reducing the observed C:nutrient ratios (Hedley and Stewart, 1982; Jenkinson et al., 2004). To our knowledge, the fumigation technique has only been evaluated in regards to total concentrations of C, N, and P and not their ratios. Such a bias may contribute to differences between the direct analyses of cells and whole communities. Biochemical considerations of the macromolecular composition of cells can be used to corroborate estimates of cellular stoichiometry. Assuming microbial cells are composed of approximately 50% protein, 25% nucleic acids, 20% lipids, polysaccharides, and LPS, and 5% other material (Bremer and Dennis, 1987), we can provide an estimate of expected cellular elemental ratios. The C:N:P ratio of nucleic acids is approximately 9.5:3.5:1. If nucleic acids are the major contributors to the P pool, then an N:P ratio as low as 7 can be achieved if DNA and RNA constitute approximately 50% of the cell biomass. Given such a high fraction, nucleic acids will have to contribute a high proportion of the cellular nitrogen pool as well. Thus, proteins will need to be a small fraction of the cellular biomass to maintain a C:N ratio near 8. Inorganic phosphate in the form of polyphosphates can account for up to 30% of the cellular P in some lineages and under certain environmental conditions (Rhee, 1973; Thompson et al., 1994; Reina et al., 2011). Even so, an average N:P of 7 is biochemically difficult to achieve, and empirical studies including our own rarely observe such low cellular N:P ratios (e.g., Geider and La Roche, 2002; Zimmerman et al., 2014). Thus, an average microbial community N:P ratio of 7 appears to be difficult to reach from a biochemical standpoint.

Our study reveals intriguing inconsistencies between the likely biochemical composition of cells, direct measures of cellular stoichiometry in cultures, and whole-community elemental ratios based on chloroform fumigation. This gap warrants caution in the elemental ratios used in terrestrial biogeochemical models (Manzoni et al., 2010). In such models, microbial ratios are key to the representation of organic matter decomposition, patterns of nutrient limitation, and links between fluxes of C, N and P. Potential biases in our approach mean that additional studies are needed to confirm the elemental stoichiometry of individual microbial taxa across a much wider range of growth conditions and ecosystems. However, we also recommend further examination of elemental ratios in terrestrial microbial communities and possible methodological biases. It would be particularly useful to compare the fumigation technique with other more direct approaches like cell-sorting or X-ray analysis. In this way, we may improve the quantification of elemental stoichiometry, ensure accurate characterization of the elemental variation in terrestrial ecosystems, and expand our knowledge of controls on the elemental stoichiometry of microbial communities.

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