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The importance of amino sugar turnover to C and N cycling in organic horizons of old-growth Douglas-fir forest soils colonized by ectomycorrhizal mats

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Abstract Amino sugar dynamics represent an important but under-investigated component of the carbon (C) and nitrogen (N) cycles in old-growth Douglas-fir forest soils. Because fungal biomass is high in these soils, particularly in areas colonized by rhizomorphic ectomycorrhizal fungal mats, organic matter derived from chitinous cell wall material (or the monomeric building block of chitin, *N*-acetylglucosamine (NAG)) could be a significant source of C or N to the soil microbiota, and thus an important driver of microbial C and N processing. This paper reports the results of incubation experiments initiated to measure chitin degradation, NAG utilization, and the contribution of these substrates to soil respiration and N mineralization rates in mat-colonized and non-mat soil organic horizons. Amendments of chitin and NAG stimulated respiration, N mineralization, and biomass accumulation in mat and non-mat soils, and responses to NAG amendment were stronger than to chitin amendment. NAG-induced respiration was consistently two-fold higher in mat soils than non-mat soils, but induced N mineralization was similar between the two soil patch types. Assimilation of both C and N into microbial biomass was apparent, biomass C:N ratio decreased in all treatments, and microbial N use efficiency (treatment means 0.25 ± 0.06–0.50 ± 0.05) was greater than C use efficiency (treatment means 0.12 ± 0.04–0.32 ± 0.02). NAGase enzyme response was non-linear and showed the same pattern in chitin and NAG amendments. Responses to NAG and chitin amendment differed between mat and non-mat soils, indicating different mechanisms driving NAG and chitin utilization or differences in saprotrophic community composition between the two soil patch types. Net chitin and NAG processing rates were 0.08–3.4 times the basal respiration rates and 0.07–14 times the ambient net N mineralization rates, high enough for the turnover of total soil amino sugars to potentially occur in days to weeks. The results support the hypotheses that amino sugars are important microbial C and N sources and drivers of C and N cycling in these soils.

Keywords Microbial biomass · Amino sugars · Forest floor · Organic layer soils · Ectomycorrhizae · Linked C and N cycles

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Introduction

Microbiota are generally studied as the drivers of carbon (C) and nitrogen (N) cycling in soils; however, the microbial biomass can also represent a significant

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and relatively labile pool of both C and N. Amino sugars are structural molecules of microbial cell walls, and are often used to trace microbial dynamics because they are produced predominantly by prokaryotes and fungi, but not by plants (Amelung et al. 2001; Guggenberger et al. 1999; Stevenson 1982). Carbon from amino sugars can turn over rapidly in soils (Decock et al. 2009; Roberts et al. 2007), and these molecules also comprise significant pools of N in many soils (Amelung 2003; Stevenson 1982). However, the microbial demand for amino sugar C and N is not often quantified, thus the contribution of amino sugar turnover to both soil C and N cycles is not well understood. Understanding this contribution may be particularly important in N-poor soils where amino sugar N could be in high demand (Olander and Vitousek 2000).

Old-growth forest soils are low in labile C and N compounds, but high in microbial biomass. Ectomycorrhizal (EcM) fungi represent a major source of microbial biomass in these soils, especially because EcM fungi commonly form rhizomorphic mat structures that can dominate the soil matrix in colonized patches (Cromack et al. 1979; Dunham et al. 2007; Griffiths et al. 1996; Griffiths et al. 1991a). In fact, these EcM mat-colonized soil patches have elevated respiration and N mineralization rates relative to adjacent non-mat-colonized patches with otherwise similar soil chemistry (Griffiths et al. 1990; Griffiths et al. 1991b; Phillips et al. 2012). One explanation for this pattern is that because EcM must satisfy both their own and their plant symbiont's N demand, they invest recently fixed C into enzymes that release N from soil organic matter, resulting in higher activity rates. Kluber et al. (2010) found that NAGase activity potentials are higher in EcM colonized soils; however, soil protease activity potentials do not differ between mat and non-mat patches.

Although microbial biomass or amino sugar turnover is not often measured directly, soil biologists commonly measure chitinase activity as an index of the microbial recycling of biomass or N demand (Miller et al. 1998; Sinsabaugh et al. 2002). As with other organic polymers in soil, the availability of this material for microbial uptake and processing is limited by the enzymatic release of assimilable mono- or oligomers (Schimel and Weintraub 2003; Sinsabaugh et al. 2002): for example, chitinase enzymes degrade the fungal cell wall polymer chitin into its constituent *N*-acetyl-β-D-glucosamine (NAG) monomers. In many soils, the rate of this process has been estimated by the measurement of *N*-acetyl-β-D-glucosaminidase enzyme potential activity (NAGase, commonly referred to as chitinase). In a given soil, chitinase activity can be similar or higher than protease activity, signifying the high potential microbial demand for cell wall material as a C or N substrate (Sinsabaugh et al. 2008; Zeglin et al. 2007). However, extracellular enzyme activity is affected by complex biotic and abiotic factors, and cannot be simply interpreted as a rote indicator of microbial activity or substrate turnover (Wallenstein and Weintraub 2008). Thus, to confirm the importance of fungal cell wall material to C and N dynamics in the soil, direct measurements of substrate processing are necessary.

We hypothesize that microbial cell wall material is a rapidly cycling C and N pool in old-growth Douglas-fir forest soils, and a substrate that sustains higher rates of biotic activity in EcM mat colonized soils relative to non-mat soils. In particular, amino sugars (predominantly NAG and chitin in organic layer soils (Zhang and Amelung 1996)) should be an important pool of both C and N. To directly address this hypothesis, quantify soil amino sugar cycling rates, and assess the level to which depolymerization limits the microbial availability of chitin, we conducted a series of NAG and chitin amendment experiments in soils harvested from organic horizon EcM mat and non-mat patches. To characterize the process-level and enzymatic response to total substrate availability, we ran these experiments with a series of NAG and chitin amendment levels (0.5, 1, 2 and 4 % w/w). We discuss results in the context of the microbial dynamics of linked C and N cycling in these and other forest soils.

Materials and methods

Site description and soil sampling

Organic horizon soils were collected from an old-growth stand of Douglas-fir (*Pseudotsuga menziesii*) located at the H. J. Andrews Experimental Forest, western Cascade Mountains, Oregon, USA (44°13′N, 122°15′W, 450 m elevation) in November 2008. An average of 230 cm precipitation falls here each year, primarily between November and March, and at this elevation snow rarely accumulates. Soils are coarse

loamy mixed mesic Typic Hapludands, and have a mean O horizon depth of 6 cm (Dixon 2003). The studied O horizon soil had an organic matter content of approximately 63.5 % w/w and a pH of 4.5–4.8.

The distribution of EcM mat colonization in the organic and mineral soil horizons across the H. J. Andrews Experimental Forest is well characterized. We know that *Piloderma* spp. (*Atheliaceae*) are the most common and widespread mat-forming EcM associates of old-growth Douglas-fir trees in this forest (Dunham et al. 2007). We identified established EcM mats using the same criteria as Dunham et al. (2007), and ascertained that we sampled only *Piloderma* spp. by genotyping the fungal ITS gene found in DNA extracted from infected root tips associated with a rhizomorphic mat. We then collected organic horizon soil from within the boundary of rhizomorphic penetration ("mat" soils) and paired adjacent uncolonized organic horizon soil ("non-mat" soil). Three patches of each designated type of soil were harvested. These soils were stored intact, to preserve mycelial structure, at 4 °C until the experimental incubations were started. Prior to the experiment, soils were sieved (4 mm) and let rest for 24 h. The sieved field replicate soils were combined into one composite batch of mat soil and one batch of non-mat soil, and then allotted into three independent incubation replicates per soil + treatment combination.

