Microbial mechanisms of carbon and nitrogen acquisition in contrasting urban soils

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Abstract

Urban soils play an essential role in delivering ecosystem services due to soil microbial functions but there is limited evidence of the role of urban soils in the global carbon cycle. Inorganic nitrogen (N) reduces microbial respiration of soil organic matter (SOM) in pristine and managed forest soils but there is less evidence available on the extent to which this occurs in contrasting urban soils. This study examined the ephemeral effect of inorganic N and SOM (woodland versus grassland urban soil) on microbial functions represented by extracellular enzyme activities and microbial respiration of added substrates of contrasting quality. It was hypothesized that inorganic N stimulates extracellular enzyme activities and microbial respiratory responses to the addition of substrates varying in SOM quantity or quality. Results showed significantly higher SOM content, DOC and dissolved phenolic compounds in the woodland compared to grassland soil. In the woodland soil only, N addition increased β-glucosidase and *N*-acetyl-glucosaminidase enzyme activities and decreased microbial respiration responses to substrates. This suggests a microbial requirement for C acquisition dependent on N availability that reduced overflow respiration of the microbial community due to the composition of the woodland SOM pool. In conclusion, urban soils that contrast in vegetation types and hence OM content will likely differ mechanistically in response to increased N deposition and climate change altering their potential ability to store soil C in the future.

1. Introduction

In urban ecosystems, soils play an essential role in delivering ecosystem services via the soil microbial community. However, the microbial community is not generally taken into consideration in the sustainable development of urban ecosystems [1]. Urban land-use change has been identified as one of the major components of environmental change because of its effects on climate, water, biodiversity, carbon (C), and nutrients across large areas of the globe [2]. Despite the growing body of literature [3] many aspects of urban ecosystem services have not been studied conclusively and empirical evidence is still scarce [4].

There is considerable interest in understanding the biological processes that determine C storage in soils in order to better understand mechanisms to limit anthropogenic climate change [5]. Soil organic matter (SOM) provides C to a range of soil organisms, being utilised as the basis for a range of organic molecules and is essential for providing the energy at the base of food webs [6,7]. Decomposition of SOM and ultimately carbon dioxide (CO_2) release depend on the combined response of extracellular and intracellular (microbial), enzymatically mediated reactions [8]. Extracellular enzymes catalyze the initial hydrolysis of a variety of complex polysaccharides in soil to simple monomers that can be transported actively and passively into microbial cells and catabolized by intracellular enzymes producing CO_2 [8]. The quantity and quality of SOM is known to affect soil enzyme activities, microbial respiration, and microbial biomass, and these in turn will impact on soil C storage via greenhouse gas production [5,9,10]. Edmondson et al [11] have shown that organic C storage may be significantly greater in urban soil than in regional agricultural land at equivalent depths. However, the microbial mechanisms explaining C storage in contrasting urban soils are not fully understood.

76 The nitrogen (N) cycle has been perturbed since post-industrial times through
77 enhanced reactive N in the form of anthropogenic sources such as fossil fuel burning
78 and agricultural fertilizers with implications for microbial functions and C dynamics
79 within urban soils [12,13,14]. It has been argued that N deposition is a fundamental
80 driver of increased C sequestration in forest ecosystems, significantly affecting the C
81 balance of temperate and boreal forests [15]. Janssens et al. [16] proposed that the
82 mechanism for this increased C sequestration has occurred from N stimulating woody
83 biomass at the expense of below ground C allocation. As a result, two simultaneous
84 effects occur: (1) an increase of recalcitrant sources of C into forest soil ecosystems as
85 the additional woody biomass enters soil C pools as leaf litter and (2) decreasing
86 labile C through root exudation. This results in a decrease of microbial biomass and
87 respiration that increase soil C storage.
88 Kleber [17] and Schmidt et al [18] recently discussed in detail how the
89 molecular structure (recalcitrance) of SOM alone does not control SOM stability.
90 Labile organic carbon has been defined [19] as being both chemically degradable and
91 physically accessible by soil microbes whilst availability of SOC has been defined
92 [20] as the biochemical recalcitrance of organic compounds, that is, their
93 susceptibility to enzymic degradation with further uptake of reactive products by soil
94 microorganisms. Soil microbial communities require C and nutrients to synthesize
95 extracellular enzymes to breakdown recalcitrant SOM [21]. Therefore, nutrients such
96 as N can become a limiting factor for growth of microorganisms reliant on recalcitrant
97 sources of SOM [22]. However, the production of extracellular enzymes by microbes
98 represents an energetic cost [14,23] and enzymes will only be synthesized when
99 available nutrients in simple forms are scarce resulting in the utilisation of more
100 complex and stable forms [14]. In C rich soils, N availability can limit extracellular

