# Root exudates increase N availability by stimulating microbial turnover of fast-cycling N pools

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**Abstract**

Theory and experiments suggest that rhizodeposition can accelerate N-cycling by stimulating microbial decomposition of soil organic matter (SOM). However, there are remarkably few experimental demonstrations on the degree to which variations in root exudation alter rhizosphere N dynamics in the field. We conducted a series of *in situ* substrate addition experiments and a modeling exercise to investigate how exudate mimics and enzyme solutions (at varying concentrations) influence rhizosphere SOM and N dynamics in a loblolly pine (*Pinus taeda*) plantation (Duke Forest). Exudates were added semi-continuously to unfertilized and fertilized soils in summer and fall; enzymes were added during the following summer. Exudate additions enhanced the microbial biomass specific activities of enzymes that degrade fast-cycling N pools (i.e., amino acids and amino sugars), and increased microbial allocation to N-degrading compounds. More, such effects occurred at low exudate concentrations in unfertilized soil and at higher concentrations in fertilized soil. Direct additions of a subset of enzymes (amino sugar- and cellulose-degrading) to soils increased net N mineralization rates, but additions of enzymes that cleave slow-cycling SOM did not. We conclude that exudates can stimulate microbes to decompose labile SOM and release N without concomitant changes in microbial biomass, yet the investment of plants to trigger this effect is much higher in N-rich soils.

*Keywords:* Extracellular enzymes, labile SOM pools, N fertilization, microbial decomposition simulation model, rhizosimulators, root exudation

## 1. Introduction

It is well established that plants can accelerate N-cycling in the rhizosphere by releasing root exudates that stimulate microbial growth, activity and turnover (reviewed in Badalucco and Kuikman, 2001; Jackson et al., 2008; Frank and Groffman, 2009). Studies of root-induced N-cycling generally fall into one of three categories: comparisons of N-cycling in rhizosphere vs. bulk soil (Norton and Firestone, 1996; Herman et al., 2006; Phillips and Fahey, 2006; Finzi et al., 2015), comparisons of N-cycling in the presence vs. absence of root inputs (Vitousek et al., 1982; Weintraub et al., 2007; Kaiser et al., 2011; Brzostek et al., 2013), and lab incubation studies of N transformations following additions of microbial substrates (Landi et al., 2006; Kieloaho et al., 2016). While these studies provide strong evidence of links between root-derived C and N-cycling, the mechanisms that drive these dynamics are poorly understood owing to challenges of experimentally manipulating exudate inputs *in situ* (but see Drake et al., 2013*a*). Theory predicts that exudates should enhance N-cycling by stimulating microbial growth – a process that can induce microbes to release extracellular enzymes that depolymerize SOM via priming effects (Cheng et al., 2014). However, the factors that control the magnitude and direction of priming effects and their seasonality are still debated (Rousk et al., 2015; Georgiou et al., 2016).

One important source of uncertainty regarding priming effects is which components of SOM are primed. One theory suggests that priming effects result from microbial mining of N from recalcitrant, slow-cycling SOM pools. Soil organic matter consists of multiple compounds, many of which are chemically recalcitrant or energetically unviable as microbial substrates (Kemmitt et al., 2008). In forest soils, this slow-cycling pool of SOM contains abundant quantities of organic N in the form of phenol-protein complexes (Rillig et al., 2007; Majuakim and Kitayama, 2013). Root exudates released by plants could thus provide an energy subsidy to microbes, enabling

them to produce extracellular enzymes to mine N from SOM (Jones et al., 2004; Kuzyakov, 2010; Phillips et al., 2012; Kuzyakov and Xu, 2013). If most of the N that is primed comes from slow-cycling SOM, ecosystems that have a nearly inexhaustible supply of N (e.g., high latitude forests) may be less constrained by N limitation.

An alternative mechanism for priming is that the N released comes from the accelerated turnover of fast-cycling SOM pools. A significant portion of SOM, particularly in forests, is comprised of moderately fast-turnover, N-rich pools such as amino sugars (Roberts et al., 2007). Chitin, the polymeric form of the amino sugar *N*-acetyl-glucosamine, forms the cellular walls of most soil fungi so that when fungi die, much of the necromass is degraded by *N*-acetyl-glucosaminidase (NAG) (Zeglin et al., 2013). While chitin and *N*-acetyl-glucosamine are generally believed to turn over slower than proteins (Roberts and Jones, 2012), it is unknown whether the turnover of this SOM pool is stimulated by the addition of root exudates. If priming effects occur owing to the accelerated release of N from the medium stable, amino sugar pool of N, then microbial mining of N may be a transient process, as most soils have much less N in amino sugars (5-10% of all N) relative to proteins and amino acids (30-45% of total N; Myrold, 1998). Thus, as nutrient demand increases (owing to competition or elevated $CO_2$), plants that do not exude much C or support a large fungal biomass, may be more prone to experiencing N constraints to plant productivity.

Multiple $CO_2$ enrichment studies, which increase plant demand for N, provide further evidence that rhizosphere priming effects may exert a major impact on both soil C loss and N availability (Hoosbeek et al., 2004; Heath et al., 2005; Carney et al., 2007; Langley et al., 2009; Phillips et al., 2011; Zak et al., 2011; Phillips et al., 2012). In most of these studies, rhizosphere priming effects were not directly measured and root-induced changes in N-cycling were generally inferred by calculating the mass balance for N (Drake et al., 2011; Zak et al., 2011). At the Duke Forest

free-air carbon enrichment (FACE) site, multiple indirect lines of evidence suggest that trees respond to elevated $CO_2$ by increasing C fluxes belowground (Pritchard et al., 2008; Jackson et al., 2009; Drake et al., 2011), and increasing C fluxes from roots to soil (Phillips et al., 2011). The increases in belowground C fluxes were linked to enhanced enzyme activities in the rhizosphere (Phillips et al., 2011; Meier et al., 2015) – a process that putatively released N and sustained plant productivity under elevated $CO_2$ (Drake et al., 2011; Phillips et al., 2012; Cheng et al., 2014). While belowground dynamics were clearly key drivers of ecosystem N-cycling at the site, there was conflicting evidence about the source of the N that was being used to support the sustained NPP response. In Phillips et al. (2011), increases in exudation under $CO_2$ were positively correlated with rhizosphere NAG activity, suggesting that root exudates were fueling the turnover of fungal necromass. This finding was supported in a follow-up study that found that the enhanced turnover of mycorrhizal fungi under elevated $CO_2$ could account for the N used to sustain NPP (Phillips et al., 2012). However, Phillips et al. (2011) and Meier et al. (2015) also reported that not only was fungal necromass in the rhizosphere decaying faster under elevated $CO_2$, but so too was slow-cycling SOM. This suggests that the microbes may be targeting recalcitrant N compounds in the SOM (e.g., protein-phenol complexes) as well. The latter finding was supported by Cheng et al. (2014) who used a modeling analysis to determine that the slow-cycling pool of SOM (with a turnover time of 2050 years) may be the one pool that contains enough N to sustain plant growth under elevated $CO_2$.

In the current study, we experimentally added root exudate solutions or extracellular enzymes to unfertilized and fertilized forest soil at the Duke Forest, NC, USA, as a means for better understanding how root-microbe interactions control N dynamics. We hypothesized that accelerated N-cycling under elevated $CO_2$ previously observed at the site results more from microbial priming of fungal necromass than of the slow-cycling SOM pool.

## 2. Materials and methods

### *2.1 Study site*

This research was conducted at the Duke free-air carbon enrichment (FACE) experiment, North Carolina, USA. At this site a loblolly pine (*Pinus taeda* L.) plantation has been established from 3-year-old seedlings in 1983 following clear-cutting and burning, and has subsequently been recruited from hardwood tree species (i.e., *Liquidambar styraciflua, Ulmus alata, Cornus florida, Acer rubrum, Cercis canadensis*) in the understory. Loblolly pine has comprised 98% of the basal area of this forest (DeLucia et al., 1999). The site is located in the region of the Piedmont plateau on low-fertility, slightly acidic Hapludalfs (Enon series) derived from diabase (Oh and Richter, 2005). The climate is humid subtropical, characterized by warm, humid summers and relatively moderate winters. Mean annual precipitation is 1140 mm and mean annual temperature 15.5°C.