Experimental setup

To measure the rates of utilization of the polymer, chitin, and the monomer, NAG, at different supply levels in mat and non-mat soils, a series of four substrate concentrations and an unamended (control) treatment were set up for each soil and substrate, in triplicate, for a total of 60 incubation chambers. For each incubation replicate, 10 g dry (approximately 30 g wet at field water holding capacity) soil was incubated in an airtight 500-mL canning jar with a gas sampling septum. Substrates were amended to each replicate jar either in solution (NAG, A3286, Sigma-Aldrich, Inc., St. Louis, MO, USA) or suspension (crabshell chitin powder, C7170, Sigma-Aldrich, Inc., St. Louis, MO, USA) at levels of 0.5 %, 1 %, 2 % and 4 % w/w dry soil. These levels bracket the ambient total soil amino sugar concentration of 1.3–1.6 % w/w dry soil (Table 1). Control soils received an equal amount of water (3 mL) as the substrate-amended

Table 1 Soil chemical and microbiological characteristics, mean (SE)

	FWC	C	N	C:N	NO_3^-–N	NH_4^+–N	DON	DOC	AS	MBC	MBN	F:B	Net nitr.	Net N min.	Respiration	Chitinase
Mat soil	2.2 (0.03)	41.0 (0.7)	1.21 (0.03)	39.6 (1.6)	4.4 (0.8)	12.5 (1.7)	11.0[a] (3.3)	799[a] (18)	15.8 (0.2)	7.0[a] (0.4)	0.78[a] (0.05)	0.15 (0.003)	11.1[a] (2.3)	20.9[a] (5.4)	28.9[a] (0.8)	23.0[a] (5.4)
Nonmat soil	2.1 (0.06)	40.1 (1.8)	1.11 (0.04)	42.2 (2.0)	6.1 (0.6)	15.9 (0.7)	26.8[b] (1.6)	547[b] (27)	13.2 (1.4)	4.6[b] (1.3)	0.51[b] (0.12)	0.14 (0.008)	−0.6[b] (0.3)	1.4[b] (0.4)	20.1[b] (1.9)	4.1[b] (0.8)

Field water content (FWC) in g g^{-1} dry soil; total C and total N in soil dry mass %; molar C:N ratio; extractable nitrate (NO_3^-–N), ammonium (NH_4^+–N), dissolved organic N (DON) and dissolved organic C (DOC) concentrations in µg g^{-1} dry soil; amino sugars (AS), microbial biomass C (MBC) and N (MBN) in mg g^{-1} dry soil, ratio of fungal:bacterial (F:B) phospholipid fatty acids (mol %); net nitrification and net N mineralization over 7 days in µg N g^{-1} dry soil day^{-1}, respiration in µg C g^{-1} dry soil h^{-1}; NAGase potential activity in µmol pNP g^{-1} dry soil h^{-1}. When present, significant differences in properties between mat and non-mat soils are designated by lower case letters

incubations. NAG incubations were run for 1 week (7 days) and chitin incubations were run for 5 weeks (35 days), because the NAG-induced respiration response was more rapid than the chitin-induced response. Respiration was monitored consistently in all incubation chambers over the experimental time course. At the end of each experiment, soils were harvested from all incubation replicates and divided for analysis of extractable inorganic N (IN), organic carbon (DOC) and organic nitrogen (DON), microbial biomass C and N (MBC and MBN), and NAGase activity. Soil nutrient chemistry and microbial biomass samples were processed immediately, while the soils for NAGase activity were frozen and stored at −20 °C until the assays were run.

CO_2–C concentration and respiration rate

Incubation chamber headspace CO_2–C concentration was measured using a GC–TCD (Carle Instruments Inc., Fullerton, CA, USA) with CO_2 in Ar as a standard calibrant. CO_2–C data were collected at approximately 2, 6, 18 and 24 h after the beginning of the experiment, then every 12 h until 7 days had passed (and NAG incubations were harvested), then every 24–72 h until 35 days had passed (and chitin incubations were harvested). Incubation chamber headspace was flushed with room air after every data collection point. Cumulative respiration was calculated as the summed accumulation of CO_2–C in the headspace of incubation chambers over the course of each incubation, relative to the mass of soil incubated. Cumulative CO_2–C respired as a percent of substrate added was calculated as the total CO_2–C respired in each amendment incubation minus the mean total CO_2–C respired in the control incubations, then divided by the total C added as NAG or chitin for each amendment level. Because we cannot directly trace the fate of the amended NAG or chitin molecules, all cumulative calculations assume that C mineralization above control levels was derived from the added substrate.

Nitrate- and ammonium–N concentrations and N mineralization rates

Inorganic N was extracted from soils by shaking in a 0.05 M K_2SO_4 solution (40 mL per 3 g dry soil) for 1 h. Inorganic N concentrations in the extract solutions were measured colorimetrically using an autoanalyzer (Astoria-Pacific, Clackamas, OR, USA). After reduction to nitrite on a cadmium column, nitrate + nitrite (NO_3^-–N) was quantified using the *N*-1-naphthylethylenediamine dihydrochloride chromophore. Ammonium (NH_4^+–N) was measured as ammonia using the alkaline salicylate-hypochlorite method. N mineralization rates were calculated from the amount of total IN accumulated over the course of each incubation, i.e. ((harvest time [IN] − time zero [IN])/total time elapsed). Total N mineralized as a percent of substrate added was calculated as the difference in total N mineralized in the amended minus control incubations, divided by the total amount of N added as NAG or chitin for each amendment level. Because we cannot directly trace the fate of the amended NAG or chitin molecules, cumulative calculations assume that N mineralization above control levels was derived from the added substrate.

DOC, DON and microbial biomass

Microbial biomass C and N was estimated as the DOC and DON liberated after a 24 h chloroform fumigation of the soil (Brookes et al. 1985; Vance et al. 1987). Organic C and N was extracted from fumigated and unfumigated soils by shaking in a 0.05 M K_2SO_4 solution (40 mL per 3 g dry soil) for 1 h, and was quantified via combustion/chromatography analysis with a Shimadzu total C and N analyzer (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA). Extractable DOC and DON of the unfumigated soils are reported as standalone variables, and the excess DOC or DON in amended soils was calculated as the difference between the amount of extractable organic C or N in that replicate and the mean amount in control soil incubations at the end of the experiment. Final MBC and MBN values were calculated using the extraction efficiency coefficients $k_{EC} = 0.45$ and $k_{EN} = 0.56$ (Brookes et al. 1985; Vance et al. 1987). Carbon and N assimilated as a percent of substrate added was calculated as the amount of MBC or MBN measured in each amendment incubation soil minus the mean control value, divided by the amount of C or N added as substrate. Again, cumulative assimilation calculations must assume that recovery of C and N in biomass above control levels was derived from the added substrate. In addition, C and N growth yield efficiency (GYE) was calculated as the amount of biomass C or N assimilated divided by the total C or N

utilized (Six et al. 2006; Thiet et al. 2006). Cell wall phospholipid fatty acids (PLFA) were extracted from 2 g soil and their relative abundance used to estimate fungal and bacterial biomass ratios (Bligh and Dyer 1959; Brant et al. 2006).

