



# Carbon use efficiency and microbial functional diversity in a temperate Luvisol and a tropical Nitisol after millet litter and N addition

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## Abstract

Tropical soils often contain less soil organic C (SOC) and microbial biomass C (MBC) than temperate soils and, thus, exhibit lower soil fertility. The addition of plant residues and N fertilizers can improve soil fertility, which might be reflected by microbial C use efficiency (CUE) and functional diversity. A 42-day incubation study was carried out, adding leaf litter of the C<sub>4</sub> plant finger millet (*Eleusine coracana* Gaertn.) and inorganic <sup>15</sup>N fertilizer. The aim was to investigate amendment effects on CUE and functional diversity in a tropical Nitisol and a temperate Luvisol. At day 42, 28% of the millet litter-derived C (C<sub>4</sub>) added was mineralised to CO<sub>2</sub>C<sub>4</sub> in the temperate Luvisol and only 18% in the tropical Nitisol, averaging all N treatments. In contrast, none of the different fractions used for calculating CUE values, i.e. CO<sub>2</sub>C<sub>4</sub>, MBC<sub>4</sub>, microbial residue C<sub>4</sub>, and particulate organic matter C<sub>4</sub>, differed between the soils in the N<sub>0</sub> (no N addition) treatment. CUE values considering microbial residues varied around 0.63, regardless of soil type and sampling day, which needs further evaluation. Millet litter increased autochthonous SOC-derived CO<sub>2</sub>C<sub>3</sub> production, but N addition did not. This priming effect was apparently not caused by N mining. The respiratory response to most substrates added by multi-substrate-induced respiration (MSIR) and, thus, functional diversity was higher in the Luvisol than in the Nitisol. Millet litter had positive and N addition negative effects on the functional diversity of Nitisol, indicating that MSIR is a useful tool for evaluating soil fertility.

**Keywords** Microbial biomass · <sup>13</sup>C/<sup>12</sup>C ratio · CO<sub>2</sub> mineralisation · Particulate organic matter · Multi substrate-induced respiration · Microbial necromass · N uptake

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## Introduction

Soil organic matter (SOM) and soil microorganisms are central components for soil fertility (Joergensen 2010), i.e. the ability of soils to maintain key ecological functions, such as decomposition of plant residues and provision of nutrients for plant growth (Craswell and Lefroy 2001; Joergensen and Castillo 2001; Cerri et al. 2006). Soil microorganisms maintain the majority of enzymatic processes in soil and preserve energy and nutrients in their biomass (Jenkinson and Ladd 1981). As these processes are controlled by temperature, the turnover rates of microbial biomass C (MBC) and soil organic C (SOC) are higher in tropical regions than in temperate climates (Jenkinson and Ayanaba 1977; Diels et al. 2004; Sierra and Desfontaines 2018). This shortens the response time to changes in management practices and increases the risk of permanent damage to soil fertility (Cerri et al. 2003; Bationo et al. 2007).

In contrast to humid temperate areas, the tropical monsoonal climate of India is characterised by extreme changes

between dry and rainy seasons (Srivastava and Singh 1991; Manna et al. 2007; Vineela et al. 2008). Dystric Nitisols are the dominating soil type of the old landscapes around tropical Bangalore in South India (Murugan et al. 2019). These soils are characterised by high contents of Al and Fe oxides, which reduce P availability, as well as low contents of SOC, which intensify drought effects. Consequently, these Nitisols currently exhibit considerably lower soil fertility in comparison with Luvisols of the young landscapes in Central Germany. However, SOC contents, and thus fertility, of tropical Nitisol soils can be improved by adding plant residues (Agegnehu et al. 2016; Sierra and Desfontaines 2018), especially in combination with N fertilizers (Geisseler and Scow 2014).

SOC sequestration can be promoted by increasing the C input or by decreasing microbial turnover, which is the product of maintenance coefficient  $\times$  C use efficiency (CUE). Often CUE values are calculated as MBC growth/MBC uptake (Manzoni et al. 2012; Spohn et al. 2016a, b; Geyer et al. 2019). There, MBC growth is usually substrate C incorporated into the microbial biomass, while MBC uptake is the sum of substrate-derived MBC and CO<sub>2</sub> (Manzoni et al. 2012). Accurate CUE estimates using this approach depend upon a relatively narrow window of incubation time that ensures complete metabolisation of an added substrate (Joergensen and Wichern 2018).

The view of Manzoni et al. (2012) and Spohn et al. 2016a, b was challenged by Joergensen and Wichern (2018), who asked for all microbial metabolites to be included in the CUE calculation. Research has been focused on CUE of <sup>13</sup>C- and <sup>14</sup>C-labelled sugars, mainly glucose (Bardgett and Sagar 1994; Bremer and Kuikman 1994), and other simple organic components (Jones et al. 2018) but rarely SOM (Spohn et al. 2016a, b). Less information is available on the CUE of complex plant residues (Muhammad et al. 2006; Rottmann et al. 2010; Sauvadet et al. 2018), which can be determined by the recovery of added substrate as particulate organic matter (Magid and Kjærsgaard 2001). This approach is often combined with the difference in  $\delta^{13}\text{C}$  of C4 plants and SOC mainly originated from C3 plants (Ryan and Aravena 1994; Balesdent and Mariotti 1996; Faust et al. 2019). However, nothing is known about the effects of soil type and incubation time on this type of CUE value. As the metabolisation of added complex substrates is usually incomplete at a specific sampling date (Faust et al. 2019), CUE values of complex plant litter might vary over incubation time, like that of simple glucose (Joergensen and Wichern 2018).