The Duke FACE experiment consisted of eight randomly selected 30-m-diameter plots, with one prototype reference site, three fully instrumented control plots receiving ambient air, and four treatment plots receiving elevated atmospheric $CO_2$. Since 2005 a nitrogen fertilizer ($NH_4NO_3$) treatment was added to half of each plot in two applications in spring to achieve a total annual fertilization rate of 11.2 g N $m^{-2}$ $yr^{-1}$ (Drake et al., 2008). The experimental design was a split-plot in a randomized complete block design; $CO_2$ treatment was the whole-plot factor and N treatment was the subplot factor.

### *2.2 Experimental exudate substrate addition*

We conducted a series of four and eight-week experiments in summer and fall, 2009, respectively, in the unfertilized and fertilized half of each FACE ring. Sterile model exudate substrates were semi-continuously delivered to soil at ecologically relevant rates via a sustained, low-volume drip from an artificial root (i.e. a rhizosphere simulator). The simulators, which are

10 cm long and 2 mm in diameter, were placed into 0-10 cm depth of the mineral soil at random positions in a 1 x 1 m sampling spot which was located by random in each plot (minimum distance between individual simulators at least 10 cm, two simulators per plot and addition rate). The simulators were made from a chemically-inert hydrophilic polymer with a pore size of 0.15 µm, which precluded microbes from entering the simulators, and contained a stainless steel wire inside to provide mechanical support (Rhizosphere Research Products, The Netherlands). The polymer parts of the simulators were inserted completely into the soil, ensuring tight soil contact, and were covered by litter. Each simulator was connected by food-grade tubing to a 16-channel, low-flow peristaltic pump (Watson–Marlow 205U, Falmouth, UK). After installation, simulators were left to equilibrate by pumping 500 µL DI water twice per day for one week. Subsequently, the pumps were set by a timer to deliver ~500 µl of exudate solution twice per day. Previous experiments with a similar pumping system suggest that such small volumes of solutions minimize the potential for saturating (i.e. anaerobic) conditions, and have the most concentrated effect on soils within 1-2 mm of the simulator (data not shown). The exudate solution was kept cool and the source of the delivery system changed every couple of days.

Each pump delivered model exudate solutions to soil at three concentrations and a DI control. The total amount of C in the substrates represented 0, 20, 100 and 200 µg C $cm^{-2}$ simulator surface area $day^{-1}$ (Fig. S1). This approximates medium to up to twenty times higher exudation rates from pine roots (2 – 11 µg C $cm^{-2}$ fine root surface area $day^{-1}$) that we measured in April 2007 at this site. The solution contained a mixture of organic acids and simple sugars (3:1 by volume). This organic acid:simple sugar ratio approximates the relative proportions of acids and sugars in exudates collected from mature trees (Smith, 1976). Rather than using single acids and sugars, we used four low molecular weight organic acids (fumaric acid, malonic acid, oxalic acid, and shikimic acid) and three simple sugars (glucose, fructose and sucrose). The acids and sugars

were selected based on their frequency in which they were detected in a previous experiment with loblolly pine seedlings (M. Waters, personal communication), and two additional studies with *Pinus* (reviewed in Grayston et al., 1996). Although pine roots may release amino acids, we did not add these to our solution based on the fact that their rapid re-acquisition by roots (Jones et al., 2004) likely contributes to their low concentrations in most tree root exudates (Smith, 1976). After a four and eight-weeks pulse, respectively, of exudate addition to soil, soil adjacent to each of the simulators (<6 mm from the simulators) was sampled, brought back to the lab on ice, sieved (<2 mm) immediately, and stored at 4°C until processing within 48 h of collection.

*2.3 Experimental exoenzyme addition*

In the primary growing season in 2010, we conducted a four-week experiment in the unfertilized and fertilized half of each FACE ring. Enzymes were delivered to soil with the above described rhizosphere simulators. Each pump delivered three different enzyme solutions to soil and a DI control. The enzyme activities were 0.07 U $mL^{-1}$ for β-1,4-*N*-acetyl-glucosaminidase (NAG) and 0.67 U $mL^{-1}$ for both phenol oxidase (Phenox) and β-1,4-glucosidase (Glu) (Table S1). This was estimated to result in soil enzyme activities of 0.2 μmol $g^{-1}$ $h^{-1}$ of NAG and 2 μmol $g^{-1}$ $h^{-1}$ of both Phenox and Glu. A total of 40 simulators were installed. After a four-week pulse of enzyme addition to soil, soil adjacent to each of the simulators was sampled and put on ice in the field. Soil samples were sieved (<2 mm) immediately in the lab and stored at 4°C until processing.

*2.4 N mineralization rates*

The rate of potential net N mineralization was measured under field-moist conditions (Finzi and Schlesinger, 2003): one 10 g-subsample was extracted immediately in 100 mL 2 M KCl, while the second 10 g-subsample was incubated for seven days in the dark after which time it was also

extracted in KCl solution. The amount of $NH_4^+$ and $NO_3^-$ was measured by flow injection analysis (Lachat QuikChem 8500, Lachat Instruments, Hach Company, Loveland, USA) and the net N mineralization potential calculated as the difference in the amount of ammonium and nitrate between day one and seven. Gross N mineralization potential, i.e. the production of $NH_4^+$ from organic N, was determined using $^{15}N$ isotope pool diffusion (Finzi and Schlesinger, 2003). Samples were initially labeled with 0.5 mL of a 92 mg $L^{-1}$ 99-atom% enriched ($^{15}NH_4$)Cl solution in the lab. The initial and final pool size of $NH_4^+$ was determined by measuring the concentration of $NH_4^+$ by flow injection analysis. The atom% $^{15}N$ excess of the initial and incubated sample were analyzed by diffusing the N in each sample onto an acidified filter disc and analysis on a continuous-flow isotope ratio mass spectrophotometry at the Boston University Stable Isotope Laboratory (Boston, MA). Soil moisture was determined by drying (110°C, 48 h) and weighing.

*2.5 Extracellular enzyme activity assays*

A subsample of soil from the exudate addition experiment was stored at -80°C for enzyme analysis. Potential enzyme activities for the activity of six extracellular enzymes involved in the decomposition of C, N, and P containing compounds were assayed (Finzi et al., 2006). The six extracellular enzymes can be grouped into three functional groups based on their ability to decompose relatively labile C constituents (β-1,4-glucosidase (Glu)), to decompose recalcitrant C constituent (phenol oxidase (Phenox), peroxidase (Perox)), or to depolymerize organic N (β-1,4-N-acetylglucosaminidase (NAG), leucine aminopeptidase (LAP)) and P (acid phosphatase (AP)). Activities of Glu, NAG, and AP were measured with methylumbelliferone as substrate using a microplate fluorometer with 365 nm excitation and 450 nm emission filters. Activities of LAP were measured with 7-amino-4-methyl coumarin as substrate. Phenox and Perox activities were measured spectrophotometrically as absorbance at 460 nm using $L^{-1}$-3,4-dihydroxyphenylalanine

(L-DOPA) as the substrate. After correction for controls and quenching, enzyme activities were expressed in units of mol substrate cleaved g $soil^{-1}$ $h^{-1}$ and as mol substrate cleaved g $C_{microbial\ biomass}^{-1}$ $h^{-1}$.

We assayed potential proteolytic enzyme activity according to Brzostek et al. (2012; modified from Watanabe and Hayano, 1995 and Lipson et al., 1999), using trichloroacetic acid solution to halt the activity of proteolytic enzymes. The concentration of amino acids was quantified using the o-phthaldialdehyde and β-mercaptoethanol method (Jones et al., 2002). Proteolytic enzyme activity was calculated from the difference in amino acid N between incubated and non-incubated samples (Brzostek and Finzi, 2011).