101 enzyme synthesis that is required to breakdown complex C polymers to simple forms
102 that subsequently enhance microbial activity and growth [24]. However, N addition to
103 soil has been found to decrease microbial decomposition and respiration [25,26]
104 especially for the SOM pools that cycle slowly [27,28]. Spohn [29] suggested three
105 mechanisms to explain these changes: (i) microorganisms 'mine' litter for N, burning
106 readily available (or labile) C in order to gain energy to acquire N from more
107 recalcitrant forms of SOM containing a higher C/N ratio [29,30,31] – microbial C
108 limitation will be regulated by the return on investment in extracellular enzymes that
109 depends on the availability of N [14]; (ii) microorganisms uncouple respiration from
110 energy production and only respire easily available C to dispose of it via 'overflow
111 respiration' to maintain the stoichiometric ratio of C/N [32] – when growing on N-
112 poor substrate, microorganisms do not have enough N to build up as much biomass as
113 the C concentration would allow due to stoichiometry [29]; and (iii) the activity of
114 oxidative enzymes involved in the degradation of aromatic compounds decreases with
115 increasing N concentration [33] suggesting that lignin degradation is a mechanism of
116 N acquisition by mining. As urban environments are characterized by high levels of N
117 deposition [13], this raises the question of whether direct N deposition to soil (i.e.
118 mineral versus organic N), leading to decreased litter C/N ratios, might control C
119 storage by driving microbial mechanisms of C and N acquisition in specific urban
120 soils.

121 This study examined the ephemeral effect of inorganic N addition on
122 extracellular enzyme activities and microbial respiration by adding substrates of
123 varying quality in two urban soils (woodland and grassland) located in close
124 proximity and characterized by contrasting SOM pools (Table 1). Woodland soils
125 typically have sparse understorey vegetation and C accumulates from dead leaf litter

and detritus that are decomposed to produce humified recalcitrant SOM [34]. By contrast, grassland plant communities have been shown to positively affect the supply of root exudates suggesting rhizopheric microbes dominate with limited requirement for the production of C degrading enzymes [35]. Thus, woodland soils generally consist of higher amounts of SOM with recalcitrant C (i.e. aromatic phenolics) whilst grassland has a greater availability of faster cycling labile C reflecting differences in SOM quantity and quality. We compared the activities of the extracellular enzymes β-glucosidase, *N*-acetyl-glucosaminidase and phenol oxidase, as these enzymes are involved in the decomposition of cellulose (a major type of complex C compounds in soil), chitin (a significant fraction of humus-bound N in soil) and polyphenolic substances (slowly decomposing complex aromatic compounds). The substrates chosen for microbial respiration using the MicroResp™ method were carbohydrates/complex organic polymers (D-glucose, D-arabinose, D-galactose, fructose, D-trehalose, sucrose, cellulose, lignin) or amino/carboxylic acids (L-arginine, L-alanine, glycine, γ-aminobutyic acid, L-malic acid, citric acid) representing a range of important exudates and labile or recalcitrant organic substrates in soil to investigate the microbial respiration response and substrate preference by the microbial community. It was hypothesized that inorganic N would stimulate extracellular enzyme activities and microbial respiration of added labile C substrates but that this response would be dependent on soil characteristics of the grass and woodland soil (i.e. SOM content, phenolics).