Soil amino sugars concentration

Soil total amino sugars were measured colorimetrically following acid hydrolysis of soil organic matter (Amelung 2001; Bremner 1954; Vignon et al. 1986). Between 50 and 100 µg of dried, milled soil was incubated in 5 mL 6 M HCl for 18 h at 80 °C. After cooling, this mixture was neutralized with 25 mL 0.2 M sodium acetate, and a 0.25 mL aliquot of the supernatant of this mixture was moved to a 2 mL microcentrifuge tube for a 3-methyl-2-benzothiazolinone hydrazone hydrate hydrochloride (MBTH) colorimetric assay of total amino sugars content. First, the hydrolyzed soil organic matter in solution was deaminated with HNO_2 (added as 0.25 mL 5 % w/v $KHSO_4$ and 0.25 mL 5 % w/v $NaNO_2$ and incubated at room temperature for 15 min). Then, excess HNO_2 was removed by diazotization, by adding 0.25 mL 12.5 % w/v ammonium sulfamate and shaking for 5 min at room temperature. To this solution, 0.25 mL 0.5 % w/v freshly prepared MBTH was added and incubated for 1 h at room temperature. Finally, 0.25 mL 0.5 % $FeCl_3$ was added, and color development after 30 m was measured at 653 nm on a spectrophotometer. A reducing sugars control (parallel assay with no deamination step) was run for each hydrolyzed sample to subtract any background free aldehyde signal.

NAGase potential activities

Potential *N*-acetyl-*β*-D-glucosaminidase (NAGase, EC 3.2.1.52) enzyme activity, commonly referred to as chitinase, was measured colorimetrically using a *p*-nitrophenol-*N*-acetyl-*β*-D-glucosaminidine (pNP-NAG, N-9736, Sigma Chemicals, St. Louis, MO) substrate. Soil slurries with pNP-NAG substrates added at saturating concentrations, and buffer-only controls, were incubated at 30 °C for 1–2 h, and color development was measured as the absorbance at 410 nm on a spectrophotometer (Parham and Deng 2000). A pNP standard solution was used as calibrant, and the amount of color accumulation over the assay incubation time was used to calculate the potential NAGase activity rate in µmol pNP g^{-1} dry soil h^{-1}.

Statistical analysis

Ambient soil characteristics in mat and non-mat soils were compared using one-way analysis of variance (ANOVA). To measure the effects of concentration and substrate type (chitin and NAG) on microbial process rates in mat and non-mat soils, we used a three-way ANOVA with concentration, substrate and soil as groups. Within each concentration series, response variables were compared using one-way ANOVA and Bonferonni post hoc comparisons. All data fit assumptions of normality and had similar variance, except for NAGase data. NAGase data was not normally distributed even when ln-transformed, and was analyzed using non-parametric Wilcoxon tests of the effect of soil, substrate and concentration separately. Unless otherwise noted, all tests were evaluated using an α-value of 0.05. Statistics were run using SPSS 11 for Mac OSX and Systat 5 for Windows.

Results

Ambient soil chemical and microbial characteristics

Water content, total C and N, total amino sugars and extractable inorganic N levels were similar in mat and non-mat soils, but microbial biomass, dissolved organic nutrient concentrations, and microbial activities differed (Table 1). Microbial biomass C and N, soil extractable DOC, N mineralization, respiration and NAGase potential activity were all higher in mat soil than non-mat soil, whereas soil extractable DON was lower in mat soil than non-mat soil (ANOVA, $P < 0.05$). The amount of total amino sugars released from soil via acid hydrolysis was high, between 13.2 and 15.8 g kg^{-1} dry soil, meaning that the substrate amendment levels of 0.5 %, 1 %, 2 % and 4 % w/w (i.e., 5–40 g kg^{-1} dry soil) bracket ambient levels of soil amino sugars. Amino sugar concentration in extractable solution was not detectable using this spectrophotometric method. Also, the experimental amendment levels of C (2.2–17.4 g kg^{-1} dry soil) and

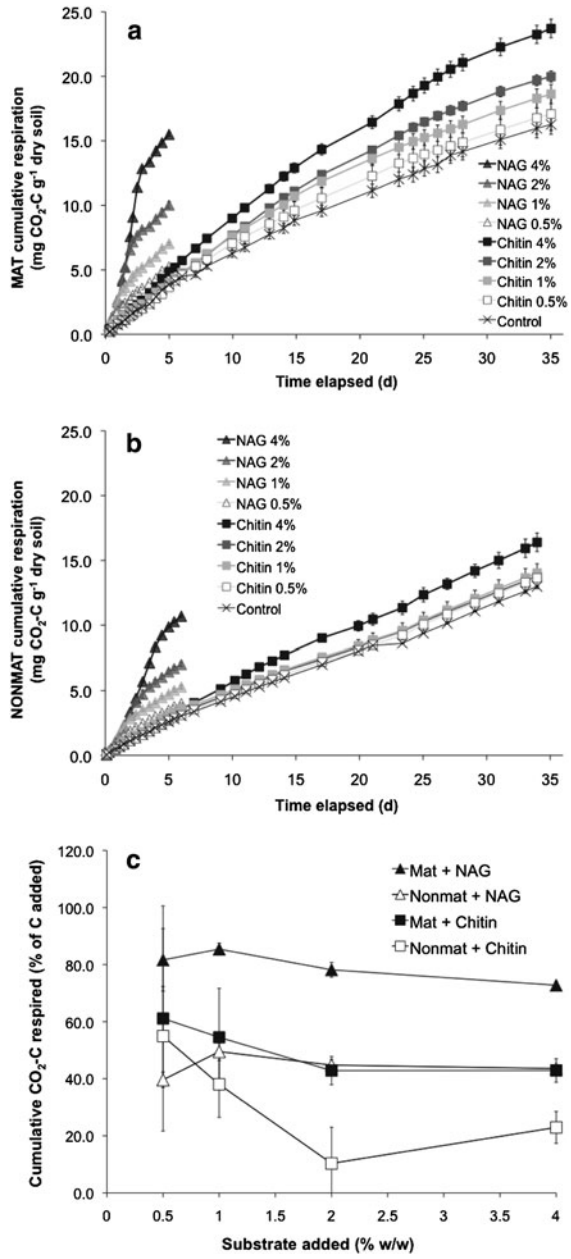

Fig. 1 Total cumulative C respired over time in all **a** mat soil and **b** non-mat soil incubations, mg C g^{-1} dry soil. NAG-induced responses are denoted by triangles, chitin-induced responses are denoted by squares and control conditions are denoted by *times* symbols. Substrate amendment concentrations (w/w) are denoted as follows: 0.5 %, *open symbols*; 1 %, *light grey symbols*; 2 % *dark grey symbols*; 4 %, *black symbols*. Trends in cumulative % C respired as a function of amino sugar C added **c** as a function of substrate addition level. Mat soils are denoted by *filled symbols* and non-mat soils are denoted by *open symbols*; NAG-induced responses are denoted by *triangles* and chitin-induced responses are denoted by *squares*. All data are shown as mean ± 1 SE; *error bars* are present on all data points but are not visible if the size of the corresponding mean symbol is larger than the SE value

N (0.3–2.5 g kg^{-1} dry soil) bracket ambient levels of MBC (4.6–7.0 g kg^{-1} dry soil) and MBN (0.51–0.78 g kg^{-1} dry soil). Fungal:bacterial ratios from PLFA analysis ranged from 0.14 to 0.15, values typical of this ecosystem (Brant et al. 2006).