*2.6 Microbial biomass in the experimental rhizosphere*

In the exudate mimics addition experiment, microbial biomass was measured using a modification of the substrate-induced respiration method (Anderson and Domsch, 1978): we added 10 mL of yeast extract (4 g autolyzed yeast per liter nanopure water) to 2 g of soil in air-tight vials with rubber septa caps and mixed to slurry. A 5 mL aliquot of headspace gas was sampled immediately and 2 and 4 h after yeast addition and injected into a LI 6200 infra-red gas analyzer (Licor Inc., Lincoln, NE, USA) for $CO_2$ analysis. Between sampling, vials were put onto a table shaker and incubated at room temperature. Average respiration rates over the 4 h incubation (μg C-$CO_2$ $g^{-1}$ $h^{-1}$) were converted to microbial biomass C (μg C $g^{-1}$) using the equation from Anderson and Domsch (1978): microbial biomass C = 40.04x+0.37,where *x* is the maximum rate of $CO_2$ respiration.

In the enzyme addition experiment, microbial biomass C and N was analyzed by a modification of the chloroform fumigation direct extraction-method (Brookes et al., 1985; Högberg and Högberg, 2002). Non-purgeable organic C and total N were measured on a total organic carbon

analyzer (Shimadzu TOC-V CPH/CPN, Shimadzu Scientific Instruments, Columbia, USA). Microbial biomass C or N was calculated from the difference in C and N between fumigated and non-fumigated samples, divided by the extractable part of microbial biomass C ($k_{EC}$ = 0.45; *cf.* Beck et al., 1997).

*2.7 Theoretical modeling of N mineralization*

Given that we were not able to monitor changes in N mineralization that occurred during the course of the exudate addition experiments (since soil samples were only collected at the end of the experiment), we used a stoichiometrically-constrained microbial decomposition simulation model (the *Mi*crobial *C*arbon and *N*itrogen *P*hysiology or MCNiP; for details of the model refer to Drake et al., 2013a, 2013b; Finzi et al., 2015) to explore the dynamic relationships between root substrates and microbial processes. Specifically, we used measured data from our field site (e.g., pools of microbial biomass C, microbial biomass C:N, enzyme C, enzyme N, mineral N, mineral C:N, DON, SOM, and soil temperature) to validate the predictions of the MCNiP simulation model and make predictions on the expected level of N mineralization in unfertilized and fertilized Duke Forest FACE stands in response to exudate addition. While the model enabled us to explore theoretical linkages between SOM depolymerization, microbial physiology, root exudation, and C:N stoichiometry, the model only contains a single SOM pool, and so it was not used to make predictions relative to our overarching hypothesis.

MCNiP (i) utilizes reverse Michaelis-Menten kinetics to model the depolymerization of SOM into dissolved organic carbon (DOC) and dissolved organic nitrogen (DON); (2) Michaelis-Menten kinetics to model DOC and DON uptake (Allison et al., 2010); (iii) first-order kinetics to model inorganic N loss (Drake et al., 2013a); (iv) simulates immobilization of N when microbial biomass is N limited and mineralizes it otherwise; and (v) assumes the rate of exoenzyme

production is proportional to microbial biomass (Allison et al., 2010). We adjusted the baseline model by the incorporation of microbial and exoenzyme data from the Duke Forest and assumed constant and continuous (that means daily) C exudate mimics addition to the DOC pool during a 60-day experimental period. Four scenarios for each unfertilized and fertilized half of the eight Duke Forest FACE rings were simulated: daily addition of (i) 0 µg, (ii) 20 µg, (iii) 100 µg, and (iv) 200 µg exudate mimics C, respectively. Subsequently, we calculated means from the eight plots per exudate and enzyme addition simulation, respectively, and fertilization level.

We explored how the modeled responses of N mineralization varied with the amount of exudate addition by running the model with different amounts of exudate C additions. The sensitivity of the modeled responses of microbial biomass, exoenzyme N activities, gross N mineralization, and microbial N immobilization to daily exudate additions was calculated as in Allison et al. (2010), to normalize the change in the response of these variables to the size of the change in exudate mimics addition:

$$\text{Sensitivity} = \frac{|\log|high\ response| - \log\ |low\ response||}{|\log|high\ C\ addition| - \log\ |low\ C\ addition||} \qquad \text{(Eq. 1).}$$

The sensitivity of the modeled responses was calculated separately comparing (i) no and low (0 and 20 µg C), (ii) low and moderate (20 and 100 µg C), and (iii) moderate and high (100 and 200 µg C) exudate addition.

*2.8 Statistical analyses*

Statistical analyses were conducted with the package SAS, version 9.3 (SAS Institute Inc., Cary, NC, USA). We refer to a *P* value of $\leq 0.05$ as statistically significant. Means and standard errors of soil parameters were calculated from each eight and four rhizosimulators (sample size) per exudate and enzyme addition treatment, respectively, and fertilization level. We concentrated our

analyses on N availability effects on the mechanistic link between root exudates and microbial activity for N mineralization and, thus, refrained from incorporating atmospheric $CO_2$ concentrations as a factor to our analyses.

The probability of a fit to a normal distribution was tested using a Shapiro–Wilk test. Treatment means of normally distributed data were compared by analyses of variance (ANOVA) to analyze differences between treatments and the treatment means of non-normally distributed data by one-way Kruskal–Wallis single-factor analyses of variance (ANOVA) and non-parametric multiple comparison tests after Wilcoxon to locate the differences.

## 3. Results

### *3.1 Experiment one – Adding exudate mimics to soils*

There was a positive effect of exudate additions on the specific proteolytic activity of microbes to cleave amino acids in unfertilized soil (Fig. 1a): the addition of exudate mimics significantly increased the proteolytic activity by more than twice from 37.2 to 90.4 mg AA-N g $C_{mic}^{-1}$ $d^{-1}$ with an addition of 100 µg C $cm^{-2}$ $d^{-1}$ (significant; Fig. 1b). An increase in the amount of C addition to 200 µg C $cm^{-2}$ $d^{-1}$ did not result in a further increase in proteolytic activity. The background proteolytic activity of microbes in fertilized soil was generally higher and did not respond to the addition of exudate mimics (54.8-72.2 mg AA-N g $C_{mic}^{-1}$ $d^{-1}$).

The effect size on specific NAG activity decreased with increasing C exudate addition in unfertilized soil, while it increased in fertilized soil (Fig. 2a): exudate mimics doubled amino-sugar degrading NAG activity in unfertilized soil from 12.5 to 25.5 mg N g $C_{mic}^{-1}$ $d^{-1}$ with an addition of 20 µg C $cm^{-2}$ $d^{-1}$ (significant increase), but did not increase NAG activity with higher additions of C (Fig. 2b). The increase in NAG activity with exudate mimics was twice as high in

fertilized soil (12.6 to 49.8 mg N g $C_{mic}^{-1}$ $d^{-1}$) and the positive effect increased with increasing amounts of exudate C additions (Fig. 2b).

The addition of exudate mimics did not significantly influence Phenox, Perox, LAP, or Glu activities (Figs. S2a, S3). This led to a significant increase in the hydrolytic enzyme N:C stoichiometry (the relationship of the specific activities of NAG and LAP to the specific activity of Glu) with exudate additions to fertilized soils (0.19 to 0.87 mmol $mol^{-1}$; Fig. 3a), but to no significant increase in unfertilized soil. Despite this, a high positive effect size was found at the lowest exudate addition rate in unfertilized soil (effect size: 1.10), while in fertilized soil the highest positive effect was found at the intermediate exudate C addition rate (effect size: 0.78; Fig. 3b).