151

152 **2. Materials and Methods**

153 *2.1. Site selection and soil sampling*

154 The study area, Stoke Park Estate, is an urban, public area, located in close
155 proximity to the M32 motorway in the north of Bristol, UK (Figure 1). Soil was
156 collected from a seasonally wet semi-natural grassland pasture (*Arrhenatherum*
157 *elatius*) measuring approximately 16 ha and ancient lowland mixed broadleaf
158 woodland (*Quercus robur, Fraxinus excelsior, Fagus sylvatica*, *Aesculus*
159 *hippocastanum*) measuring approximately 8 ha (UK grid reference: 51.494827, -
160 2.553233). The grassland area was likely originally part of the woodland in the past
161 as can be seen from the regular shape of the grassland. These two habitats were
162 chosen as they are located in close proximity that minimizes variability in
163 confounding factors such as weather and physicochemistry. Despite the differences in
164 aboveground vegetation and hence SOM that was of interest as a factor, the sites were
165 also chosen as the soil types are both defined as the Denchworth vegetation type
166 (712b) (Stagni-Vertic Cambisol under FAO classification). This soil type is defined as
167 slowly permeable, seasonally waterlogged, clayey soils with similar fine loamy over
168 clayey soils [36,37,38].

169 On the 6th of November 2014, surface leaf litter, detritus and/or grass
170 leaves/roots were removed from a representative area in each habitat measuring
171 approximately 10 m^2. Soil samples were pooled from 4 spatial replicates to 15 cm
172 below the O horizon. These sites were chosen due to the likelihood of differences in
173 SOM quantity (i.e. loss on ignition) and quality (i.e. phenolics, SOM / inorganic N)
174 appropriate for this laboratory study rather than as a comparison of habitat types.
175 Sixteen samples of 220 grams of soil (wet weight) were homogenised through a 1 mm

sieve and placed in 16 x 1 litre containers (8 x woodland and 8 x grassland soils). The soils were prepared within 24 h and incubated at field temperature (13 °C). Within each vegetation type, at week 1 and week 3, four randomly selected replicates were treated with 30 ml of deionised water and the remaining four treated with 30 ml of 0.125 M NH_4NO_3. This amount was chosen according to DEFRA fertilizer guideline application rates [39]. The applications of N were split in two applications to prevent osmotic shock and applied uniformly across the soil surface. The experimental design was fully factorial with SOM (site) x NH_4NO_3 treatment allowing for interaction effects.

Gravimetric water content was adjusted to 65% in all jars until week 4 when moisture was reduced to 44% for the MicroResp™ assay [40]. Soil moisture was determined every 72 h for moisture loss by weight and moisture was replaced by equivalent amounts of deionised water. Jars were open to prevent CO_2 build up and incubated for five weeks in total. Soil moisture content was determined by drying in an oven at 105 °C for 24 h. SOM was determined by loss on ignition in a furnace at 500 °C for 24 h. The following soil physicochemical characteristics were determined using soil extracts prepared by dissolving 2 g of wet soil in 50 ml deionised water. Relative soil pH was measured using a standard electrode. A proxy for aromatic dissolved organic carbon (DOC) was determined by centrifuging a sample at 12,000 rpm for 10 minutes and measuring UV absorbance at 254 nm [41] and also dissolved phenolics by colorimetric analysis at 750 nm following reaction with Folin-Ciocalteau reagent [42]. Nitrate and ammonium were determined using 1 g wet soil in 50 ml of 2 M KCL solution using the methods of Griffin et al 1995 [43].