Lower MBC and SOC levels of Nitisols might be caused by lower CUE values of an N limited microbial community (Kaiser et al. 2014; Poeplau et al. 2019). Microbial C and N limitation can be reflected by a variable, but often lower respiratory response to simple low-molecular-weight organic substrates added to soil, depending on the metabolic demand (Struecker and Joergensen 2015). This creates differences in

functional diversity of the soil microbial community, using multi-substrate-induced respiration (Campbell et al. 2003). This approach has been shown to respond sensitively to soil type (Moscatelli et al. 2018) and to the addition of organic N fertilizers (Sradnick et al. 2013; Murugan et al. 2014; Podmirseg et al. 2019). However, nothing is known about the immediate effect of the combined addition of plant litter and inorganic N fertilizers on functional diversity. Microbial C limitation is certainly reduced by the addition of plant litter (Struecker and Joergensen 2015), but the response to inorganic N fertilisation remains largely unknown.

The central objective of the current incubation study was to investigate the following hypotheses: (1) CUE of millet litter does not depend on the incubation time if all microbial metabolites are included. (2) The Nitisol with less MBC has a lower CUE of millet litter than the Luvisol with more MBC. (3) Microbial functional diversity is increased by millet litter and reduced by inorganic N addition. (4) Amendment effects on CUE and functional diversity are stronger in the Nitisol than in the Luvisol.

## Materials and methods

### Sites, soils, and litter

The temperate soil is a Haplic Luvisol (IUSS Working Group WRB 2015). Samples were taken at 0–15 cm from the site Teileranger I in Neu-Eichenberg, Germany (51° 22' 50.8" N 9° 54' 34.3" E) in March 2018. The site was cultivated with a mixture of alfalfa (*Medicago sativa* L.) and Italian rye grass (*Festuca perennis* Lam.) under a non-ploughing system. The soil had a pH-H<sub>2</sub>O of 6.83 with a texture of 3% sand, 83% silt, and 13% clay. The soil contained 11.4  $\pm$  0.3 mg SOC and 1.48  $\pm$  0.03 mg total N g<sup>-1</sup> soil with a  $\delta^{13}\text{C}$  of  $-27.4 \pm 0.1\text{‰}$  and a  $\delta^{15}\text{N}$  of  $8.2 \pm 0.3\text{‰}$ . Mean annual rainfall is 579  $\pm$  165 mm (2013–2017). Mean annual air temperature is 10.5  $\pm$  0.8 °C.

The tropical soil is a Dystric Nitisol (IUSS Working Group WRB 2015). Soil samples were taken from the irrigated field experiment (FOR2432) at the University of Agricultural Sciences (UAS) in Bangalore, India (13° 05' 23.9" N 77° 33' 58.8" E) (Hoffmann et al. 2017). Soils were sampled at 0–20 cm in four replicated plots after harvest of lablab (*Lablab purpureus* (L.) Sweet) under a non-ploughing system in November 2017. The soil had a pH-H<sub>2</sub>O of 6.69 with a texture of 58% sand, 10% silt, and 32% clay. The soil contained 8.9  $\pm$  0.6 mg SOC and 0.90  $\pm$  0.04 mg total N g<sup>-1</sup> soil with a  $\delta^{13}\text{C}$  of  $-22.6 \pm 0.2\text{‰}$  and a  $\delta^{15}\text{N}$  of  $5.4 \pm 0.3\text{‰}$ . Long-term mean annual rainfall is 935 mm and mean annual air temperature is 24.0 °C.

All soil samples were sieved (< 2 mm), homogenised, and, after the removal of roots and stones, stored in plastic bags at

4 °C in the dark until the start of the experiment. Soil pH was measured electrochemically at a soil to water ratio of 1 to 2.5. Water holding capacity was determined by weighing 10 g moist soil into a glass tube (2 × 10 cm) with a porous membrane at the bottom that allows water flow. The tubes were transferred into a water bath with a filling level of 1 cm height, followed by water addition to the tubes until the soil was completely covered, and left for 3 h. Then, the tubes were removed from the water bath, placed onto absorbent tissue paper for 10 min, and weighed. Finally, the tubes were dried at 105 °C for 24 h and weighed again (Wilke 2005).

Finger millet (*Eleusine coracana* Gaertn.) is a C4 plant and common in India. The millet leaf litter used in this experiment originated from the rainfed field experiment of the DGF project FOR2432 at the UAS, Bangalore (Hoffmann et al. 2017; Murugan et al. 2019). The millet litter contained  $384.7 \pm 1.4$  mg total C and  $6.8 \pm 0.1$  mg total N g<sup>-1</sup> dry weight (DW) with a  $\delta^{13}\text{C}$  of  $-13.4 \pm 0.4\text{‰}$  and a  $\delta^{15}\text{N}$  of  $15.0 \pm 1.0\text{‰}$ .