Similar to the response of NAG activity, specific AP activity of microbes to degrade organic P increased significantly from 103.6 to 169.3 mg P g $C_{mic}^{-1}$ $d^{-1}$ with an addition of 20 µg C $cm^{-2}$ $d^{-1}$ (effect size: 1.60) but did not increase further with higher additions of C (Fig. S2b). AP activity did not significantly increase in fertilized soil with an increase in exudate mimics addition, but had a positive effect size (1.05) at the high exudate addition rate (Fig. S2d).

*3.2 Nitrogen mineralization rate of microbial biomass in response to exudate mimics*

Gross N mineralization per microbial biomass (i.e., the specific activity of microbes to mineralize N) did not significantly increase with the addition of exudate mimics in unfertilized or fertilized soil (Fig. S4a, c). The background gross N mineralization rate was higher in fertilized than in unfertilized soil (80 mg g $C_{mic}^{-1}$ $d^{-1}$). There was a positive effect of the addition of 100 µg C $cm^{-2}$ $d^{-1}$ on gross N mineralization in fertilized soil (effect size: 0.66). Net N mineralization generally had a high variability and did not relate to exudate mimics additions (Fig. S4b, d). Net N mineralization was about eight times lower than gross N mineralization in unfertilized soil and up

to about 140-times lower in fertilized soil, indicating significant amounts of microbial N assimilation (immobilization), particularly in fertilized soil. In the fall, N immobilization appeared as negative net N mineralization rates with all exudate addition rates (Fig. S7).

*3.3 Experiment two – Adding extracellular enzymes to soil*

Gross and net N mineralization rates changed in response to extracellular enzyme additions merely due to changes in the microbial activity (Fig. 4) and not due to changes in microbial biomass: microbial biomass decreased significantly with the addition of NAG yet did not change with the additions of Phenox or Glu (Fig. S5a). The addition of NAG to unfertilized soil did not significantly increase gross N mineralization, but its addition to fertilized soil led to a significant decrease (4.6 to 1.0 mg g $C_{mic}^{-1}$ $d^{-1}$; Fig. 4a) and a negative effect on gross N mineralization (effect size: -0.79; Fig. 4c). By contrast, NAG addition had a positive effect on net N mineralization in unfertilized soil (1.29; Fig. 4c): it significantly increased net N mineralization from -1.1 to 1.0 mg g $C_{mic}^{-1}$ $d^{-1}$ (Fig. 4b). In fertilized soil, NAG addition had no influence on net N mineralization.

The addition of Glu did not significantly increase gross N mineralization in unfertilized soil, but significantly decreased its rate to almost zero when added to the fertilized soil (4.6 to 0.1 mg g $C_{mic}^{-1}$ $d^{-1}$; Fig. 4a, d). On the contrary, Glu addition had a positive effect on net N mineralization in unfertilized soil which significantly increased (-1.1 to 0.1 mg g $C_{mic}^{-1}$ $d^{-1}$; Fig. 4b, d). When added to the fertilized soil, Glu did not significantly increase net N mineralization despite a positive effect size (effect size: 0.99). Due to the concurrent decrease in gross N mineralization, net N mineralization was 21-times higher than its gross rate in fertilized soil, which may indicate an elevated nitrification rate in N-rich soil with the addition of Glu.

*3.4 Modeled responses of N decomposition and N depolymerization*

The parameter sensitivity analysis of modeled responses showed for all factors the greatest responses with a change from no to low exudate additions, both in unfertilized and fertilized soil (Table 1). The greatest sensitivity was predicted for microbial biomass (8.0), followed by gross N mineralization (6.6-6.8) and finally soil organic nitrogen (SON) depolymerization with much smaller sensitivity (0.02). The parameter sensitivity of microbial biomass C and gross N mineralization to a change from no to low and to a change from low to moderate exudate additions was slightly higher in the unfertilized soil than in the fertilized (higher by 0.01-0.05), while at higher exudate additions the sensitivity was higher in fertilized soil (higher by 0.09-0.10).

Simulated gross N mineralization per soil mass increased exponentially with an increase in exudate C additions in unfertilized and fertilized soil (Fig. 5a) due to an increase in microbial biomass C (Table S2). This simulated increase in microbial biomass differs from the unaltered microbial biomass observed in the experiment (Fig. S7). The specific activity of microbes to mineralize N was modeled to decrease with the addition of exudate C to about 9 mg g $C_{mic}^{-1}$ $d^{-1}$ and remain at this specific activity with any concentration of exudate C addition (Fig. 5b). Again, the simulated decrease in specific gross N mineralization differed from the mineralization rates in the experiment, which were not significantly different but rather increased by trend in fertilized soil with an increase in exudate additions (Fig. S4a).

## 4. Discussion

Understanding the process by which exudates impact microbial decomposition represents a key first step towards predicting how nutrient limitation may be further exacerbated or overcome by plants exposed to rising $CO_2$ (Zaehle et al., 2014; Medlyn et al., 2015). In this study, we found

evidence that microbial extracellular enzyme activities provide a mechanistic link between root exudates and accelerated long-term N-cycling as was reported from previous observational FACE studies (e.g., Drake et al. 2011). Specifically, we found while exudates can accelerate the decay of fast- and slow-cycling SOM pools, much of the N that becomes available to plants is likely derived from turnover of amino acids (catalyzed by protease) and amino sugars (catalyzed by NAG), putatively from fungal necromass.

Notably, the strongest effect on enzyme activities was triggered by the lowest exudate supply rate in soil with the greatest N deficiency. However, our field results indicated that the accelerated enzyme activity resulted more from microbial shift in the enzyme C:N acquisition activities towards fast-turnover N-degrading enzymes and not from changes in microbial biomass – a finding that differed from our model results. Consequently, this highlights the need to use field data to confront models and suggests that future efforts to model rhizosphere dynamics such as priming effects should consider other mechanistic linkages between substrates, microbes and their interactive effects on decomposition.

*4.1 Microbial activity as a function of the quantity of exudate C*

In contrast to earlier studies (e.g., Blagodatskaya and Kuzyakov, 2008; Sullivan and Hart, 2013), accelerated enzyme activity was not mediated by an increase in microbial biomass. This could be a result of both (i) a change in the microbial community composition and (ii) a functional change in community-level physiology. Generally, the community composition and activity of soil microbes is closely dependent on the release of rhizodeposits: rhizosphere microbes derive more than half of their C from root exudates (van Hees et al., 2005). But even fundamental changes in microbial community structure can be independent of community-level physiology, if microbial processes are sufficiently resilient (McFarland et al., 2013). Likewise, exudates can also invoke a

functional change in community-level activity or physiology without a compositional change (van Hees et al., 2005; Goldfarb et al., 2011). While we cannot prove a direct influence of exudate additions on microbial community structure and physiology with our current study, the relationships between root exudation rates and microbial community composition and functioning should be at the attention of future research.

The activation level for the stimulation of microbes to scavenge for N was higher when soil N was abundant. It is generally expected that the stimulation of SOM decomposition by root exudates is lower in soil with higher nutrient availability (nutrient mining theory; Fontaine et al., 2003, 2004, 2011; Blagodatskaya and Kuzyakov, 2008; Guenet et al., 2010; Paterson and Sim, 2013; Sullivan and Hart, 2013), but contrasting results have been found as well (Hoosbeek et al., 2006; Chen et al., 2014; Chowdhury et al., 2014), which may be partly due to the stoichiometry of substrates and microbial demand for substrates (Craine et al., 2007). Here we show that, at higher exudate C additions, the stimulation response of SOM decomposition can be similar in N-rich soil as in N-poor soil, indicating support of basic stoichiometric decomposition theory. Yet since many plants decrease belowground C allocation and exudation in response to N enrichment (Phillips et al., 2009), SOM decomposition may either not be stimulated or even decreased in these soils (Phillips and Fahey, 2006).