2.2. Extracellular enzyme activities

201 Hydrolytic enzyme activities were determined by the method of DeForest
202 [44]. Fluorogenic methylumbelliferyl (MUF) substrates: MUF-glucopyranoside and
203 MUF-N-acetylglucosaminide are substrates for the determination of β-glucosidase
204 and *N*-acetyl-glucosaminidase enzyme activities respectively. MUF substrates (1 mM
205 – saturating concentration) and MUF standard (100 µM) were pre-dissolved for 24 h
206 in 1ml ethylene glycol monomethyl ether. A buffer of sodium acetate trihydrate/acetic
207 acid was prepared to maintain a pH of 5.5 in the soil suspensions. 1.39 g of soil (dry
208 weight) from each experimental replicate were placed in 50 ml of buffer and 150 µl of
209 each replicate substrate pipetted into a 300 µl well. 100 µl of substrate were added in
210 a specific order on a black 96 well plate with blanks, controls and quench standards.
211 The plates were incubated for one hour at 13 °C and 50 µl of NaOH added to
212 terminate the reactions and maximise fluorescence [44]. As substrate fluorescence can
213 diverge with NaOH and time, plates were read immediately. The plates were read at
214 365 nm excitation and 450 nm emission wavelengths on a SpectraMax M2 Micro-
215 mode Microplate reader (Molecular Devices). The calculations of DeForest [44] were
216 used to calculate enzyme activity as nmol MUF g^{-1} dry weight soil h^{-1}.
217 Phenol oxidase enzyme activities were determined by adding 0.75 ml of a 10
218 mM solution of L-3,4-dihydroxyphenylalanine (L-DOPA) to 0.75 ml of the 1.39 g
219 dry weight per 50 ml buffered soil extract in 1.5 ml centrifuge tubes [45]. Deionised
220 water was used in place of the L-DOPA solution as a blank. Final activity was
221 determined after 1 h incubation at 460 nm on a SpectraMax M2 Micro-mode
222 Microplate reader (Molecular Devices). Phenol oxidase activity (minus the blank) was
223 determined as nmol dicq g^{-1} dry weight soil h^{-1}.
224
225 *2.3. Microbial respiration*

226 The MicroResp™ method as outlined by Campbell et al [40] was used to
227 assess respiration of added C substrates. One week prior to the assay, soils were dried
228 to 44% moisture. Deep well plates were filled with 6.9 g per 16 wells to an equal level
229 to give approximately 0.3 g of soil dry weight per well. Soil was collected from each
230 soil container using a corer as to take a representative sample over the depth of each
231 jar (10 cm). The deep well plates were incubated for 24 h to minimise disturbance
232 effects. Detection plates were prepared by pipetting 150 µl of 1% agar and cresol red
233 indicator solution at a ratio of 1:2 into clear 96 well plates. Plates were placed in a
234 dark desiccator at room temperature (20 °C) and equilibrated for 48 h. Plates were
235 read at 570 nm prior to the assay. Fifteen different substrates were pipetted in 25 µl
236 aliquots delivering 30 mg of substrate per gram soil water to the 96 deep well plates.
237 The substrates were carbohydrates/complex organic polymers (D-glucose, D-
238 arabinose, D-galactose, fructose, D-trehalose, sucrose, cellulose, lignin) or
239 amino/carboxylic acids (L-arginine, L-alanine, glycine, γ-aminobutyic acid, L-malic
240 acid, citric acid) representing a range of important labile and recalcitrant organic
241 substrates. After the addition of substrates, detection plates and deep well plates were
242 clamped together with an airtight seal and incubated at 13 °C for 6 h. Detection plates
243 were then removed and read at 570 nm for colour change on a SpectraMax M2 Micro-
244 mode Microplate reader (Molecular Devices). Measured values were then converted
245 to % CO_2 by curve fitting experimental data relating absorbance to % CO_2 where %
246 CO_2 = -0.2265 – 1.606 / (1 – 6.771 abs). The data were converted to a flux of µg CO_2
247 g^{-1} h^{-1} and normalized as outlined by Campbell et al. [40]. Shannon's diversity index
248 ($H' = -\Sigma p_i \log 2\ p_i$) was computed as a measure of functional diversity [46] where p_i is
249 the ratio of respiration rate of each single C-substrate specific substrate to the sum of
250 all SIR.