NAG- and chitin-stimulated respiration rates

NAG and chitin amendment caused significantly elevated respiration in both mat and non-mat soils (Fig. 1a, b; Table 2). The cumulative percentage of substrate induced CO_2–C respired after the 7-day NAG incubation was relatively consistent across substrate amendment levels, and was approximately 2 times higher in mat soils (85 ± 2 %) than non-mat soils (40 ± 3 %; Fig. 1c). Cumulative CO_2–C respired above control levels after the 35-day chitin incubation decreased from 55 ± 17 % to 61 ± 39 % at the lowest (0.5 % w/w) amendment level to 23 ± 6 %–43 ± 5 % at the highest (4 % w/w) amendment level, and was also higher in mat soils than non-mat soils (Fig. 1c).

NAG- and chitin-stimulated N mineralization rates

NAG and chitin amendment also increased N mineralization in both mat and non-mat soils (Fig. 2a, b; Table 2). NAG (7-day) N mineralization was very high, but did not differ strongly or consistently between mat and non-mat soils (Fig. 2a). Chitin (35-day) N mineralization was 2–7 times higher in mat soils than non-mat soils (Fig. 2b). Nitrification appeared to be important in mat soils, as nitrate was produced at near-unamended rates over the 7-day NAG incubation period and at elevated rates over the 35-day chitin incubation period (Fig. 2a, b). A significant proportion of the N added was mineralized over the course of the soil incubations, and this amount was statistically equivalent or greater with NAG amendment than chitin amendment (Fig. 2c; Table 2; NAG: 26 ± 7 %–57 ± 1 %, chitin: 20 ± 1 %–42 ± 13 %).

Table 2 Results of analysis of variance tests for all response variables (*P*-values)

	Direct responses (rates or pool sizes)							Summary responses (substrate utilization efficiencies)						
	Total resp.	Nitr.	N min.	MBC	MBN	MB C:N	NAGase	% C resp.	% N min.	% C assm.	% N assm.	C GYE	N GYE	% C util.	% N util.
Soil (ambient)	**0.001**	**0.000**	**0.000**	**0.002**	**0.004**	0.582	**0.024**	–	–	–	–	–	–	–	–
Soil (response)	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	0.060	**0.006**	0.853	0.984	0.115	0.055	0.096	0.949	0.662	0.095
Substrate	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.025**	0.771	0.417	**0.000**	**0.000**	**0.000**	**0.003**	0.171	0.323	**0.000**
Concentration	**0.000**	0.412	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	0.086	0.913	**0.035**	0.058	0.457	0.689	0.124	0.225
Soil × substrate	**0.000**	**0.000**	**0.000**	0.076	**0.003**	**0.001**	n/a	**0.000**	**0.000**	0.070	**0.001**	0.682	**0.002**	**0.000**	0.483
Soil × concentration	**0.000**	0.230	**0.002**	0.827	0.993	0.130	n/a	**0.019**	0.651	0.095	0.251	0.575	0.900	**0.010**	0.449
Substrate × concentration	**0.000**	**0.007**	**0.000**	**0.001**	**0.001**	0.527	n/a	**0.045**	**0.004**	**0.001**	**0.001**	**0.008**	**0.018**	**0.037**	0.179
Soil × substrate × concentration	0.689	**0.002**	**0.007**	0.989	0.672	**0.001**	n/a	**0.045**	0.130	0.067	0.059	0.705	0.210	0.092	0.101

Ambient process values were tested using a one-way ANOVA; experimental response values were tested using a three-way ANOVA, except for NAGase data

NAGase data could only be evaluated using non-parametric, non-interactive Wilcoxon tests, so interactive effects are not applicable (n/a). A *bold number* denotes a significant *P* value (< 0.05)

Total resp total cumulative respiration, *nitr* nitrification, *N min* N mineralization, *assm* assimilation, *util* total utilization

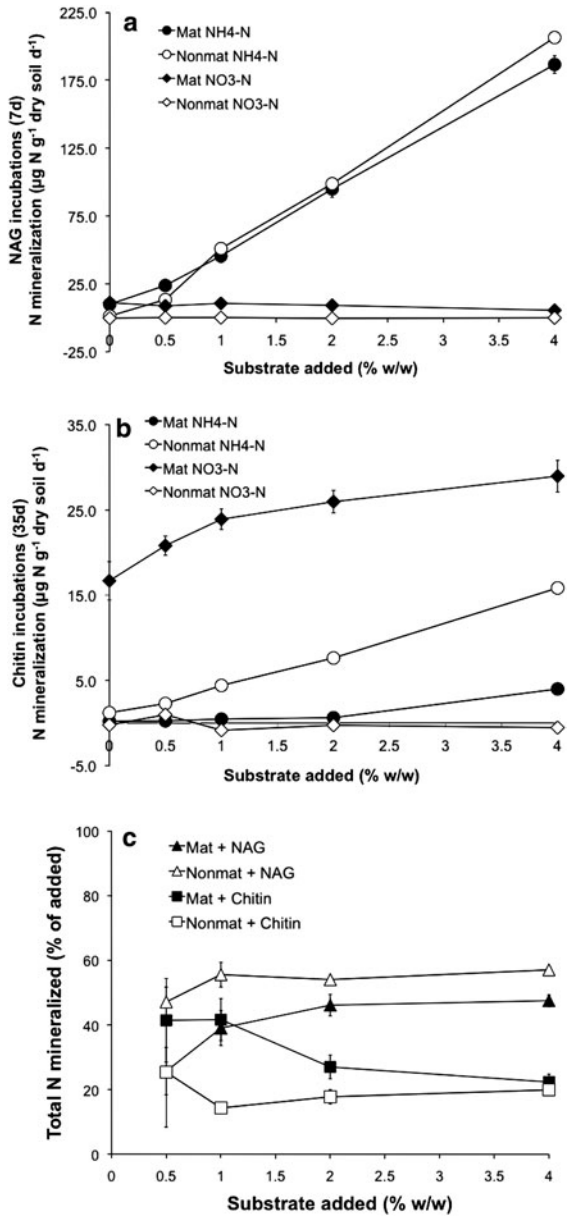

Fig. 2 Net N mineralized per day as nitrate (NO_3–N, diamonds) or ammonium (NH_4–N, *circles*) in all **a** mat soil (7 days) and **b** non-mat soil (35 days) incubations, µg N g^{-1} dry soil as a function of w/w % concentration of amino sugars added. Control incubations are noted as "0 % substrate added" data points. Mat soils are denoted by *filled symbols* and non-mat soils are denoted by *open symbols*. Trends in cumulative % N mineralized as a function of amino sugar N added **c** as a function of substrate addition level. All data are shown as mean ± 1 SE; *error bars* are present on all data points but are not visible if the size of the corresponding mean symbol is larger than the SE value