Above a specific maximum, we found declines in enzyme activities with increasing exudate addition rates; this maximum occurred at higher exudate addition rates in fertilized than in unfertilized soils. In a meta-analysis, Blagodatskaya et al. (2011) reported that labile C additions increased the degradation of SOM at low concentrations but decreased it at higher concentrations, as the microbial community preferentially utilized the labile substrates (Cheng, 1999). In the MCNiP model, modest fluxes of root-derived C (6% of NPP) induce disproportionately large effects on microbial decomposition and priming (Finzi et al., 2015), with the largest losses of soil

organic carbon (SOC) occurring when the C:N ratio of exudates is <7 because of an increase in the efficiency of SOM decomposition. At wider C:N, exudation still primes SOM decomposition, but at a slower rate owing to a larger pool of microbial biomass rather than an increase in efficiency. Adding to the earlier finding by Finzi et al. (2015), the result of our study seems to indicate that an optimal exudate-C to soil-N ratio results in similar effects on microbial enzyme production.

Most process-based models consider SOM decomposition a function of first-order rate constants (Jenkinson and Coleman, 2008; Manzoni and Porporato, 2009). A number of more recent studies found that the relationship between the magnitude of SOM degradation enhancement and the substrate addition rate is non-linear (Guenet et al., 2010; Paterson and Sim, 2013). The cumulative priming effect tended to level off when the C addition rate was higher than a soil-specific C concentration (saturation effect; Wang et al., 2015) and the ratio of priming effect to C addition rate was simulated by a first-order exponential decay relationship. Among the explanations for the non-linearity of the relationship are (i) an increasing limitation of soil microbes by other factors than C, (ii) spatial separation (Feeney et al., 2006; Salomé et al., 2010) or insolubility (Kemmitt et al., 2008) of SOM making it less accessible to microbes, and (iii) rapid exhaustion of newly added substrate preventing priming effects from increasing in an equal proportion. In support of the non-linearity of the relationship between exudate C addition and SOM degradation the results of the current study suggest a hump-shaped pattern for the relationship between root exudation and the decomposition of N fractions from SOM.

*4.2 Seasonal effects of root exudate additions on SOM decomposition and N mineralization*

In the current study we decoupled root exudation rates from seasonality and, thus, observed the potential of season for affecting the interplay between exudation rates with the active microbial

biomass, amount of SOM, and net N mineralization directly. Season did not change the influence of root exudation on neither the amount of active microbial biomass, nor SOM, nor net N mineralization rates, but had a strong direct impact on mineralization and immobilization: net N mineralization prevailed during summer while net N immobilization dominated during fall.

Previous studies at the Duke Forest FACE site have reported that the extracellular activities of enzymes involved in N mineralization were at their maximum during early to mid-season in the Duke Forest (Phillips et al., 2011; Meier et al., 2015). This has consequences for the seasonality of mineralization and immobilization: the growing season mineralization phase is characterized by high N mineralization activity, low microbial N uptake, and high N availability in the soil (Kaiser et al., 2011). By contrast, during the immobilization phase (non-growing season) gross N mineralization is low and is exceeded by microbial N uptake, which leads to high levels of N in the microbial biomass and low N availability in the soil. This immobilization phase can be interpreted as protection of the dissolved N from being lost from the soil as drainage when plant uptake is generally low. Here we show that this seasonality is not a direct result of differences in the amount of root exudation; it may be due to changes in the microbial community composition, belowground litter inputs and/or temperature as suggested by earlier studies (Bardgett et al., 2005; Yarwod et al., 2009; Kaiser et al., 2010).

### *4.3 Conclusion*

We conclude that while exudation may stimulate microbes to decompose fast-cycling N pools, microbial growth may be unaffected. Much of this N likely comes from the turnover of amino acids and amino sugars, with the latter mainly deriving from fungal necromass. This effect cannot be further increased by greater C exudation above a fertilization-specific maximum owing to stoichiometric constraints and the size of the microbial pool. Thus, plants that do not support a

large fungal biomass may be prone to experiencing nutrient constraints to plant productivity as nutrient demand increases, e.g., under elevated $CO_2$.

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**Figure captions**

**Fig. 1** Specific proteolytic enzyme activity per SIR microbial biomass C is increased with low and moderate exudate mimics additions with root simulators to intact forest soil in unfertilized loblolly pine (*Pinus taeda)* stands in summer 2009, but is unaffected in fertilized soil. Shown are **(a)** standardized effect sizes (Hedges' g) and **(b)** means and error bars (± 1 SE). Significant differences at $P \leq 0.05$ between exudate additions are indicated for the unfertilized FACE treatments by different upper case letters.

**Fig. 2** Specific *N*-acetyl-glucosaminidase (NAG) activity per SIR microbial biomass C is increased with low exudate mimics additions with root simulators to intact forest soil in unfertilized loblolly pine (*Pinus taeda)* stands in fall 2009, and with high exudate additions in fertilized soil. Shown are **(a)** standardized effect sizes (Hedges' g) and **(b)** means and error bars (± 1 SE). Significant differences at $P \leq 0.05$ between exudate additions are indicated for the unfertilized FACE treatments by different upper case letters and for the fertilized treatments by different lower case letters.

**Fig. 3** The response of hydrolytic enzyme N:C stoichiometry [(*N*-acetyl-glucosaminidase + leucine aminopeptidase) : β-1,4-glucosidase] to enzyme additions with root simulators to intact forest soil in loblolly pine (*Pinus taeda)* stands in the Duke Forest in fall, 2009. Shown are **(a)** standardized effect sizes (Hedges' g) and **(b)** means and error bars (± 1 SE). Significant differences at $P \leq 0.05$ between exudate additions are indicated for the fertilized FACE treatments by different lower case letters.

**Fig. 4** The response of **(a)** gross and **(b)** net N mineralization activity per microbial biomass C to enzyme additions with root simulators to intact forest soil in loblolly pine (*Pinus taeda)* stands in the Duke Forest in summer, 2010. Shown are **(a)** means and error bars (± 1 SE) for all enzymes