2.4. Statistical analyses

IBM SPSS® Statistics (Version 23) and Minitab (Minitab ® 17.2.1) software were used for all statistical analyses. Data were tested for normality using the Anderson-Darling test and equality of variance using an *F* test. Two-way ANOVA using the General Linear model with interaction terms were used to determine differences between Site (woodland versus grassland) and N addition (control versus NH_4NO_3) treatments in soil physicochemistry, enzymes and MicroResp™ data. Community Level Physiological Profiles (CLPPs) were compared using MANOVA with all substrates, carbohydrates/complex organic polymers or amino/carboxylic acids. Post-hoc pairwise comparisons were used to determine specific differences between treatments using Bonferroni tests.

3. Results

The woodland soil was significantly higher in SOM ($P < 0.001$ – 24 vs 17%), showing higher UV absorbance at 254 nm as a proxy for aromatic DOC ($P< 0.001$ – 0.29 vs 0.03 abs units) and dissolved phenolic compounds ($P < 0.01$ – 9.3 vs 5.1 μg L^{-1}) compared to the grassland soil (Table 1). There were no significant differences in nitrate, ammonium, pH or gravimetric moisture content between the incubated grass and woodland soils (Table 1). β-glucosidase activity was significantly higher in the woodland soil ($P < 0.001$) and enhanced by inorganic N in the woodland soil only (Bonferroni $P < 0.01$) (Fig 2). Site and N had a significant interactive effect on *N*-acetyl-glucosaminidase activity ($P < 0.01$) (Fig. 2). Nitrogen addition significantly reduced the SOC / inorganic N ratio ($P < 0.001$) in both woodland (2.93 to 0.30) and

grassland (4.80 to 0.25) but there were no significant effects of site or N on phenol oxidase activity.

MANOVA showed distinctions in respiration when categorised into carbohydrates/complex organic polymers or amino acids/carboxylic acids. The respiratory response to carbohydrates showed significant main effects of Site and N ($P < 0.001$ and $P < 0.01$; Fig. 3) except for an insignificant effect of N on respiration of sucrose. Bonferroni post-hoc results for each carbohydrate showed distinct univariate effects of the treatments on specific respiration - the interactive effect of Site and N on D-arabinose ($P < 0.001$) and lignin ($P < 0.05$) (Fig. 4). MANOVA for the respiratory response to amino acids/carboxylic acids showed a significant effect of Site ($P < 0.01$). As with the carbohydrates, this multivariate main effect was due to distinct univariate effects of Site and N on the amino acids/carboxylic acids. Bonferroni post-hoc results for each carbohydrate are shown in Fig. 5. Overall, these results suggest an inhibitive effect of inorganic N on respiration of substrates in the woodland soil but not the grassland soil (except for fructose and Y-aminobutyric acid). There were no significant differences in microbial respiration patterns using MANOVA between woodland and grassland soils using the 13 substrates and no significant difference between treatments in functional diversity represented by the Shannon diversity index.

4. Discussion

This study suggests that rhizospheric soil systems such as grasslands may show a distinct response in microbial mechanisms of C and N acquisition relative to more complex SOM systems such as woodland soils that have higher detrital inputs from leaf fall. Results from natural and agricultural systems have reported increased

301 enzyme activities upon the addition of N [31,47] and this effect may depend on SOM
302 quantity and/or quality (e.g. C/N ratio, phenolics). The increase in hydrolase activities
303 likely occurred as the addition of N induced the microbial production of enzymes as
304 'cost effective' in terms of return in C and nutrients [23]. This was to be expected as
305 microbes use labile sources of C and shift to complex or stable forms that require
306 catabolism by extracellular enzymes [48]. While the rate at which SOM is processed
307 is strongly controlled by the quality of the material, the extent of C limitation to the
308 microorganisms is controlled by the dynamics of exoenzymes [22], as opposed to
309 SOM quality [49] or soil microbial dynamics [50]. Allison and Vitousek [14] showed
310 that the addition of simple nutrients on their own does not necessarily affect soil
311 enzyme activities, attributed to the prerequisite of C and N for microbial enzyme
312 synthesis. The significant increase in activities in only the woodland soil suggests
313 limited accessibility to simple C (i.e. higher root exudation of labile C in grassland or
314 higher phenolic concentration in woodland), hence the microbial synthesis of
315 enzymes in response to N availability. β-glucosidase and *N*-acetyl-glucosaminidase
316 catalyse the hydrolytic release of glucose from the most abundant soil
317 polysaccharides, cellulose and chitin, so activities are closely related to soil C content
318 [51] and are induced by the availability of their substrate in excess [14]. However,
319 despite the significant difference in SOM quantity between woodland and grassland,
320 which is known to influence soil microbial functioning [7], the levels of SOM were
321 both elevated given the ecosystem types (24% and 17% respectively). Therefore, the
322 driver for enzyme production in this case was most likely SOM quality (i.e.
323 phenolics) or limited root exudation although we acknowledge that this requires
324 further investigation.

