Addition of residues with different C/N ratio in soil over time individually or as mixes - effect on nutrient availability and microbial biomass depends on amendment rate and frequency

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Abstract

Residues with different properties may be added to soil simultaneously (as mixes) or after each other. In most previous studies on the effect of mixes on decomposition and nutrient release, residues were added once at the start of the experiment. Less is known about the effect of mixtures added more than once on soil respiration, microbial biomass and nutrient availability and how the addition frequency influences interactions between high C/N (H) and low C/N (L) residues in mixes. In the 48-day incubation experiment with total amendment rate in all treatments was 20 g kg⁻¹, treatments differed in addition frequency (twice, four or eight times), rate (10, 5 or 2.5 g kg⁻¹) as well as order of H and L. Soil was sampled every 12 days. Treatments had similar total cumulative respiration, but differed in distribution over the 12 day periods. Available N and MBN changed strongly over time in treatments with 10 or 5 g kg⁻¹, depending on the C/N ratio of the residue added before sampling. Frequent addition of small amounts of residues (2.5 g kg⁻¹) limited microbial N uptake initially compared to higher amendment rates, but resulted in similar available N irrespective of the C/N ratio of the residue added. It can be concluded that with less frequent residue addition, microbes decompose mainly the recently added residue. With more frequent addition, microbes decompose recently added residue together with residue added before the most recent amendment.

Keywords: Amendment, addition frequency, C/N ratio; MBN, microbial biomass; mixes, N availability.

1. Introduction

It is well-known that composition of organic amendments determines decomposition rate and nutrient availability (Tian *et al.*, 1992; Bending and

Turner, 1999). One of the most important properties is the N concentration of the organic materials, commonly expressed as C/N ratio. Addition

of organic materials with high N concentration (C/N < 20) results in net N mineralization (Hadas *et al.*, 2004) whereas amendments with low N concentration (high C/N) induce net N immobilization (Moritsuka *et al.*, 2004). However, other residue properties such as organic C composition also influence decomposition rate and nutrient release (Carteri *et al.*, 2018). Nevertheless, the C/N ratio is considered as a good indicator of N availability and immobilization.

Plant residues differing in C/N ratio can be added to soil one after another, e.g. in intercropping. In previous studies, we found that nutrient availability after the second residue addition is influenced by the C/N ratio of the first and the second residue amendment, termed legacy effect (Marschner *et al.*, 2015). For example, N availability was higher after high C/N residue amendment when it followed low C/N residue than if high C/N residue was added to unamended soil. The extent of the legacy effect depended on the amount of the first residue left in the soil when the second residue is added (Zheng and Marschner, 2017). In another study, repeated slurry application compared to a single addition increased soil respiration and the proportion of CO₂ derived from slurry, but reduced the proportion of CO₂ from SOC (Cavalli *et al.*, 2014). Bonanomi *et al.* (2017) reported that repeated applications of organic materials increased inhibition of germination and growth of soil-borne fungi.

Residues differing in C/N ratio can not only be added sequentially, but also together. The effect of simultaneous addition of plant residues of different composition has been studied extensively (Gartner and Cardon, 2004; Xiang and Bauhus, 2007; Cobo *et al.*, 2008). In such residue mixes, expected decomposition or nutrient availability can be calculated based on decomposition/nutrient availability with each residue separately and the ratio of residues in the mixes. For example, in a 360-day study where a 1:1 mix of *Hedera helix* leaf litter with low C/N ratio (21) with

cellulose strips that had high C/N (440), measured decomposition in the 1:1 mix was higher than expected due to N transfer from low C/N litter to high C/N cellulose (Bonanomi *et al.*, 2014). In another study, low C/N litter (20) and high C/N litter (50-80) were incubated singly or as 1:1 mixes in litter bags which were placed in soil for two or nine months (Cuchietti *et al.*, 2014). Measured decomposition in mixes was usually higher than expected because high C/N litters appeared to increase decomposition rate of low C/N litters. In a shorter term study, with litters differing in C/N ratio (high in poplar leaves and low in soybean leaves) mixed at a 1:1 ratio, mass loss and N release after 84 days were higher than expected in the mix (Mao and Zeng, 2012).