## Experimental treatments

The experimental set-up was designed as a three-factorial experiment with the following factors in quadruplicate: (1) soil (temperate Luvisol and tropical Nitisol), (2) millet litter addition (with and without), and (3) N level: N0 (no N addition), N20 (= 20 µg N g<sup>-1</sup> soil) and N50 (= 50 µg N g<sup>-1</sup> soil) applied as 10 atom% NH<sub>4</sub>-<sup>15</sup>NO<sub>3</sub> (Chemotrade, Leipzig, Germany). The amount of millet leaf litter (5 mm cuttings) corresponding to 1 mg C g<sup>-1</sup> soil was added to the soil of ML + treatments. Litter and soil were mixed in the plastic bag and transferred into 50-ml Falcon tubes. The water content was adjusted to 50% water holding capacity (WHC) by adding the NH<sub>4</sub>NO<sub>3</sub> solution to the N20 and N50 treatments and water to the N0 treatment, respectively. Two Falcon tubes per treatment, vial A (15 g moist soil for sampling day 7) and vial B (35 g moist soil for sampling day 42), were placed into a Mason jar, equipped with sealing rings. Soils were incubated at 25 °C. For measuring particulate organic matter (POM), moist soil of 100 g per replicate was mixed with 1 mg C g<sup>-1</sup> soil of millet litter and transferred into 250-ml plastic vessels, placed in another set of Mason jars, placed alongside the Mason jars with the Falcon tubes. The POM experiment was restricted solely to the N0 treatment, due to limited quantity of the Nitisol.

## CO<sub>2</sub> evolution

The CO<sub>2</sub> evolved was trapped during the incubation in 0.25 M NaOH solution, which was changed after 2, 4, 7, 14, 21, 28, 35, and 42 days. The trapped CO<sub>2</sub> was precipitated with 5 ml of a saturated BaCl<sub>2</sub> solution (Zibilske 1994) and stored under CO<sub>2</sub>-free atmosphere. Then, the NaOH not consumed was

back titrated with 0.25 M HCl, using a TITRONIC 500 (Xylem Analytics, Weilheim, Germany) system to the transition point of phenolphthalein at a pH of 8.3. For the determination of  $\delta^{13}\text{C}$  values, BaCO<sub>3</sub> samples from the titration events day 2, 7, and 42 were centrifuged (3000 g for 10 min at 20 °C), washed three times with H<sub>2</sub>O to remove excess ions, and freeze-dried before analysis. The  $\delta^{13}\text{C}$  values of the titration events day 4, 14, 21, 28, and 35 were estimated by linear interpolation between the sampling days.

## Microbial biomass

Soil microbial biomass C (MBC) and N (MBN) were determined by fumigation extraction (Brookes et al. 1985; Vance et al. 1987) at day 7 (vial A) and day 42 (vial B) of incubation. To reduce the amount of inorganic N in the sample, 15 g moist soil were pre-extracted for 30 min by oscillating shaking at 200 rev min<sup>-1</sup> with 40 ml 0.05 M K<sub>2</sub>SO<sub>4</sub> (Widmer et al. 1989). Non-fumigated and fumigated 5-g portions were extracted for 30 min by oscillation shaking at 200 rev min<sup>-1</sup> with 20 ml 0.05 M K<sub>2</sub>SO<sub>4</sub> (Potthoff et al. 2003), centrifuged (3000 g for 10 min at 10 °C), filtered (hw3, Sartorius Stedim Biotech, Göttingen, Germany), and stored at -18 °C before analysis. Organic C and total N in the extracts were determined with a Multi N/C 2100S analyser (Analytik Jena, Germany). MBC was calculated as  $E_C/k_{EC}$ , where  $E_C$  = (organic C extracted from fumigated soils) - (organic C extracted from non-fumigated soils) and  $k_{EC}$  = 0.45 (Wu et al. 1990). MBN was calculated as  $E_N/k_{EN}$ , where  $E_N$  = (total N extracted from fumigated soils) - (total N extracted from non-fumigated soils) and  $k_{EN}$  = 0.54 (Brookes et al. 1985). About 14 ml of the soil extracts were freeze-dried for isotope analysis (Alpha 1-4 LD plus, Christ, Osterode, Germany).

## Particulate organic matter

POM was recovered as described by Magid and Kjærgaard (2001) at day 0, 7, and 42 of incubation. Briefly, 100 g moist soil was dispersed in 400 ml 5% NaCl solution, stirred by hand, and allowed to stand overnight (Muhammad et al. 2006). Samples were poured gradually onto a 400-µm sieve and washed with tap water. Soil aggregates were destroyed by pushing them through the sieve during the washing process. A 5% NaCl solution was added to the washed soil and the procedure was repeated until organic particles were no longer visible in the mineral fraction and the washing water was clear. POM was transferred into crucibles, dried at 60 °C, and weighed.