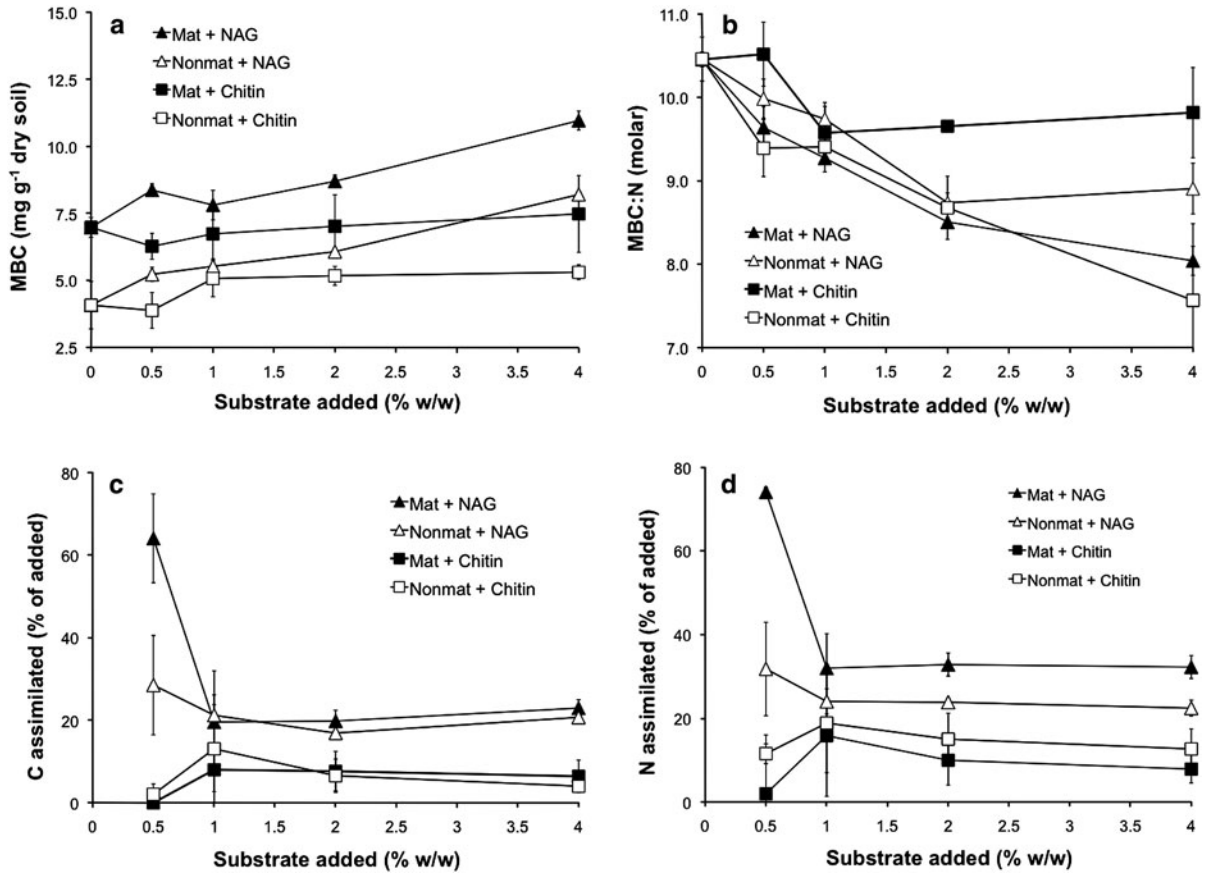

Fig. 3 Soil microbial biomass **a** C and **b** molar C:N, **c** microbial biomass C in excess of the control mean value as a % of the amount of amino sugar C added, **d** microbial biomass N in excess of the control mean value as a % of the amount of amino sugar N added. Control data, where relevant, are noted as "0 % substrate added" data points. All data points are mean ± 1 SE; *error bars* are present on all data points but are not visible if the size of the corresponding mean *symbol* is larger than the SE value. Mat soils are denoted by *filled symbols* and non-mat soils are denoted by *open symbols*; NAG-induced responses are denoted by *triangles* and chitin-induced responses are denoted by *squares*

C and N assimilation

Assimilation of C and N into biomass was apparent in most incubations (Fig. 3a; Table 2), but was more evident in NAG than chitin incubations, with a statistically insignificant response to chitin amendment in mat soils (Table 2). Similarly, C:N in biomass decreased in all incubations (Fig. 3b), indicating relatively greater assimilation of N than C from the added NAG or chitin, but was least dynamic in mat soils with chitin amendment. Accordingly, a greater percentage of the added C and N was assimilated from NAG (C: 21 ± 1 %–64 ± 11 %; N: 22 ± 2 %–74 ± 1 %) than from chitin (C: negligible to 8 ± 5 %; N: negligible to 19 ± 15 %) (Fig. 3c, d). In addition, the highest assimilation levels per unit substrate added were observed at the lowest (0.5 % w/w) NAG amendment level, and mat soils amended with NAG showed the highest amount of N assimilation per unit substrate added (Fig. 3c, d; Tables 2, 3).

The microbial growth yield efficiency (GYE) showed that C and N GYE tended to be highest at low NAG amendment levels and lowest at low chitin amendment levels (0.5 % w/w; Fig. 4a, b), reflecting the pattern of C and N assimilation. Also, N utilization efficiency (0.05 ± 0.17–0.75 ± 0.05) was similar to or higher than C utilization efficiency (zero to 0.43 ± 0.04); C:N GYE averaged 1:1 in non-mat soils with NAG added but was closer to 1:2 in the other

Table 3 Parameters (±SE) from linear regression analysis of net substrate induced C and N mineralization

	Pearson's R^2	P value	Slope (N:C)	Intercept	Slope^{-1} (C:N)
Mat soil + NAG	0.975	<0.0001	0.089 ± 0.0004	−8.30 ± 2.78	11.3
Nonmat soil + NAG	0.992	<0.0001	0.166 ± 0.0049	−3.23 ± 1.78	6.0
Mat soil + chitin	0.698	0.0098	0.059 ± 0.0011	5.81 ± 4.22	16.8
Nonmat soil + chitin	0.512	0.0401	0.083 ± 0.025	2.75 ± 5.22	12.0

treatments (Fig. 4a–c). The relationship between N and C mineralized also reflected this difference between treatments, as the slope of this relationship was < 0.125 (equivalent of C:N = 8, the molar ratio of the added substrate) only in non-mat soils with NAG added (Fig. 4d; Table 3).

NAGase enzyme activity

Patterns of NAGase potential activity response to substrate amendment did not fit the expected trends (Fig. 5): NAGase response was very similar with both NAG and chitin amendment, but showed significant differences based on soil and concentration (Table 2). In non-mat soils, NAGase activity increased at higher NAG amendment levels (2 % and 4 % w/w). In mat soils, NAGase activity decreased relative to unamended rates at low chitin and NAG amendment levels (0.5 % and 1 % w/w) and was equal to the ambient potential at higher substrate amendment levels. Biomass-specific chitinase activity reflected a dampened span of potential rates but showed the same pattern (data not shown).

Total C and N budgets

Assuming that the measured net changes in C and N mineralization and assimilation in excess of control levels were a direct consequence of degradation of the added NAG or chitin, substantial amounts of substrate utilization were measured in all incubations (Fig. 6a, b). The sum of C utilized was near 100 % of the substrate C added in mat soil with NAG amendments, but the mass balance for other treatments and for N utilization was less than 100 % (Fig. 6a). Small amounts of DOC were detected in excess of control levels in the chitin amendments (40 ± 38–260 ± 30 μg C g^{-1} dry soil, data not shown), and these were included in the summation of total substrate utilized. Presumably, the mass of NAG or chitin C and N that was not measured in the mineralized, assimilated or solubilized pools was not degraded or was incorporated into the non-extractable soil organic matter pool.