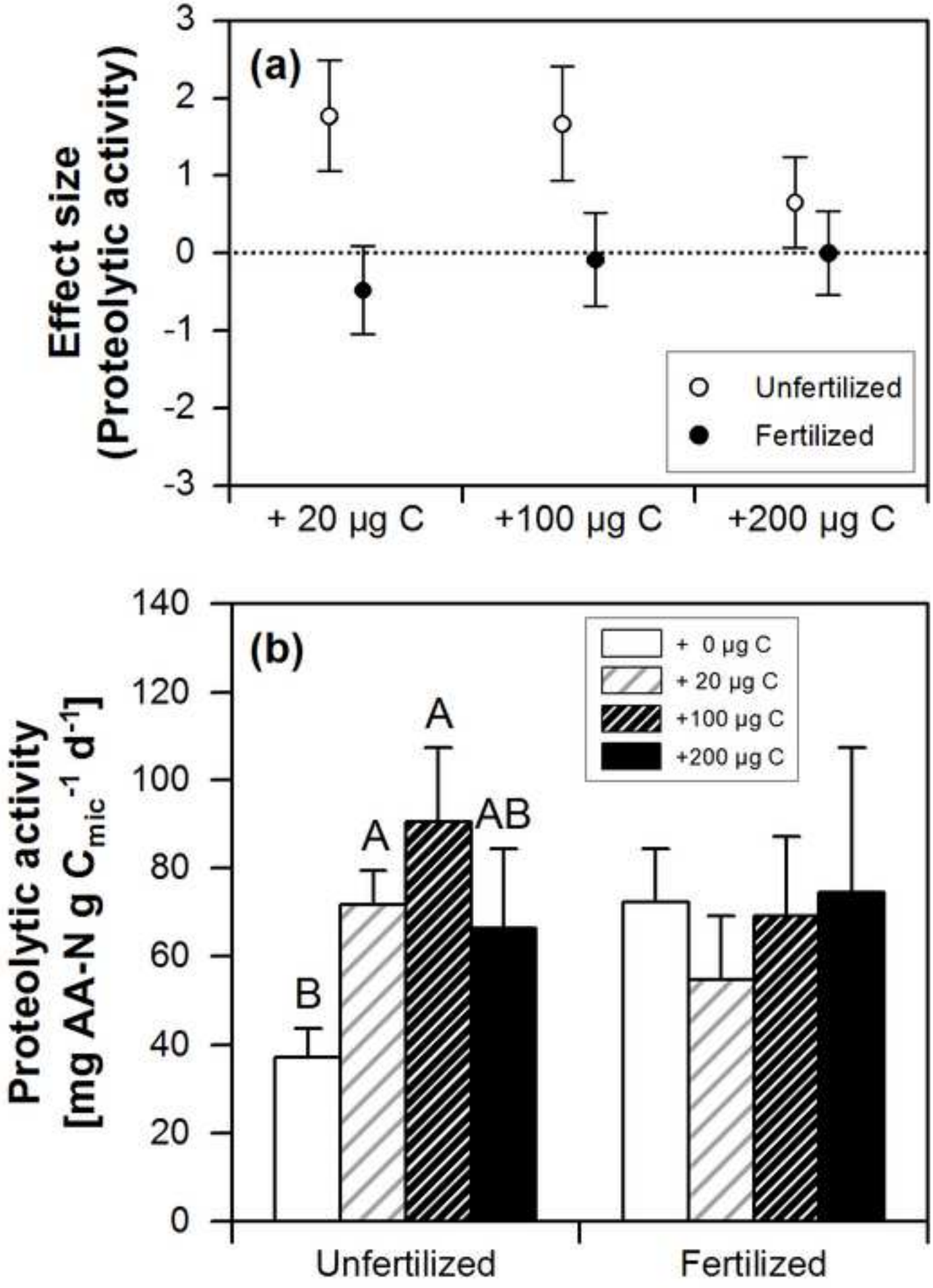
and **(b)** standardized effect sizes (Hedges' g) for the addition of *N*-acetyl-glucosaminidase (NAG) and β-1,4-glucosidase (Glu). Significant differences at $P \leq 0.05$ between exudate additions are indicated for the unfertilized FACE treatments by different upper case letters and for the fertilized treatments by different lower case letters.

**Fig. 5** Simulated response of **(a)** absolute gross N mineralization per soil mass and **(b)** specific gross N mineralization activity per microbial biomass C to exudate additions in loblolly pine (*Pinus taeda)* stands in the Duke Forest.

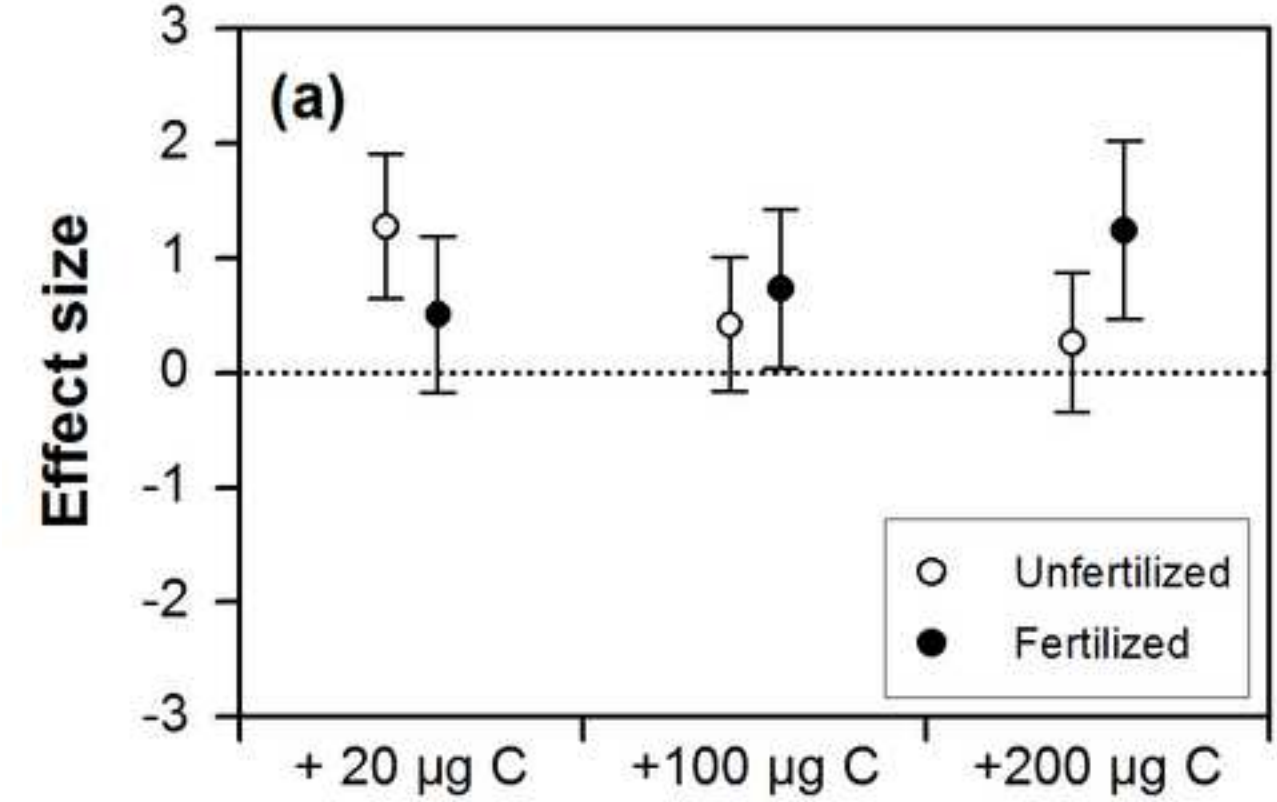
**Table 1** Parameter sensitivity analysis of modeled responses of microbial biomass, SON depolymerization, and gross N mineralization to daily exudate additions in unfertilized (C) and fertilized (F) loblolly pine (*Pinus taeda)* stands in the Duke Forest. Exudate mimics at 0, 20, 100, and 200 µg C $cm^{-2}$ $d^{-1}$ are indicated as no, low, moderate and high.