## C and N analyses and calculations

For analyses of total C,  $\delta^{13}\text{C}$ , total N, and  $\delta^{15}\text{N}$ , samples were dried for 24 h at 105 °C (soil) and 60 °C (POM and litter), respectively, and ball-milled. The  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  in K<sub>2</sub>SO<sub>4</sub>

extracts as well as  $\delta^{13}\text{C}$  of  $\text{BaCO}_3$  were analysed in freeze-dried samples. Isotope values were measured by elemental analyser–isotope ratio mass spectrometry and are expressed in  $\delta$ -notation relative to the Vienna Pee Dee Belemnite (VPDB) for  $^{13}\text{C}$  and in atom% relative to air for  $^{15}\text{N}$ . The fraction of millet-derived C in the  $\text{K}_2\text{SO}_4$  extracts of fumigated and non-fumigated samples in  $\text{CO}_2\text{C}$  as well as in POMC was calculated for each individual replicate of all treatments from the  $\delta^{13}\text{C}$  data according to a two pool-mixing model (Balesdent and Mariotti 1996) using the following equation:

$$\text{C4-fraction (\%)} = (\delta^{13}\text{C}_{\text{sample}} - \delta^{13}\text{C}_{\text{control}}) / (\delta^{13}\text{C}_{\text{millet}} - \delta^{13}\text{C}_{\text{control}}) \times 100$$

where  $\delta^{13}\text{C}_{\text{sample}}$  represents the millet treatments,  $\delta^{13}\text{C}_{\text{control}}$  the no millet treatments at the respective sampling days 7 and 42. The fraction of addition-derived  $^{15}\text{N}$  in the  $\text{K}_2\text{SO}_4$  extracts of fumigated and non-fumigated samples was calculated using the following equation (Dijkstra et al. 2006; Zareitalabad et al. 2010):

$$\text{MB}^{15}\text{N (\%)} = ({}^{15}\text{N}_{\text{sample}} - {}^{15}\text{N}_{\text{control}}) / ({}^{15}\text{N}_{\text{addition}} - {}^{15}\text{N}_{\text{control}}) \times 100$$

where  $^{15}\text{N}_{\text{sample}}$  represents the N20 and N50 treatments,  $\delta^{15}\text{N}_{\text{control}}$  the N0 treatment at the respective sampling days in atom%, and  $^{15}\text{N}_{\text{addition}} = 10$  atom%.

## CUE calculations

CUE values of millet litter were calculated at sampling days 7 and 42 in the N0 treatment according to Joergensen and Wichern (2018), considering all microbial metabolites, i.e. millet-derived microbial residue C (MRC4):

$$\text{CUE} = (\text{MBC4} + \text{MRC4}) / (100 - \text{POMC4})$$

$$\text{MRC4} = 100 - \text{POMC4} - \text{CO}_2\text{C4} - \text{MBC4}$$

Millet-derived C4 in MBC, POM, and  $\text{CO}_2\text{C}$  are abbreviated as MBC4, POMC4, and  $\text{CO}_2\text{C4}$ . MRC (Joergensen and Wichern 2018) or microbial necromass (Liang et al. 2019) embraces all freshly synthesised microbial products that leave the cells as non-biomass metabolites, such as exoenzymes (Burns et al. 2013), extracellular polymeric substances (Redmile-Gordon et al. 2014, 2015), e.g. the glomalin-related protein (Wright and Upadhyaya 1996; Spohn and Giani 2011), other secondary metabolites, e.g. antibiotics (Boruta 2018; Keller 2019), and dead tissue remains (Joergensen and Wichern 2018). In addition, CUE of millet litter at sampling days 7 and 42 in the N0 treatment was calculated in the classical way, as proposed e.g. by Manzoni et al. (2012) and Spohn et al. (2016a, b). This calculation approach solely

considers the incorporation of millet-derived C4 into the MBC4 and is thus abbreviated as  $\text{CUE}_{\text{MB}}$ :

$$\text{CUE}_{\text{MB}} = \text{MBC4} / (\text{CO}_2\text{C4} + \text{MBC4})$$

All millet litter-derived fractions were considered in the calculations as % of the added substrate.

## Multi-substrate-induced respiration

Multi-substrate-induced respiration (MSIR) was determined according to Campbell et al. (2003). MSIR was conducted on the soil from vial B, which was stored after the incubation experiment at 4 °C in the dark until analysis. The soil samples were dried from 50 to 40% WHC by monitoring the gravimetric reduction, before 300 mg soil was weighed in triplicate per substrate, divided on different deep-well plates (Nunc, Langensfeld, Germany). Then, the soil was pre-incubated for 7 days in the dark at 25 °C prior to MSIR analysis.

The physiological profiles were determined by applying  $\text{H}_2\text{O}$ , 6 amino acids [ $\gamma$ -aminobutyric acid (Aba), L-alanine (Ala), L-arginine (Arg), DL-aspartic acid (Asp), L-glutamine (GluN), L-leucine (Leu)], 2 amino sugars [N-acetyl glucosamine (NAG), D-glucosamine (GlcN)], 5 neutral sugars [L-arabinose (Ara), D-galactose, D-glucose (Glc), D-fructose (Fru), D-trehalose (Tre)], 3 carboxylic acids [citric acid (Cit), L-malic acid (Mal), oxalic acid (Oxa)], and phenolic protocatechuic acid (ProCa). These substrates present a cross section of root exudates (Campbell et al. 2003) and microbial components and products (Amelung et al. 2001) and have been used in similar studies before (Campbell et al. 2008; Sradnick et al. 2013; Murugan et al. 2014; Struecker and Joergensen 2015). To achieve a final concentration of 8 mg substrate  $\text{g}^{-1}$  soil, 20  $\mu\text{l}$  substrate solution was added to 300 mg moist soil. Exceptions were aspartic acid (0.04 mg  $\text{g}^{-1}$  soil), L-leucine (1.33 mg  $\text{g}^{-1}$  soil), and protocatechuic acid (0.8 mg  $\text{g}^{-1}$  soil), due to their lower solubility in water. The colorimetric (12.5 ppm cresol red, 150 mM KCl, and 2.5  $\text{NaHCO}_3$ )  $\text{CO}_2$  trap was produced according to Campbell et al. (2003) and stored under  $\text{CO}_2$  free atmosphere at 25 °C in the dark for at least 72 h prior to measurement (Murugan et al. 2014). Addition of aqueous substrates was performed 40 min prior to the initial ( $t_0$ ) measurement, to avoid the first wetting peak. Detection plates were measured immediately before sealing ( $t_0$ ) and after 6 h of incubation ( $t_6$ ) at 25 °C in the dark with a microtiter plate reader (FLUO Star Omega, BMG, Ortenberg, Germany) at 572 nm (Sradnick et al. 2013). Absorbance was converted to  $\text{CO}_2$  concentration, using the following equation:

$$\mu\text{lCO}_2\text{g}^{-1}\text{soil h}^{-1} = 51 \times (0.2 + \text{ABS})^3$$

where ABS is the difference in absorption (572 nm) between  $t_6$  and  $t_0$  (Murugan et al. 2014,  $r = 0.98$ ). Finally,  $\mu\text{g CO}_2$  was

calculated by multiplying  $\mu\text{l CO}_2$  by 0.49. The Shannon diversity index ( $H$ ) was calculated according to Zak et al. (1994):

$$H = -\sum p_i \times (\ln p_i)$$

where  $p_i$  is the ratio of the respiration rate on a particular substrate minus the blank of the plate to the sum of respiration rates on all substrates.

### Statistical analysis

Data are presented as arithmetic means on an oven-dry weight basis. All data used for ANOVA analysis exhibited normality of residuals and homogeneity of variances according to the Shapiro-Wilk test and Levene test, respectively. The significance of soil, millet litter, and N addition effects on autochthonous SOC-derived  $\Sigma\text{CO}_2\text{C}_3$ , MBC3, total MBN, and  $\text{MB}^{15}\text{N}$  were tested using a 3-way ANOVA. The significance of soil and N effects on  $\Sigma\text{CO}_2\text{C}_4$  and MBC4 were tested using a 2-way ANOVA. The significance of soil effects on POMC4, the POM-C/N ratio, and all fractions required for CUE calculations were tested using an unpaired  $t$  test. The significance of changes between days 7 and 42 was tested using a paired  $t$  test. ANOVA and  $t$  test analyses were carried out using SigmaPlot 13.0 (Systat, San José, USA). For MSIR, discriminant function analysis was used to identify classification success according to respiratory response of 17 substrates and  $\text{H}_2\text{O}$ , using SPSS 21.0 (IBM, Armonk, USA).

## Results

### Millet litter decomposition

At the end of the incubation, 28% of the added millet litter C was mineralised to  $\text{CO}_2$  ( $\Sigma\text{CO}_2\text{C}_4$ ) in the temperate Luvisol, but only 18% in the tropical Nitisol, averaging all N treatments (Table 1). In contrast, similar percentages of millet litter C were incorporated as MBC4 in the two soils, approximately 3% at day 7, significantly ( $P < 0.04$ , paired  $t$  test) increasing to 4% at day 42. N addition did not generally affect  $\Sigma\text{CO}_2\text{C}_4$  and MBC4. However, in the N50 treatment, the lower MBC4 of the Luvisol ( $25 \mu\text{g g}^{-1}$  soil) in comparison with the Nitisol ( $53 \mu\text{g g}^{-1}$  soil) caused a significant soil  $\times$  N interaction. In contrast to  $\Sigma\text{CO}_2\text{C}_4$ , the recovery of particulate organic matter C did not differ between the soils (Table 2). The C/N ratio of the recovered millet litter was always higher in the Luvisol than in the Nitisol, but this difference declined during the incubation.

MBC3 and total MBN in the Luvisol were roughly twice those in the Nitisol at day 7 (Table 3). MBC3 and total MBN generally ( $P < 0.01$ , paired  $t$  test) increased in both soils from day 7 to 42, so that the relative differences between the two

**Table 1** Cumulative  $\Sigma\text{CO}_2\text{C}_4$  and MBC4 in soils amended with millet litter over all N treatments after 7 and 42 days of incubation at 25 °C; probability values of a two-way ANOVA, using soil and N addition as factors

Treatment	$\Sigma\text{CO}_2\text{C}_4$ ( $\mu\text{g g}^{-1}$ soil)		MBC4 ( $\mu\text{g g}^{-1}$ soil)	
	Day 7	Day 42	Day 7	Day 42
Luvisol + Millet	80	277	29	36
Nitisol + Millet	59	177	32	45
Probability values				
Soil	0.01	0.01	NS	NS
N	NS	NS	NS	NS
Soil $\times$ N	NS	NS	NS	0.03
CV ( $\pm$ %)	25	22	50	37

CV mean coefficient of variation between replicates ( $n = 4$ ); NS not significant

soils declined. Millet litter addition did not affect MBC3, but moderately increased total MBN. In contrast, millet litter addition strongly increased cumulative  $\Sigma\text{CO}_2\text{C}_3$  production. The absolute increase in the evolution of autochthonous  $\text{CO}_2$  was roughly the same in both soils at days 7 ( $+ 73 \mu\text{g C g}^{-1}$  soil) and 42 ( $+ 234 \mu\text{g g}^{-1}$  soil), although the basal respiration rate of the Luvisol was twice that of the Nitisol. However, the significant soil  $\times$  millet interactions on  $\Sigma\text{CO}_2\text{C}_3$  production at day 42 and on MBN at day 7 were in both cases caused by the significantly larger differences in the Nitisol than in the Luvisol.