Discussion

Chitin and NAG amendment stimulated respiration, N mineralization, and C and N assimilation in both EcM mat colonized and non-mat old-growth Douglas-fir forest soils, and both basal and induced biomass levels, respiration, N mineralization and NAGase rates were higher in mat soils than non-mat soils (Table 2). These results fit our predictions, which were based on previous observations of higher microbial activity in EcM mat colonized forest soils (Griffiths et al. 1990; Griffiths et al. 1991b; Kluber et al. 2010; Phillips et al. 2012). The results may also reflect a pre-defined difference in fungal community composition, i.e. the presence or absence of *Piloderma* spp., independent from fungal:bacterial ratio or microbial biomass C:N (Table 1). Collectively these experimental results may help explain spatial variability in soil C and N biogeochemistry, by confirming that nutrient cycling rates differ between mat and non-mat patches of organic horizon soils. In addition, the significance of many interaction terms in the three-way ANOVA hint at the complexity of the results: although standardized substrate utilization and mass balance responses were generally similar in mat and non-mat soils (Table 2), some notable differences were found, particularly when contrasting C and N dynamics. The data also show strong differences in utilization dynamics between chitin and NAG, in terms of both absolute rates and summary variables, and many concentration-dependent responses (Table 2). These contrasting responses allow some insight into the availability and depolymerization dynamics of microbial cell wall material in the study soils.

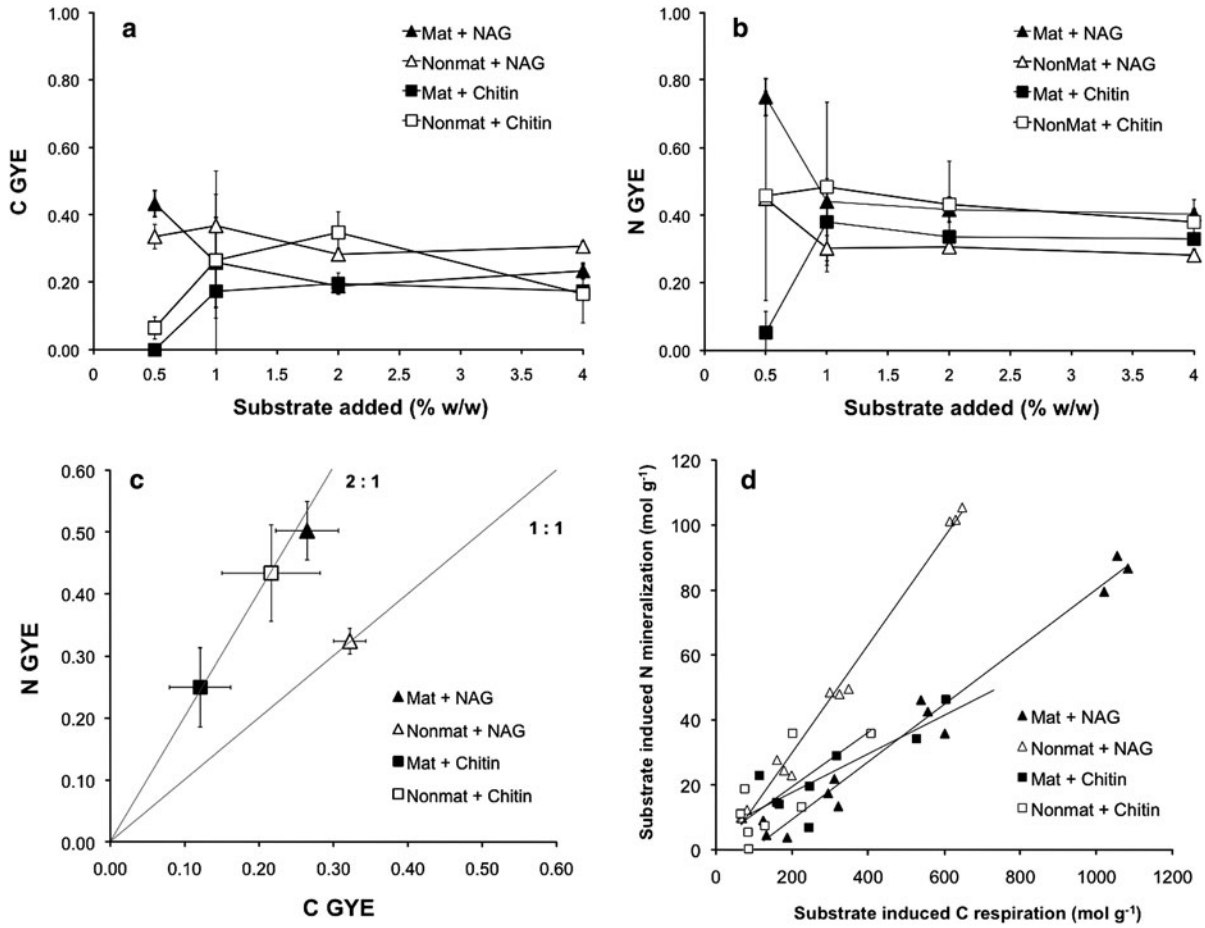

Fig. 4 Growth yield efficiencies of **a** C and **b** N, mean ± 1 SE as a function of w/w % concentration of amino sugars added, **c** comparisons of C and N growth yield efficiencies, and **d** total mineralization (moles C or N g^{-1} dry soil). Mat soils are denoted by *filled symbols* and non-mat soils are denoted by *open symbols*; NAG-induced responses are denoted by *triangles* and chitin-induced responses are denoted by *squares*

Synthesis of C and N response dynamics in mat and non-mat soils

The relative fates of C and N derived from chitin and NAG amendment suggest that the soil microbiota were primarily N limited. In most cases, a greater percentage of added C was mineralized than added N (Figs. 1c, 2c, 4d), more N was assimilated into biomass relative to C (Fig 3a–d) and N growth yield efficiencies (treatment means 0.25 ± 0.06–0.50 ± 0.05) were higher than C growth yield efficiencies (treatment means 0.12 ± 0.04–0.32 ± 0.02; Fig. 4a–c). In grassland mineral soils the C GYE of glucosamine was 0.55–0.85 (Roberts et al. 2007), implying significantly higher microbial C limitation in that system. Relative microbial N limitation in this old-growth forest organic horizon is expected given the high soil C:N ratio (40–42) (Hart and Stark 1997). Also, while there is evidence that microbial GYE is higher for N than C across many environments (N:C GYE ratio of ∼ 1.3 (Doi et al. 2010; Herron et al. 2009)), in our study most N:C GYE ratios fell close to 2 (*i.e.* N was assimilated twice as efficiently as C), indicating a strong tendency for amino sugar N to be retained within the microbial biomass (Fig. 4c). This also suggests that, in most experiments, the decomposition of chitin and NAG was driven by microbial demand for N, and C was released in the process.