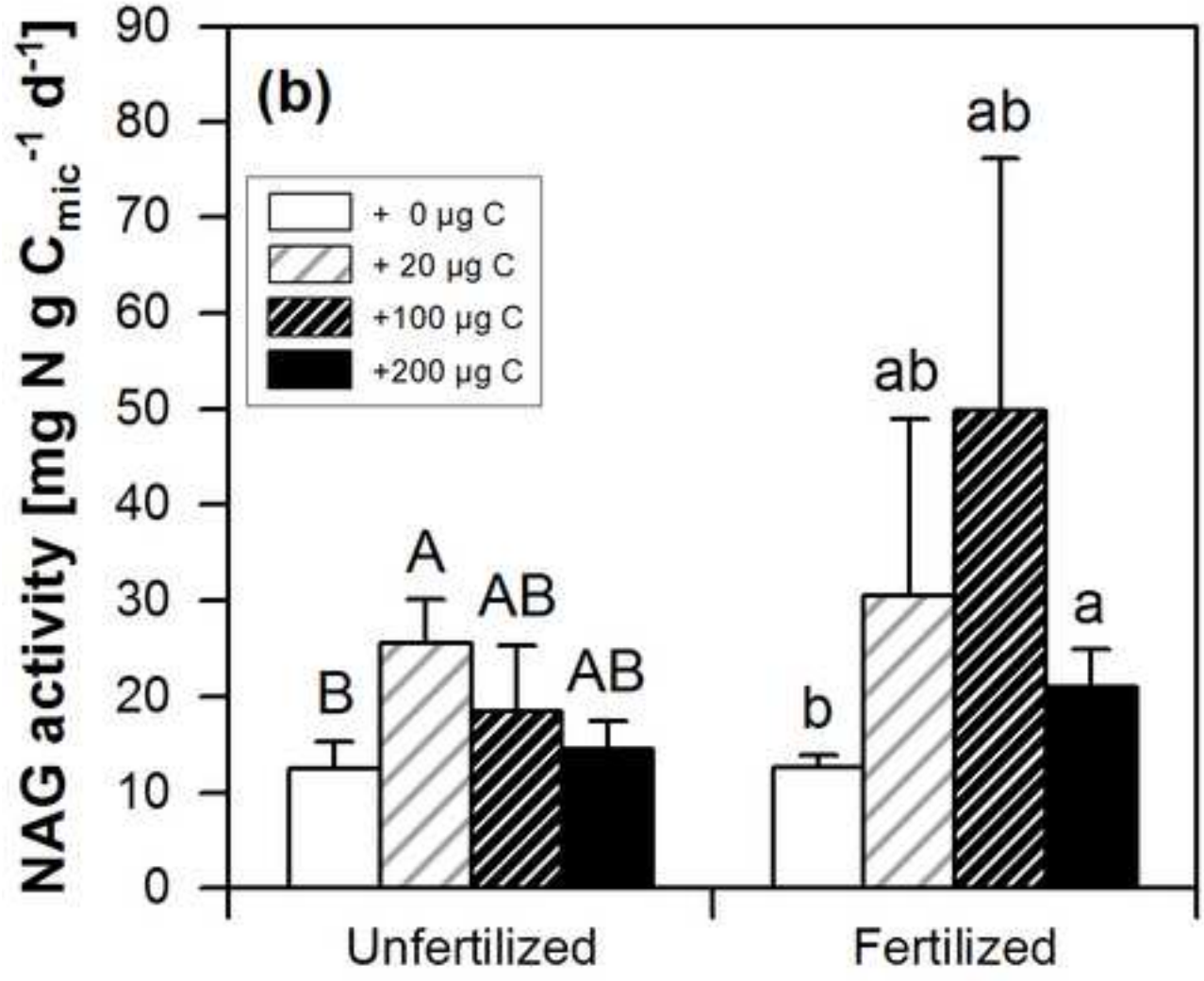
| | **Parameter sensitivity** | | | | | |
|---|---|---|---|---|---|---|
| **Change in exudate mimics addition from** | **No to low** | | **Low to moderate** | | **Moderate to high** | |
| **Fertilization treatment** | **C** | **F** | **C** | **F** | **C** | **F** |
| Microbial biomass C | 8.04 | 8.01 | 1.03 | 0.98 | 0.96 | 1.06 |
| SON depolymerization | 0.0187 | 0.0196 | 0.0013 | 0.0012 | 0.0004 | 0.0005 |
| Gross N mineralization | 6.75 | 6.56 | 1.03 | 0.98 | 0.96 | 1.07 |

Figure

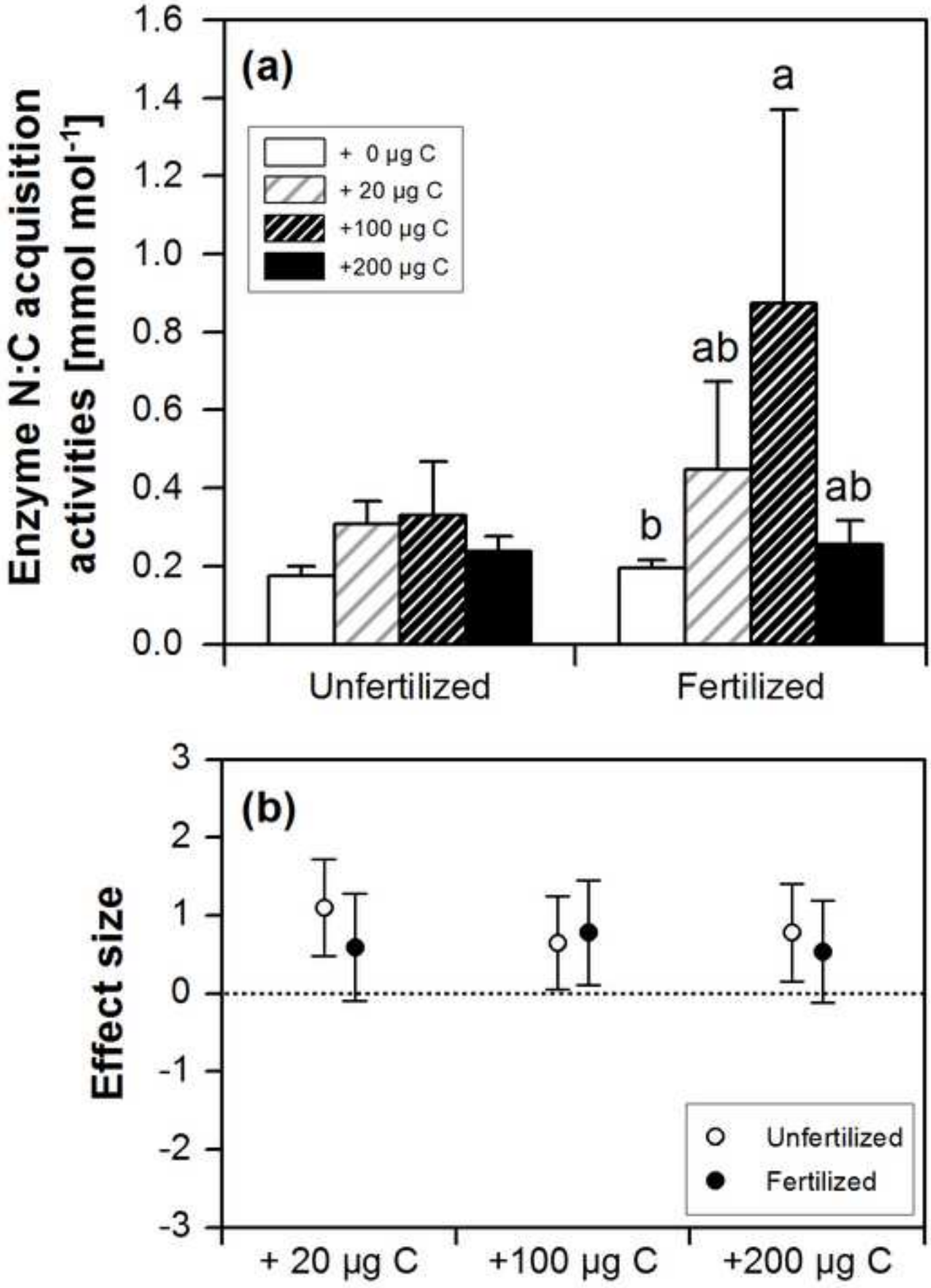

(a)
Effect size
(Proteolytic activity)
3
2
1
0
-1
-2
-3
Unfertilized
Fertilized
+ 20 µg C
+100 µg C
+200 µg C
(b)
Proteolytic activity
[mg AA-N g C$_{mic}^{-1}$ d$^{-1}$]
140
120
100
80
60
40
20
0
+ 0 µg C
+ 20 µg C
+100 µg C
+200 µg C
A
A
AB
B
Unfertilized
Fertilized