N addition did not generally affect  $\Sigma\text{CO}_2\text{C}_3$  production, MBC3 and MBN. However, the significant soil  $\times$  N interaction at day 7 was caused by 47 and 22% higher  $\Sigma\text{CO}_2\text{C}_3$  production in the N0 treatment of the Luvisol compared with the N20 and N50 treatments, respectively. Microbial uptake of  $^{15}\text{N}$  was proportionate to the addition rate (Table 4), i.e. approximately 3.5% of the added N was incorporated as  $\text{MB}^{15}\text{N}$  without significant ( $P = 0.14$ , paired  $t$ -test) changes from day 7 to 42. However, significantly less  $^{15}\text{N}$  was incorporated into the MBN of the Nitisol at day 7 in the N50 treatment, whereas

**Table 2** Recovery of POMC4 and POM-CN ratio in soils of the N0 treatment amended with millet litter after 7 and 42 days of incubation at 25 °C

Treatment	POMC4 (% added)			POM-CN		
	Day 0	Day 7	Day 42	Day 0	Day 7	Day 42
Luvisol + Millet	92	73	32	88 a	55	29 a
Nitisol + Millet	97	84	29	63 b	42	24 b
CV ( $\pm$ %)	8	8	8	11	16	7

CV mean coefficient of variation between replicates ( $n = 4$ ); different letters within a column indicate a sampling day-specific difference between the two soils (unpaired  $t$  test,  $P < 0.05$ )

**Table 3** Cumulative  $\Sigma\text{CO}_2\text{C}_3$ , MBC3, and total MBN over all N treatments after 7 and 42 days of incubation at 25 °C; probability values of a three-way ANOVA, using soil, millet litter, and N addition as factors

Treatment	$\Sigma\text{CO}_2\text{C}_3$ ( $\mu\text{g g}^{-1}$ soil)		MBC3 ( $\mu\text{g g}^{-1}$ soil)		MBN ( $\mu\text{g g}^{-1}$ soil)	
	Day 7	Day 42	Day 7	Day 42	Day 7	Day 42
Luvisol	59	324	153	235	31	32
Luvisol + Millet	136	508	168	235	33	37
Nitisol	32	156	81	162	14	23
Nitisol + Millet	100	439	104	143	19	26
Probability values						
Soil	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Millet	<0.01	<0.01	0.10	NS	0.05	0.02
N	NS	NS	NS	NS	NS	NS
Soil $\times$ Millet	NS	0.04	NS	NS	0.05	NS
Soil $\times$ N	0.01	NS	NS	NS	NS	NS
Millet $\times$ N	NS	NS	NS	NS	NS	0.08
CV ( $\pm$ %)	22	21	30	17	23	20

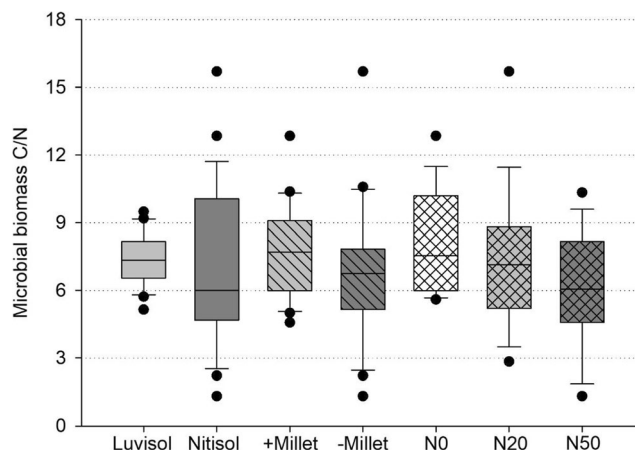
CV mean coefficient of variation between replicates ( $n=4$ ); NS not significant

all differences between the two soils diminished at day 42. At day 7, the MB-C/N ratio of the Nitisol generally exceeded that of the Luvisol (Fig. 1). At this day, the MB-C/N ratio was significantly increased by millet litter, but decreased by N

**Table 4** MB<sup>15</sup>N in the N20 and N50 treatments over the treatments with and without millet litter addition after 7 and 42 days of incubation at 25 °C; probability values of a three-way ANOVA using soil, millet litter, and N addition as factors

Treatment	MB <sup>15</sup> N ( $\mu\text{g g}^{-1}$ soil)	
	Day 7	Day 42
Luvisol-N20	0.7	0.6
Luvisol-N50	2.8	1.8
Nitisol-N20	0.7	0.7
Nitisol-N50	1.5	1.4
Probability values		
Soil	0.01	NS
Millet	NS	NS
N	<0.01	<0.01
Soil $\times$ Millet	NS	NS
Soil $\times$ N	0.01	NS
Millet $\times$ N	NS	0.05
CV ( $\pm$ %)	34	65

CV mean coefficient of variation between replicates ( $n=4$ ); NS not significant

**Fig. 1** Main effects on the microbial biomass C/N ratio at day 7 in the two soils and different treatments of the incubation experiment at 25 °C; probability values of a three-way ANOVA, using soil, millet litter, and N addition as factors: soil:  $P=0.04$ , millet:  $P=0.04$ ; N addition:  $P=0.01$ ; interactions were all not significant

addition. At day 42, all differences diminished and the MB-C/N ratio varied around 7.6, 23% larger than the overall average at day 7 ( $P<0.01$ , paired  $t$  test).