In previous studies on mixing litters with different C/N ratio, the litters were added only once at the start of the experiment. However, litter mixes may also be added to soil repeatedly, e.g. through the action of ants or earthworms. It is unclear if the effect of residue mixture on soil respiration, microbial biomass and nutrient availability is influenced by addition frequency and how the addition frequency influences interactions between high C/N and low C/N residues in mixes. In the present study, soil was amended with high and low C/N residues or their 1:1 mixture to a total amendment rate of 20 g kg⁻¹ over a period of 42 days. Residues were added twice (day 0, 24) at 10 g kg⁻¹, four times (day 0, 12, 24, 36) at 5 g kg⁻¹ or eight times (day 0, 6, 12, 18, 24, 30, 36, 42) at 2.5 g kg⁻¹.

We tested the following hypotheses which are about available N and MBN because they responded most to C/N ratio of amendment in our previous studies.

(i) At a given sampling time, available N and MBN will be less influenced by the C/N ratio of the residue added last before sampling with frequent addition of low C/N (L) and high C/N (H) residue at low rate (four or eight times) than less frequent amend-

ment (two times) at high rate. This hypothesis assumes that with frequent addition of L and H at low rate, changes in available N and MBN after a given amendment will be small due to the small amount of residue added. Further, due to the short time between residue additions, the soil contains previously added residues that are only partially decomposed. With less frequent amendment, previously added residues are largely decomposed by the time the next residue is added and microbes are likely to nutrient-starved. This will lead to rapid decomposition of the freshly added amendment.

(ii) In 1:1 mixes of H and L, available N and MBN will initially increase with amendment rate, but differences between rates will become smaller over time as lower rates are added more frequently leads to a build-up of the amount of residue in the soil during incubation.

(iii) In the mixes, differences between measured and expected values, if any, will be greater when residues are added at high rates twice than if they are added at lower rates more frequently. When residues are added at high rate, but less frequent, L and H will decompose separately whereas if they are added as mix, they will be decomposed together. With frequent amendment at low rate, the small amendments will induce small changes in measured properties and due to the short interval between additions, both H and L will be present in the soil even when added sequentially. This will occur earlier the more frequent the residues are added.

2. Materials and Methods

2.1. Soil and plant residues

The sandy clay loam used in this study was collected from 0 to 10 cm at Waite Campus, The University of Adelaide (Longitude 138°38'3.2" E, Latitude

34°58'0.2"S). The area is in a semi-arid region and has a Mediterranean climate with cool, wet winters, and hot and dry summers. The soil is a Red-brown Earth in Australian soil classification and a Rhodoxeralf according to US Soil Taxonomy. The soil has been managed as permanent pasture for over 80 years. The soil was collected from several randomly selected sites on the plot. After removal of plants and surface litter, five samples of topsoil were collected at each sampling site. The soil was then air dried at 40 °C in a fan-forced oven. During summer, top soil in this area are often reach temperatures of 40-50 °C. After air-drying, visible plant debris was removed and the soil sieved to < 2 mm. Soil from all sampling sites was pooled and thoroughly mixed before subsamples were taken for the experiment. It has the following properties (for methods see section 2.3 below): sand 54%, silt 20% and clay 25%, pH (1:5 soil:water) 6.3, electrical conductivity (EC 1:5 soil:water) 143 $\mu\text{S cm}^{-1}$, total N 1.5 g kg^{-1} and total P 371 mg kg^{-1} , total organic carbon (TOC) 17 g kg^{-1} , available N 15 mg kg^{-1} , available P 10 mg kg^{-1} , maximum water holding capacity (WHC) 378 g kg^{-1} and bulk density 1.3 g cm^{-3} .

Two types of plant residues were used: young faba bean (*Vicia faba* L., referred to as L) as low C/N residue, and mature wheat straw (*Triticum aestivum* L., referred to as H) as high C/N residue (Table 1). Legumes and cereals are often grown together in intercropping systems. The residues were dried at 40 °C in a fan-forced oven, finely ground and sieved to particle size 0.25-2 mm. Total N and total P were 4-8 times higher in low C/N ratio residue (young faba bean) than in high C/N ratio residue (wheat straw). Therefore, low C/N residue had significantly lower C/N ratio and C/P ratio than high C/N residue. Total organic C was about 10% higher, but water extractable organic C was about 60% lower in high C/N residue than in low C/N residue.