Phenol oxidase is a key enzyme in the degradation of lignin [52]. Lignin has been shown to be highly stable and a complex substrate to degrade [53]. Therefore, less complex or lower molecular weight OM was primarily decomposed by hydrolase enzymes despite potentially higher phenolic content. We only examined a single time event and so it is likely that a reduction in phenol oxidase activity by N addition may occur in later stages as the microbial community shifts from labile to recalcitrant decomposition and microbial biomass increases from total enzyme investment. Both cellulolytic [54] and lignolytic microbes [55] induce exoenzyme production under conditions of C limitation although we suggest that the woodland was also N limited as described by the model of Schimel and Weintraub [22]. Phenol oxidase can be down-regulated upon additions of low molecular weight N [33,56,57]. This is likely to arise from inefficient C use and slow growth of lignolitic fungi that results from competition with species that can thrive in abundant N supply [58]. These results for this woodland urban soil are consistent with others [33,57] that show that inorganic N accelerates the extracellular decomposition of cellulose and chitin but has no effect on decomposition of lignin and polyphenolics.

The negative effect of inorganic N on microbial respiration and the positive effect of N on extracellular enzyme activities supports model [19] and experimental results [14,29,30,47,56,59]. For example, Dalmonech et al. [47] found that after 55 days of incubation a woodland soil treated with 75 kg N ha^{-1} application of NH_4NO_3 had increased enzyme activities and microbial biomass but suppressed microbial respiration. Given that we observed no change in phenol oxidase, overflow respiration may be the most parsimonious explanation. Meyer et al [59] found evidence suggesting that “stoichiometric decomposition” and “microbial N mining” do not mutually exclude each other but operate at different temporal scales. In addition,

Meyer et al [59] reported that SOC-derived CO_2 was higher in unfertilized than in fertilized treatments that supports Qiao et al [60] that high N supply suppresses the priming effect. Both availability and lability of SOC may therefore explain the contrast in enzyme activities between the grassland and woodland soils. Limited access to C in the woodland compared to grassland soil may occur due to physical protection of SOM, complexation with soil minerals, chemical recalcitrance (i.e. phenolics) and/or low availability of low molecular weight C (i.e. rhizodeposition) [17,18]. The insignificant investment in lignin degrading enzymes is supportive of physicochemical mechanisms of SOM stabilisation over different rates of turnover of SOM pools [61]. These results suggest ephemeral investment in hydrolase enzymes due to N addition was to increase the availability of C as a microbial energy source and that this C would have reduced overflow respiration.

Conclusion

These results support observations from natural and agricultural systems that inorganic N addition may stimulate an increase in extracellular enzyme activities for C acquisition resulting in reduced overflow respiration of the microbial community due to the physicochemical nature of the SOM pool. Further long-term research is required to confirm linkages between vegetation types, rhizodeposition rates, SOM inputs and chemistry effects on microbial functioning in urban soils. In particular, there is a need to determine how priming effects via plant exudation and shifts in the microbial community structure and function may regulate the decomposition of SOM in contrasting urban habitats exposed to elevated N deposition. In conclusion, urban soils that contrast in vegetation types and hence OM content will likely differ

mechanistically in response to increased N deposition and climate change altering their potential ability to store soil C in the future.

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