### Carbon use efficiency

None of the different fractions used for calculating CUE differed between the soils in the N0 treatment (Table 5). This was also true for the  $\Sigma\text{CO}_2\text{C}_4$  evolution. For this reason, the microbial CUE values of millet litter varied around 0.63 (Table 5), regardless of soil type. The CUE values did not differ between sampling days 7 and 42, whereas those obtained by the classical CUE<sub>MB</sub> approach declined with incubation time. They were, thus, 20 and 40% smaller than the CUE values, considering MRC4. However, the CUE<sub>MB</sub> also did not differ between the two soils.

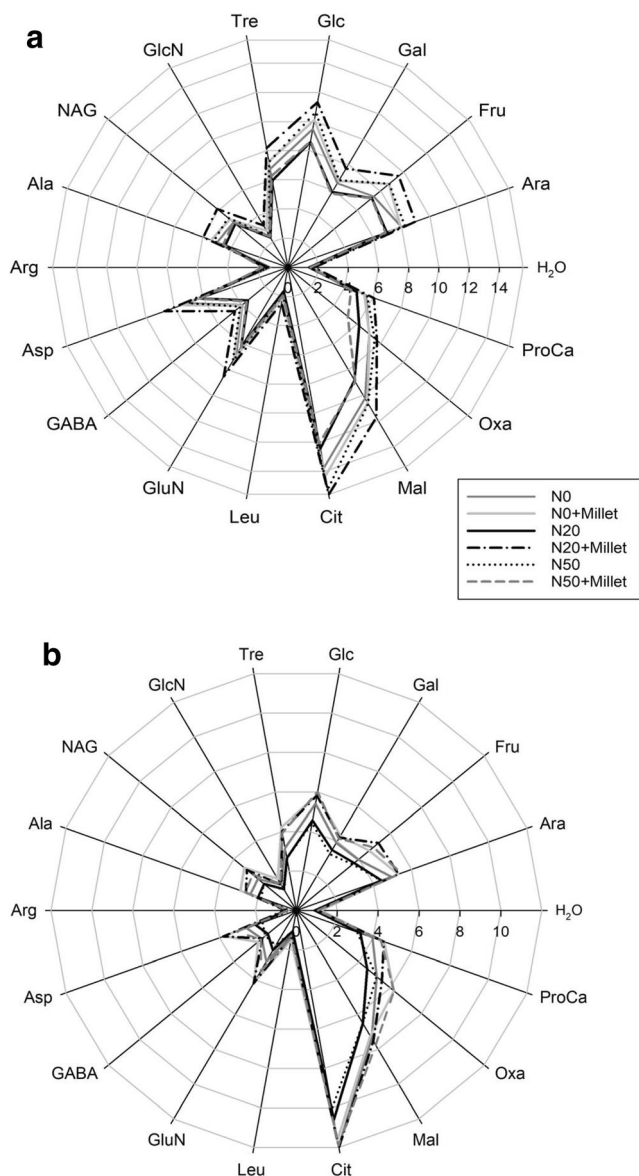
### Multi-substrate-induced respiration

All substrates and water additions resulted in a significantly ( $P<0.01$ ) stronger respiratory response in the Luvisol than in the Nitisol (Fig. 2). Arginine (Arg) addition created the lowest respiratory response, with  $1.43 \mu\text{l CO}_2\text{C g}^{-1} \text{soil h}^{-1}$  in the Luvisol (Fig. 2a) and  $0.44 \mu\text{l CO}_2\text{C g}^{-1} \text{soil h}^{-1}$  in the Nitisol (Fig. 2b). Citric acid (Cit) addition led to the highest respiratory response, with  $13.9 \mu\text{l CO}_2\text{C g}^{-1} \text{soil h}^{-1}$  in the Luvisol (Fig. 2a) and  $11.4 \mu\text{l CO}_2\text{C g}^{-1} \text{soil h}^{-1}$  in the Nitisol (Fig. 2b). Millet addition significantly ( $P<0.03$ ) increased the respiratory response of most substrates on average by 14% in both soils, except that of arginine and oxalic acid (Fig. 2). In contrast, N addition had no effect on the respiratory response of most substrates, except a mean significant ( $P<0.04$ ) reduction of glutamine (GluN), leucine (Leu), asparagine (Asp), and protocatechuic acid (ProCA) by approximately 7%. In

**Table 5** Calculation of CUE values at sampling days 7 and 42 in the N0 treatment; CUE has been calculated as:  $(MBC4 + MRC4)/(100 - POMC4)$ ,  $CUE_{MB}$  has been calculated as  $MBC4/(CO_2C4 + MBC4)$

Soil	$\Sigma CO_2C4$ (% added substrate C)	MBC4	MRC4	POMC4	CUE	$CUE_{MB}$
Day 7						
Luvisol	7.1	3.3	14.4	73.2	0.73	0.33
Nitisol	5.0	4.0	8.2	83.8	0.68	0.42
Day 42						
Luvisol	25.8	4.7	38.1	32.2	0.62	0.15
Nitisol	19.5	3.0	48.0	29.3	0.72	0.14
CV ( $\pm$ %)	27	48	48	8	14	42