Table 1. Total organic C, N, P, C/N ratio and C/P ratio, available N and P, water-extractable C, and pH of low C/N (young faba bean shoot) and high C/N (mature wheat straw) residues (n = 4). Different letters indicate significant differences between residues (P < 0.05).

	High C/N	Low C/N
Total organic C (g kg ⁻¹)	376.3 ^b	346.8 ^a
Total N (g kg ⁻¹)	4.6 ^a	38.5 ^b
Total P (g kg ⁻¹)	2.1 ^a	9.2 ^b
WEOC (g kg ⁻¹)	23.9 ^a	37.8 ^b
C/N ratio	82 ^b	9 ^a
C/P ratio	176 ^b	38 ^a
pH (1:10)	5.6 ^a	6.0 ^b

2.2. Experimental design

Before the start of the experiment, the air-dried soil was incubated for 10 days at 50% of maximum WHC at 20–25 °C in the dark to activate the soil microbes and to stabilise soil respiration after rewetting of air-dry soil. This water content was chosen because in previous studies with this soil, microbial activity was maximal at 50% WHC (Marschner *et al.*, 2015).

There were nine treatments (Table 2) differing in number of amendments (two, four or eight) and order in which the residues (L and H) were added. In treatments with two amendments, residues were added on day 0 and day 24 at 10 g kg⁻¹, either L followed by H (10-L-H), H followed by L (10-H-L) or a 1:1 mixture of L and H added twice [10-(HL)x2]. In treatments with four amendments, residues were added on day 0, 12, 24 and 36 at 5 g kg⁻¹ in the following order: first H, then L, then H, then L [5-(H-L)x2]; first L, then H, then L, then H [5-(L-H)x2]; or four times a 1:1 mixture of L and H [5-(HL)x4]. In treatments with eight amendments, residues were added on day 0, 6, 12, 18, 24, 30, 36

and 42 at 2.5 g kg⁻¹, either four times of H then L [2.5-(H-L)x4], four times of L then H [2.5-(L-H)x4] or eight times a 1:1 mixture of H and L [2.5-(HL)x8]. The treatments were designed so that (i) all treatments received both H and L, but at different rate and in different order, (ii) by day 24 and 48 the same amount of residue had been added (10 and 20 g kg⁻¹, respectively), and (iii) by day 48 all treatments had received the same amount of H and L residue. The terms 10-treatments, 5-treatments or 2.5-treatments refer to all treatments with residues added at 10, 5 or 2.5 g kg⁻¹, respectively. An unamended control was not included because the aim of the experiment was to compare different types of amendment, not the effect of amendment. The residue amendment rates were high compared to average expected amounts in the field. However, such high residue amounts are possible in the field, e.g. in windrows left by the harvester or in home gardens. The high residue mixing frequency (every eight days) may not be realistic in agriculture, but could occur in home gardens or through action of earthworms or ants.

Table 2. Experimental design with treatment names and corresponding details about residue type (high or low C/N residue (H or L) or their 1:1 mixture (HL), addition rate (10, 5 or 2.5 g kg⁻¹ soil) and amendment dates

Treatment name	Residue types and rate (g kg ⁻¹ soil) and added on day							
	0	6	12	18	24	30	36	42
10-H-L	H-10				L-10			
10-L-H	L-10				H-10			
10-(HL)x2	HL-10				HL-10			
5-(H-L)x2	H-5		L-5		H-5		L-5	
5-(L-H)x2	L-5		H-5		L-5		H-5	
5-(HL)x4	HL-5		HL-5		HL-5		HL-5	
2.5-(H-L)x4	H-2.5	L-2.5	H-2.5	L-2.5	H-2.5	L-2.5	H-2.5	L-2.5
2.5-(L-H)x4	L-2.5	H-2.5	L-2.5	H-2.5	L-2.5	H-2.5	L-2.5	H-2.5
2.5-(HL)x8	HL-2.5	HL-2.5	HL-2.5	HL-2.5	HL-2.5	HL-2.5	HL-2.5	HL-2.5