An exception was the non-mat soil amended with NAG, in which N and C were used with similar efficiencies (Fig. 4c). In these treatments, the highest percentage of the organic N added was mineralized

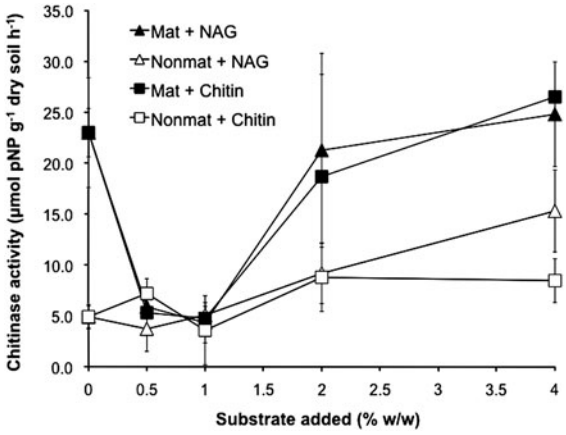

Fig. 5 Chitinase potential activity (µmol pNP g^{-1} dry soil h^{-1}) as a function of w/w % concentration of amino sugars added. Mat soils are denoted by *filled symbols* and non-mat soils are denoted by *open symbols*; NAG-induced responses are denoted by *triangles* and chitin-induced responses are denoted by *squares*. All data points are mean ± 1 SE

(47 ± 5 %–57 % ± 1 %, Fig. 2c) and NAG–N assimilation into biomass (Fig. 3d) and NAG–C mineralization (Fig. 1a, c) was also consistently lower than in mat soils. These observations are all consistent with greater microbial C limitation in the non-mat soil NAG incubations. In general, more N was mineralized per atom of C mineralized in non-mat soils than EcM soils (Fig. 4d; Table 3): with NAG added, C:N mineralized was 6.0 in non-mat and 11.3 in mat soils (with chitin added, 12.0 < 16.8). Because EcM fungi utilize primarily tree-derived C for growth, a more "leaky" C recycling mechanism might exist in mat-colonized soils. Also, though these experiments suggest that amino sugars are used by soil microbiota primarily as an N source and secondarily as a C source, it is still possible that other, more abundant components of fungal cell walls, such as beta-glucans, are also important microbial C sources.

It is important to remember that in these laboratory experiments, mycorrhizal fungi were disconnected from their C source and thus not active, unless they also have saprotrophic characteristics (Baldrian 2009; Talbot et al. 2008). Members of the dominant EcM genus in these soils, *Piloderma* spp., are not documented to capably grow as saprotrophs in the field, but can be grown in culture on glucose plus ammonium, nitrate or protein (Finlay et al. 1992). However, even with *Piloderma* spp. excluded from the taxonomic comparison, fungal community structure in mat and non-mat soils differs, as does the bacterial community structure (Kluber et al. 2011). In addition, a subset of the total microbial community may be responsible for amino sugar C or N uptake, which could also affect the observed N:C GYE ratio. For example, if bacteria, which have a lower C:N biomass than fungi, grew preferentially in mat soils but not non-mat soils, a higher net N GYE would result. Thus, physiological characteristics of the different heterotrophic taxa living in mat and non-mat soils could affect the fate of cell wall C and N.

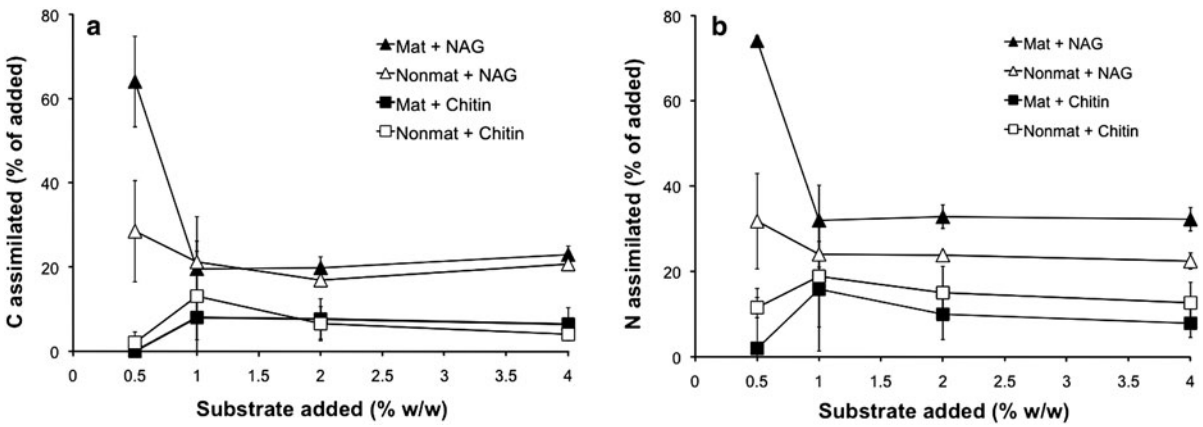

Fig. 6 Total amounts of **a** C and **b** N accumulated in the mineralized plus biomass pools in excess of control pools as a function of w/w % concentration of amino sugars added. Mat soils are denoted by *filled symbols* and non-mat soils are denoted by *open symbols*; NAG-induced responses are denoted by *triangles* and chitin-induced responses are denoted by *squares*. All data points are mean ± 1 SE; *error bars* are present on all data points but are not visible if the size of the corresponding mean *symbol* is larger than the SE value

Chitinase response dynamics

Chitinase activity responded contrary to expectations: potential activity did not increase significantly in response to an elevated supply of its polymeric substrate. Instead, chitinase activity increased in non-mat soils in response to amendment of its product, NAG. In mat soils, chitinase activity actually decreased at the lower levels of chitin and NAG amendment. Because chitin and NAG degradation and assimilation may be regulated by linked N and C demand, as discussed above, chitinase activities may not be as predictable as extracellular enzymes that reflect microbial demand for only one limiting nutrient (Allison and Vitousek 2005). Also, culture-based studies of chitinase regulation in various bacteria and fungi show that chitinase production can be induced by NAG or repressed by elevated ammonium or glucose concentrations (Duo-Chuan 2006; Felse and Panda 1999). Thus, the elevated concentration of both NAG and ammonium in amended study soils could feasibly either enhance or repress chitinase production, depending on the dominant regulatory pathways of chitinase producers in the mixed soil community (Geisseler et al. 2010). Because chitinase activity was only measured at the end of the incubation experiments, it is not known whether changes in chitinase activity were rapid, gradual, or correlated with changes in IN or DON. The straightforward induction of chitinase by similar rates of chitin amendment occurred in other soils (Rodriguez-Kabana et al. 1983), so soils from this study may support a distinctive suite of chitinase producing microbes. Also, the high ambient concentrations of microbial biomass (and presumably necromass) and amino sugars, particularly in mat-colonized soils, might be characteristic of soils that are already near saturation for chitinase activity. Overall, although the ambient level of chitinase activity does reflect a higher potential for chitin degradation in mat relative to non-mat soils, chitinase activity was not a quantitative or qualitative predictor of biogeochemical response to substrate addition in these soils. This reinforces the caution with which extracellular enzyme activity data must be interpreted (Wallenstein and Weintraub 2008).