Figure

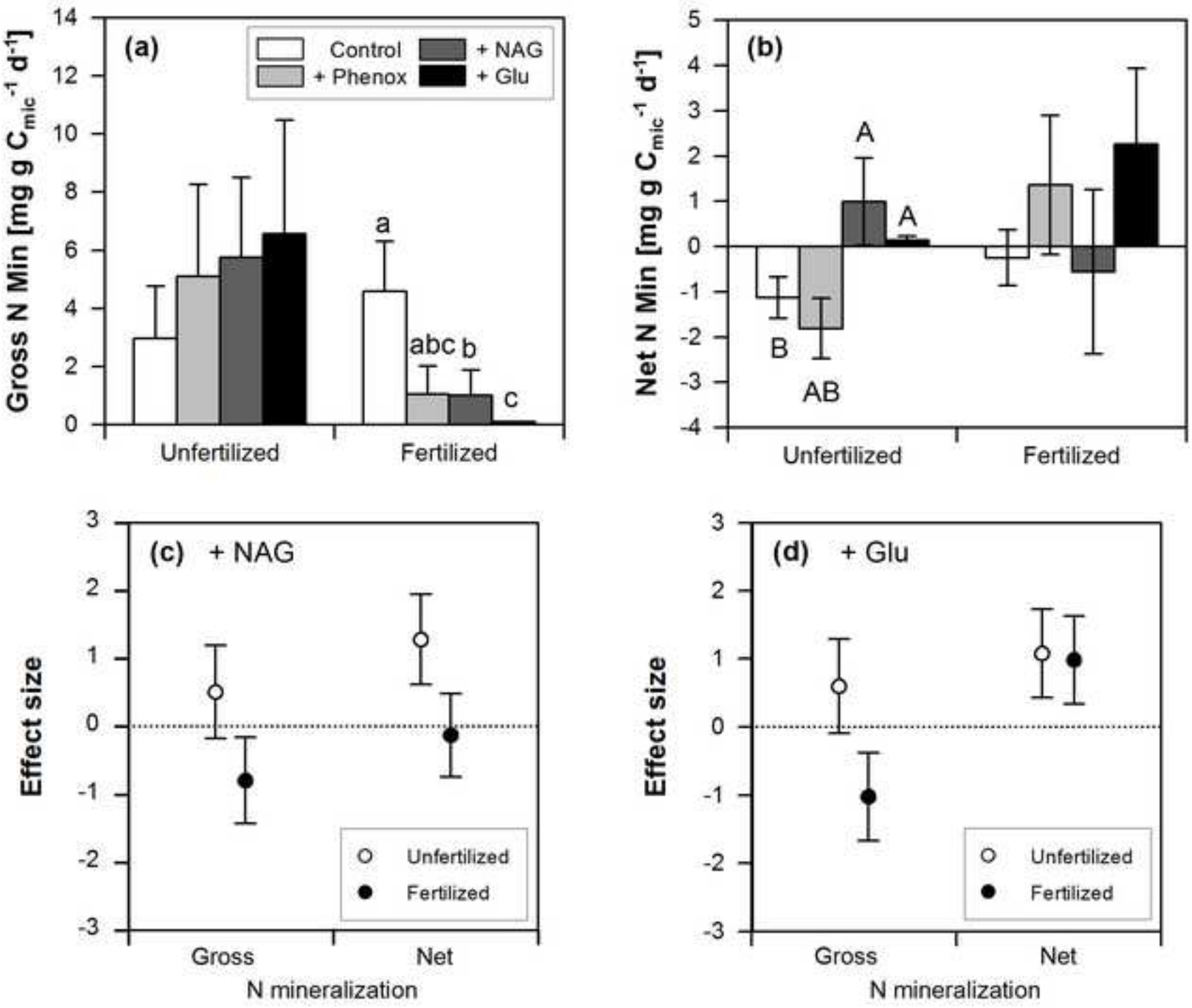

(a)
Effect size
3
2
1
0
-1
-2
-3
Unfertilized
Fertilized
+ 20 µg C
+100 µg C
+200 µg C


(b)
NAG activity [mg N g $C_{mic}^{-1}$ $d^{-1}$]
90
80
70
60
50
40
30
20
10
0
+ 0 µg C
+ 20 µg C
+100 µg C
+200 µg C
B
A
AB
AB
b
ab
ab
a
Unfertilized
Fertilized

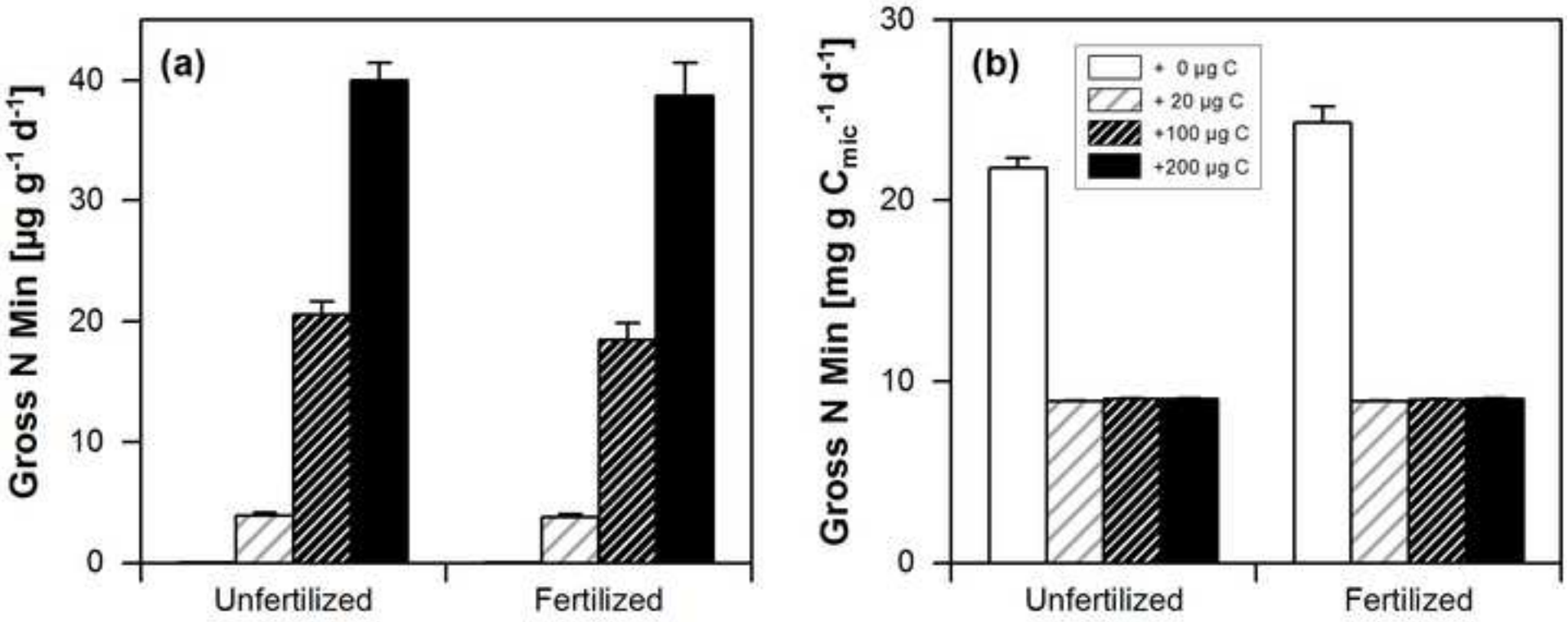
Figure


(a)
Enzyme N:C acquisition activities [mmol mol-1]
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
+ 0 µg C
+ 20 µg C
+100 µg C
+200 µg C
a
ab
ab
b
Unfertilized
Fertilized
(b)
Effect size
3
2
1
0
-1
-2
-3
Unfertilized
Fertilized
+ 20 µg C
+100 µg C
+200 µg C

Figure


(a)
Control
+ Phenox
+ NAG
+ Glu
Gross N Min [mg g C$_{mic}^{-1}$ d$^{-1}$]
a
abc
b
c
Unfertilized
Fertilized
(b)
Net N Min [mg g C$_{mic}^{-1}$ d$^{-1}$]
A
A
B
AB
Unfertilized
Fertilized
(c) + NAG
Effect size
Unfertilized
Fertilized
Gross
Net
N mineralization
(d) + Glu
Effect size
Unfertilized
Fertilized
Gross
Net
N mineralization

Figure


(a)
Gross N Min [µg g-1 d-1]
0
10
20
30
40
Unfertilized
Fertilized
(b)
Gross N Min [mg g Cmic-1 d-1]
0
10
20
30
+ 0 µg C
+ 20 µg C
+100 µg C
+200 µg C
Unfertilized
Fertilized