At each residue addition, residues were thoroughly mixed in 30 g soil (dry weight equivalent) in a small plastic bag. Then the amended soil was filled into PVC cores with 3.7 cm diameter, 5 cm height and a nylon net base (7.5 μ m, Australian Filter Specialist) and packed to a bulk density of 1.3 g cm⁻³ by adjusting the height of the soil in the cores. In 10-treatments on days 6, 12, 18, 30, 36 and 42 and in 5-treatments on days 6, 18, 30 and 42, soils were not amended but mixed similarly as the 2.5-treatments. The cores were placed individually into 1 L jars with gas-tight lids equipped with septa to allow quantification of head-space CO₂ concentration as described below. The jars were incubated in the dark at 20-24 °C. Soil moisture was maintained at 50% of WHC by checking the water content every few days by weighing the cores and adding reverse osmosis (RO) water if necessary. Cores were destructively sampled on days 12, 24, 36

and 48 for analysis of available N and P, and microbial biomass N and P.

In a given 12-day period, only the cores to be sampled at the end of the period were placed in the jars. The remaining cores were incubated under the same conditions in large plastic trays covered with aluminium foil. After removal of the cores from the jars for analysis, the cores to be harvested at the next sampling time were placed in the glass jars for respiration measurement.

Soil analyses were carried out as described in Marschner *et al.* (2015). Briefly, soil texture was determined by the hydrometer method (Gee and Or, 2002). Soil pH was determined in a 1:5 soil:water extract after 1 h end-over-end shaking at 25 °C (Rayment and Higginson, 1992). Soil water holding capacity was determined using a sintered glass funnel connected to a 1 m water column (matric potential =

-10 kPa) (Wilke, 2005). Total organic carbon content of soil and residues was determined by wet oxidation and titration (Walkley and Black, 1934). To determine total N and P in soil and residues, the material was digested with H_2SO_4 and a mixture of HNO_3 and HClO_4 , respectively. Total N was measured by a modified Kjeldahl method (Bremner and Mulvaney, 1982). Total P in the digest was measured by the phosphomolybdate method (Hanson 1950). Available N (ammonium and nitrate) concentration was measured after 1 h end-over-end shaking with 2 M KCl at 1:10 soil extractant ratio. Ammonium-N was determined after Forster (1995). Nitrate-N was determined using a modification of Miranda *et al.* (2001). Available P was extracted by the anion exchange resin method (Kouno *et al.*, 1995) and the P concentration was determined colorimetrically (Murphy and Riley, 1962). Microbial biomass N was determined by chloroform fumigation-extraction with 0.5 M K_2SO_4 at 1:4 soil to extractant ratio (Moore *et al.*, 2000). Microbial biomass N was calculated as the difference in NH_4^+ concentration between fumigated and non-fumigated samples divided by 0.57 which is the proportionality factor to convert ammonium to MBN (Moore *et al.*, 2000). Microbial biomass P was determined with the anion exchange method (Kouno *et al.*, 1995) using hexanol as fumigant. Microbial biomass P is the difference in P concentration between fumigated and un-fumigated soil (Kouno *et al.*, 1995). No correction factor was used for P because recovery of a P spike in this soil was 98% (Butterly *et al.*, 2010).

Soil respiration was measured daily by quantifying the CO_2 concentration in the headspace of the jars using a Servomex 1450 infra-red analyser (Servomex Group, Crowborough, UK) (Setia *et al.*, 2011). After each measurement (T1), jars were vented using a fan to refresh the headspace and then resealed followed by another CO_2 measurement (T0). CO_2 produced during this given interval is the difference in

CO_2 concentration between T1 and T0 (Setia *et al.*, 2011). Linear regression based on injection of known amounts of CO_2 into empty jars of the same size was used to define the relationship between CO_2 concentration and detector reading.

In treatments where residue mixes were applied, expected values for a given parameter were calculated based on nutrient availability with each organic material separately and the proportion of each organic material in the mixes (Gartner and Cardon, 2004). In this study the proportion of each residue in the mixes was 0.5.