Chitinase potential in the study soils is high compared to other soils. We measured chitinase potential activity rates of 4.1 ± 0.8–23.0 ± 5 μmol g^{-1} soil h^{-1} (Table 1) or approximately 6.7–37.7 μmol g^{-1} OM h^{-1} (if SOM is 61 % w/w (Kluber et al. 2010)). These activities are similar to or higher than chitinase activities in mineral soil of similar pH or OM content (Sinsabaugh et al. 2008), and are also on the high end of activities measured in these and other coniferous forest organic horizons (1–19 μmol g^{-1} soil h^{-1}; (Andersson et al. 2004; Dimitriu et al. 2010; Kluber et al. 2010; Niemi et al. 2007)). In fact, if these potential chitinase activities could be realized in the soil, even the high amounts of chitin added during these experiments would be depolymerized within 8–40 h. Instead, the highest net chitin degradation rate measured in these experiments was approximately 0.1 μmol g^{-1} soil h^{-1}, one to two orders of magnitude lower than chitinase potential activities. In a study of chitin degradation in an estuarine system, actual chitin degradation rates were in the same range as chitinase potentials only at certain times of year (Kirchman and White 1999), which highlights the possibility for temporal variability in potential versus realized microbial activity. Also, true utilization of chitin may be less rapid

Table 4 Estimated NAG or chitin total C and N utilization rates (μg C or N g^{-1} dry soil h^{-1}, ±SE)

Soil type + w/w % substrate added	NAG–C	NAG–N	Chitin–C	Chitin–N
Mat soil + 0.5 %	19 ± 3	10 ± 0.1	1.9 ± 1	0.2 ± 0.04
Mat soil + 1 %	27 ± 4	12 ± 0.5	3.5 ± 1	0.4 ± 0.04
Mat soil + 2 %	51 ± 3	7.7 ± 0.1	5.3 ± 1	0.6 ± 0.1
Mat soil + 4 %	99 ± 2	4.1 ± 0.6	10 ± 2	0.9 ± 0.2
Nonmat soil + 0.5 %	8.8 ± 2	1.5 ± 0.3	1.5 ± 0.4	0.1 ± 0.03
Nonmat soil + 1 %	18 ± 1	3.0 ± 0.1	2.7 ± 0.1	0.2 ± 0.1
Nonmat soil + 2 %	32 ± 1	5.8 ± 0.1	1.8 ± 2	0.5 ± 0.1
Nonmat soil + 4 %	66 ± 1	12 ± 0.3	5.8 ± 1	1.0 ± 0.1

because lab-based extracellular enzyme assay rates are run using soluble substrates under ideal conditions and generally not realized in the field. Still, the high chitinase activity potential does underpin the potential for rapid amino sugar turnover in these soils.

Amino sugar availability and turnover

In 7 days, 62 ± 3–146 ± 21 % of the C and 36 ± 4–82 ± 6 % of the N added as NAG was microbially modified; in 5 weeks, 17 ± 10–73 ± 49 % of the C and 30 ± 7–58 ± 6 % of the N added as chitin was microbially modified. On a total mass basis, this corresponds to crude estimates of microbial processing rates of 8.8–99 μg NAG–C and 1.5–10 μg chitin–C g^{-1} dry soil h^{-1}, which are 0.08–3.4 times the basal respiration rates (Table 4). For N, this corresponds to 1.5–12 μg NAG–N and 0.1–1.0 μg chitin–N g^{-1} dry soil h^{-1}, which are 0.07–14 times the basal net N mineralization rates. In other studies that directly measured amino sugar dynamics using isotopically-labeled molecules and advanced analytical methods, NAG residues were degraded and assimilated rapidly. Over a one-week incubation period in loamy sand grassland mineral soils, the soil amino sugar pool became significantly ^{13}C enriched via microbial assimilation of labeled litter material, but total amino sugar concentration did not change, implying a rapid basal rate of biomass amino sugar turnover (Decock et al. 2009). In different grassland mineral soils, glucosamine C half-life was on the order of hours at low amendment concentrations, and days at higher amendment concentrations (Roberts et al. 2007).

The estimated total amino sugars content of this soil is high in comparison to agricultural and prairie soils, and more similar but still higher than that measured in other organic horizon forest soils (Decock et al. 2009; Guggenberger et al. 1999; Roberts et al. 2007; Zhang and Amelung 1996). Amino sugar content is more typical relative to the N pool, comprising 7.5–8.2 % of total soil N (based on an amino sugar content of 6.3 % N, Table 1) (Amelung 2003; Stevenson 1982), and is actually low relative to the large pool of biomass. The ratio of amino sugar to biomass C in these soils is between 1.0–1.2 (based on an amino sugar content of 43.4 % C, Table 1), low in comparison to the average amino sugar to biomass ratio of 7.65 measured in agroecosystems (Guggenberger et al. 1999). Overall, amino sugar stabilization may be relatively low in these mineral- and aggregate-poor organic horizon soils. On the other hand, microbial cell walls can vary in amino sugar content from 1 to 20 % by mass (*Escherichia coli* is 3–10 % murien, ascomycete yeasts are 1–2 % chitin; *Aspergillus* and *Neurospora* spp. are 10–20 % chitin (Bowman and Free 2006; Lengeler et al. 1999)). If cell walls comprise 20 % of microbial cell dry mass, then approximately 0.2–4 % of microbial C and N is contained in amino sugars, and 25–500 times the microbial pool of amino sugars is recoverable from the study soil after acid hydrolysis, indicating that some stabilization of these molecules has occurred. K_2SO_4 extractable amino sugar concentrations were not detectable, and the high chitin decomposition rates measured in our experiment suggest that the total standing pools of amino sugars, if bioavailable, might be depolymerized and assimilated in a matter of days to weeks. Thus, a substantial proportion of the total amino sugars detected in the soil may be protected and unavailable for enzymatic degradation or microbial uptake, though the exact stabilization mechanism is not clear.

Ectomycorrhizal mat influences on forest floor nutrient cycling

The soil organic horizon, or forest floor, while only centimeters in depth, contains a significant amount of ecosystem C and N. In old-growth Douglas-fir forests, the forest floor has been estimated to contain 3.8 % of the total organic matter (not including coarse woody debris on the forest floor), 9.1 % of the total detrital C, and 6.1–8.3 % of the total N of the entire forest stand (Means et al. 1992; Sollins et al. 1980). Respiration from the organic horizon comprises an annual average of 73 % of the total in situ soil respiration, and EcM mat colonized soil comprises 42 % of the forest floor on an areal basis (55 % of non-woody debris covered area) (Phillips et al. 2012). Because these were laboratory experiments, the physical disturbance of the soil and treatments applied mimic mycorrhizal mortality events of different magnitudes, which in this ecosystem could feasibly occur via tree stress or death. The experiments show that saprotrophic soil microbes can readily capitalize upon a flush of amino sugars to the soil, and that organic horizon soils colonized by EcM mats might release a relatively large amount of CO_2 after such an event. However, this set of laboratory experiments did not measure in situ rates

of mycelial or microbial decomposition. These lab results raise the question of the relative influence of mycorrhizal turnover versus direct rhizosphere C and N inputs on saprotrophic activity and SOM decomposition as an intriguing and challenging field experiment.

Summary

In both EcM mat and non-mat colonized old-growth Douglas-fir forest organic soils, these experiments provided evidence that chitin and NAG are decomposed primarily as a source of N to an N-limited microbiota, and secondarily as a C source. Differences in substrate utilization dynamics between mat and non-mat soils may be related to the discrepancy in total biomass between the two soils or to differences in microbial community structure. The similar chitinase activity response to both NAG and chitin addition suggests a more complex, possibly product-induced, enzyme regulatory mechanism than expected. The existence of a large total amino sugar pool concurrent with high rates of NAG and chitin turnover suggest that amino sugars are stabilized in this organic horizon soil, though not to the extent that might be observed in an aggregated mineral soil. Overall, the potential for rapid microbial turnover in these soils is strong and likely important for microbial conservation of N in a low C:N habitat.

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