2.3. Statistical analysis

There were four replicates per treatment and sampling time, arranged in a randomized block design with destructive sampling times as blocks. After confirming normal distribution, data were analysed by two-way ANOVA (residue treatment $\times$ rate) for each sampling date separately using Genstat 15th edition (VSN Int. Ltd., UK). Tukey's multiple comparison tests at 95% confidence interval was used to determine significant differences among treatments. One-way ANOVA was used to compare the properties of the two plant residues.

3. Results

3.1. Cumulative respiration

Throughout the experiment, cumulative respiration within 12-day intervals differed little among 5- and 2.5-treatments that, by the end of the intervals, had both been amended with 5 g kg^{-1} residue (Figure 1). Except for the first 24 days in the 10-treatments, the type of residue added had little effect on cumulative respiration. Cumulative respiration in the first 12 days was higher in 10-treatments (10 g kg^{-1} added on day 0)

compared to 5- and 2.5-treatments (5 g kg^{-1} added by day 12). Although all treatments had received $10 \text{ g residue kg}^{-1}$ by day 24, cumulative respiration was about two- to three-fold higher in 5-treatments and 2.5-treatments than in the 10-treatments. Among 10-treatments, cumulative respiration was two-fold higher in 10-H-L than in 10-L-H. Cumulative respiration from day 25 to 36 was about two-fold higher in 10-treatments that were amended with 10 g kg^{-1}

residue on day 24 compared to 5-treatments (5 g kg^{-1} added on day 24) and 2.5- treatments (2.5 g kg^{-1} added on days 24 and 30). From day 37 to 48, when all treatments had received 20 g kg^{-1} , cumulative respiration was about two- to three-fold higher in 5-treatments and 2.5-treatments compared to 10-treatments which had received no residue addition since day 24. Total cumulative respiration at the end of the experiment did not differ among treatments.

Figure 1. Cumulative respiration in 12-day intervals and total over 48 days in soil amended with high (H) and low C/N (L) residues or their 1:1 mixture (HL) eight times at 2.5 g kg^{-1} , four times at 5 or twice at 10 g kg^{-1} ($n=4$). Different lower case letters indicate significant differences ($P \leq 0.05$) among treatments for a given 12-day interval. Different upper case letters indicate significant differences among treatments in total cumulative respiration. For treatment names see Table 2.

3.2. Microbial biomass N and P

In 10- and 5-treatments, MBN on day 12 was similar in treatments with H and HL addition on day 0 [10-H-L, 10-(HL)x2, 5-(H-L)x2 and 5-(HL)x4] where it was between two and four-fold higher than in the

2.5-treatments (Figure 2A). MBN did not differ between 10-L-H and 5-(L-H)x2 although two times more L had been added in 10-L-H. MBN was similar among 2.5-treatments that had all received both H and L by day 12. MBN changed little from day 12 to 24 in 10- and 5-treatments except for 5-(L-H)x2 where it

increased by about 80%. During that period, MBN increased up to three-fold in 2.5-treatments. In 10-treatments, MBN on day 24 was about 40% lower in 10-L-H which had only received L by then compared to the other two treatments that had been amended with H. On day 36, MBN in the 10-treatments was about

30% lower in 10-H-L which had received L on day 24 compared to the other two treatments. MBN differed little among 5- and 2.5 treatments. MBN on day 48 differed little among treatments at a given amendment rate, but was about 30% higher in 2.5-(H-L)x4 and 2.5-(L-H)x4 than in the 10-treatments.

Figure 2. Microbial biomass N (A) and P (B) on days 12, 24, 36 and 48 in soil amended with high (H) and low C/N (L) residues or their 1:1 mixture (HL) eight times at 2.5 g kg⁻¹, four times at 5 or twice at 10 g kg⁻¹ (n=4, vertical lines indicate standard error). Different letters indicate significant differences ($P \leq 0.05$) among treatments at a given sampling day. For treatment names see Table 2.

In all treatments, MBP was lower on day 12 than on days 36 and 48 (Figure 2B). On day 12, MBP differed little among 5- and 2.5-treatments. It was 10-20% higher in 10-L-H and 10 (HL)x2 than the other treatments. MBP on day 24 differed little among treatments except that it was 20% lower in 10-H-L than in 2.5-(L-H)x4. On day 36, MBP was about 20% higher in 10-L-H than the 2.5-treatments, but differed little among treatments at a given residue rate. On day 48, MBP was about 25% lower in 10-H-L than the 5-treatments.

3.3. Available N and P

Throughout the experiment, available N in 2.5-treatments differed little among treatments, but it was higher on day 48 than day 12 (Figure 3A). On days 12 and 24, available N was highest in 10-L-H. On day 12, available N in 10-L-H was about four-fold higher than in 10-(HL)x2 (5 g kg⁻¹ L added on day 0) and about 30-fold higher than in 10-H-L where only H was added on day 0. Available N on day 12 in the 5-treatments was highest in the treatment where only L had been added on day 0 (5-(L-H)x2) where it was about 20-fold higher than when only H had been added on day 0 (5-(H-L)x2) and two-fold higher than when both H and L had been added on day 0 (5-(HL)x4).

Available N changed little from day 12 to day 24 in the 10-treatments (no residue addition since day 0). But in the 5-treatments, available N in 5-(H-L)x2 on day 24 was about eight-fold higher than on day 12 (L added on day 12), whereas it was 50% lower in 5-(L-H)x2 (H added on day 12). On day 24, available N was about 30% higher in 5-(H-L)x2 (L added on day 12) than in the other two treatments. Available N was similar in the 10-treatments on day 36 because it increased about 10-fold from day 24 to 36 in 10-(H-L) and decreased about 60% in 10-(L-H), where L or H added on day 24, respectively. Similarly, available N increased more than two-fold from day 24 to 36 in 5-(L-H)x2 and decreased by about 70% in 5-(H-L)x2. Available N in all 10-treatments was about two-fold higher on day 48 than day 36. In the 5-treatments compared to day 36, available N increased five-fold in 5-(H-L)x2 and decreased by about 20% in 5-(L-H)x2 where L or H had been added on day 36, respectively. Available N on day 48 differed little among treatments at a given residue rate, but it was 20-50% higher in the 10- than the 2.5-treatments.

Changes over time of available P were similar as those in available N (increasing when L had been added before sampling, decreasing when H was added), but differences were less pronounced than in available N (Figure 3B).

Figure 3. Available N (A) and P (B) on days 12, 24, 36 and 48 in soil amended with high (H) and low C/N (L) residues or their 1:1 mixture (HL) eight times at 2.5 g kg⁻¹, four times at 5 or twice at 10 g kg⁻¹ (n=4, vertical lines indicate standard error). Different letters indicate significant differences ($P \leq 0.05$) among treatments at a given sampling day. For treatment names see Table 2.

3.4. Measured and expected values

The difference between measured and expected values differed among on parameters and sampling time (Table 3). In the following, only treatments and sampling times where expected and measured values differed significantly are described. In 10-(HL)x2, measured cumulative respiration was about 20% higher than expected on day 12, but 10% lower on day 48. Measured MBN was about 25% higher than expected on days 24 and 36. Measured MBP was about 25%

higher than expected on day 48. For available N in 10-(HL)x2, measured values were about half of expected values on days 12 and 24, but 10% higher on day 48. In 5-(HL)x4, measured and expected values of cumulative respiration and MBN differed only on day 12 where measured values were 10% and about two-fold higher, respectively. On day 36, measured MBP and available N in 5(HL)x4 were about 20% lower than expected. In 2.5-(HL)x8, measured cumulative respiration was slightly lower than expected on day 12. For MBN, the measured value was about 25% higher

than expected on day 24, but 25% lower on day 48. Measured MBP in 2.5-(HL)x8 on days 24 and 36 was about 20% lower than expected. Measured available

N matched expected values. Measured and expected available P matched in all three HL treatments at all sampling dates.

Table 3. Measured and expected values of measured parameters on days 12, 24, 36 and 48 for treatments where H and L were mixed. At a given sampling date, asterisks indicate significant differences between measured and expected values.

Parameter	Day	Values	10-(HL)x2	5-(HL)x4	2.5-(HL)x8
Cumulative respiration (mg CO ₂ -C g ⁻¹)	12	Measured	2.14*	1.22*	0.98*
		Expected	1.86	1.15	1.03
	24	Measured	0.61	1.33	1.57
		Expected	0.60	1.46	1.57
	36	Measured	2.27	1.40	1.23
		Expected	2.22	1.38	1.29
	48	Measured	0.62*	1.64	1.72
		Expected	0.75	1.61	1.61
	12	Measured	9.01	11.35*	3.25
		Expected	8.90	6.85	3.55
MBN (mg kg ⁻¹)	24	Measured	9.94*	9.35	9.55*
		Expected	8.13	8.27	7.48
	36	Measured	11.68*	9.30	9.94
		Expected	8.65	7.95	9.62
	48	Measured	9.52	11.70	10.20*
		Expected	9.04	8.65	13.67
MBP (mg kg ⁻¹)	12	Measured	20.01	13.84	12.76
		Expected	17.57	14.76	13.23
	24	Measured	17.27	18.11	16.71*
		Expected	15.57	17.90	18.88
	36	Measured	31.17	25.92*	23.49*
		Expected	32.46	28.73	26.90
	48	Measured	27.71*	26.97	25.24
		Expected	22.75	28.40	27.39
Available N (mg kg ⁻¹)	12	Measured	32.48*	43.74	47.41
		Expected	71.91	48.01	47.55
	24	Measured	43.58*	41.23	40.78
		Expected	79.52	52.15	42.97
	36	Measured	61.14	53.44*	53.08
		Expected	61.62	63.42	53.08
	48	Measured	122.45*	90.17	79.74
		Expected	108.77	96.51	78.19
Available P (mg kg ⁻¹)	12	Measured	17.00	13.74	13.53
		Expected	18.87	13.67	13.07
	24	Measured	17.98	16.96	18.15
		Expected	18.92	17.41	17.32
	36	Measured	24.37	21.31	22.60
		Expected	23.21	20.75	22.72
	48	Measured	28.66	29.37	28.39
		Expected	27.29	27.64	27.74

4. Discussion

This study showed that available N and MBN were influenced by residue C/N ratio, amendment rate and frequency whereas cumulative respiration in a given 12-day period was influenced only by amendment rate. Addition of H generally increased microbial N uptake which was, in some cases, accompanied by a decrease in available N. Amendment with L increased available N. But the magnitude of these changes depended on sampling time and differed among treatments.

In the 10-treatments, cumulative respiration was much higher in the first 12 days after amendment than from day 13 to 24 which is consistent with other studies (McTiernan *et al.*, 1997; Marschner *et al.*, 2015). This indicates that easily decomposable compounds were depleted in the first 12 days. In contrast, cumulative respiration differed little among 12-day periods in the 5 and 2.5 treatments. This is likely because residue addition every 12 or six days prevented or minimized depletion of easily decomposable compounds. The finding that cumulative respiration was not influenced by the C/N ratio suggests that N availability did not limit decomposition of high C/N residue in this experiment.

The difference between measured and expected cumulative respiration in the mixes depended on amendment rate. On day 12, where only either H or L had been added of H-L or L-H, measured was higher than expected which is. The higher than expected cumulative respiration on day 12 in the 10 or 5 treatments in agreement with previous studies, e.g., Mao and Zeng (2012), Cuchietti *et al.* (2014), likely due to the supply of N from L to microbes decomposing H. After day 12, expected and measured cumulative respiration generally matched, probably because H and L had been added in all treatments.

In the following discussion, we will focus on available N and MBN because available P and MBP differed little between treatments or sampling times.

The first hypothesis (at a given sampling time, available N and MBN will be less influenced by the C/N ratio of the residue added last before sampling with frequent addition of L and H at low rate four or eight times than less frequent amendment at high rate) can be confirmed. The second hypothesis (in 1:1 mixes of H and L, available N and MBN will initially increase with amendment rate, but differences between rates will become smaller over time) can be confirmed only for MBN on day 12 where it was higher in treatments where mixes were added eight times than where they were added twice. And the third hypothesis (in the mixes, differences between measured and expected values will be greater when residues are added at high rates twice than if they are added at lower rates more frequently) can be confirmed for the comparison of mix added eight times (2.5-(HL)x8) compared to added twice (10-(HL)x2), particularly for available N. In the first 24 days of the 10 and 5 treatments, available N was very low after H addition which can be explained by N immobilization which is in agreement with many previous studies, e.g. Tian *et al.* (1992), Hadas *et al.* (2004). As expected from previous studies, available N was high after L addition suggesting high net N release from the low C/N residue Moritsuka *et al.* 2004. However, MBN was quite low indicating that microbial growth was limited by C availability, likely due to the very rapid decomposition of L in the first few days after amendment. MBN may have been higher a few days after residue addition (Hoyle and Murphy, 2011; Nguyen *et al.*, 2016). But by day 12, a proportion of the microbial biomass had died. However, in the 10-treatments, after the second addition of residues on day 24, the C/N ratio of the added residue had no effect on available N and only temporarily influenced MBN. The lack of differences

between treatments in day 36 is due to a strong increase in available N in 10-H-L compared to day 24 whereas available N decreased during this period in 10-L-H. It is likely that a proportion of the residue added on day 0 was still in the soil and was decomposed together with the residue added on day 24. The decrease in available N in 10-L-H can be explained by N immobilization, as microbes decomposed H added on day 24. N transfer from low to high C/N litter was shown in previous studies (e.g. Schwendener *et al.* (2005)). A strong decrease in available N when high C/N residue is added after low C/N residue is in agreement with our previous studies, e.g. Marschner *et al.* (2015), Zheng and Marschner (2017).

In the 5-treatments, the C/N ratio of the residue added 12 days before the sampling on day 36 had a similar effect on available N and MBN as when added on day 0 (net immobilization after H addition, net release after L addition). This suggests that at the lower amendment rate, little of the previously added residue remained in the soil by the time of the next amendment. In 5-treatments, differences between treatments in available N only disappeared on day 48, probably because more residues were present in the soil than earlier and H and L decomposed together as also indicated by the smaller differences among treatments in MBN compared to day 12 and day 24.

Until day 36 compared to expected values, measured available N in 10-(HL)x2 was lower whereas MBN was generally higher. This indicates that decomposition of H and N together stimulated net immobilization compared to treatments where H and L were added sequentially. In the 5-treatments, differences between expected and measured values in available N and MBN were transient, probably because in all treatments after day 12, both H and L had been added. In the 2.5 treatments, differences between available N and MBN after H and L addition were small because before each sampling date, both H and L had

been added within the last 12 days. This also explains why measured and expected available N matched throughout the experiment and only transiently differed with respect to MBN. This is likely because soil in all 2.5-treatments contained H and L at different stages of decomposition which buffered the effect of freshly added residues. The presence of easily decomposable compounds in undecomposed or partially decomposed residues in the soil make it less likely that microbes will preferentially decompose freshly added residues. In the 5 and 10-treatments on the other hand, the freshly added residue was the main substrate source for microbes because the previously added residue left in the soil was largely decomposed. Therefore, with frequent residue addition (every 6 days) H and L are decomposed together, even when added sequentially. MBN on day 12 was lower in the 2.5-treatments than in the 5 and 10-treatments, probably because of the smaller amount of residue added on day 0. The build-up of MBN after day 12 to become similar as in the 5 and 10-treatments on days 24 and 36 can be explained by the frequent addition of residues that sustained microbial growth although only small amounts were added each time. The frequent addition can also explain the higher MBN than in the 10-treatments on day 48. Whereas available substrate was likely depleted in the latter, residues added six days before in the 2.5 treatments supplied sufficient available substrate for microbes.

5. Conclusion

This study showed that N availability and MBN with repeated addition of high and low C/N residues are influenced by amendment frequency and change over time. Amendment frequency also influenced interactions in mixes regarding N availability and MBN. Frequent addition of small amounts of residues limited microbial N uptake initially compared to higher

amendment rates, but resulted in similar available N irrespective of the C/N ratio of the residue added. Less frequent addition of large amounts of residues on the other hand resulted in large fluctuations in available N and MBN depending on the C/N ratio of the residue added previously. In the present study, the source of C, N and P in respired CO₂, microbial biomass and available nutrients could not be determined. In future studies with repeated residue additions, one of the addition could be in the form of ¹³C, ¹⁵N or ³²P-labelled residues.

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