CV mean coefficient of variation between replicates ( $n = 4$ )



**Fig. 2** Radar charts of the substrate-induced  $CO_2$  respiration rate ( $\mu l CO_2C g^{-1} soil h^{-1}$ ) for 17 substrates plus  $H_2O$  added to **a** temperate soil samples (Luvisol) and to **b** tropical soil samples (Nitisol)

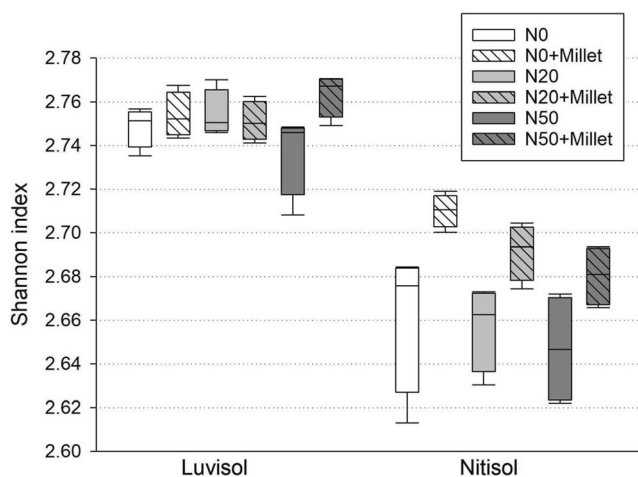
contrast to soil  $\times$  millet, numerous significant ( $P < 0.05$ ) millet  $\times$  N addition interactions were observed, because the respiratory response of the N20 treatment was significantly reduced for most substrates in the Luvisol.

The Shannon indices of all Luvisol treatments exceeded those of the respective Nitisol treatments (Fig. 3). In the Nitisol, millet addition increased the Shannon index, whereas N addition had negative effects. Discriminant function 1a strongly separated the functional diversity of the Luvisol from that of the Nitisol (Fig. 4a), whereas discriminant function 2a weakly separated the treatments with and without millet addition solely in the Nitisol. Discriminant function 1b separated the functional diversity between the N20 and N50 treatments (Fig. 4b), whereas discriminant function 2b separated the N0 from the N20 and N50 treatments.

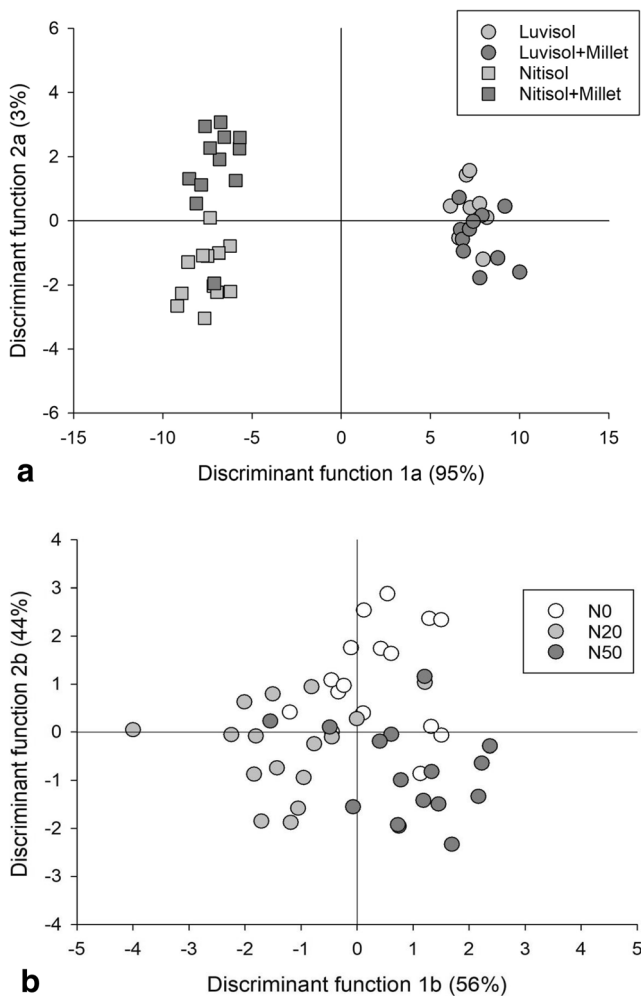
## Discussion

### N effects on litter decomposition

N addition had no effect on millet litter decomposition, indicating that neither of the soils were N limited. The lower millet decomposition rate of the Nitisol is most likely caused by the shortage of nutrients other than N, P, and K, which have all been applied as fertilizers in India. Consequently, it is more likely that SOC and MBC levels are generally low (de Castro Lopes et al. 2013; Fang et al. 2018; Luo et al. 2018). There were also no indications that N addition has general increasing effects on litter decomposition, MBC and MBN in both soils. However, the MB-C/N ratio declined with increasing N addition rate. This suggests an additional N uptake without physiological demand of soil microorganisms in both soils (Rasul et al. 2009), e.g. in



**Fig. 3** Boxplots of the Shannon diversity index for the two soils with different fertilizer levels and millet litter addition; probability values of a three-way ANOVA, using soil, millet, and N addition as factors: soil:  $P < 0.01$ , millet:  $P < 0.01$ ; N addition: not significant; soil  $\times$  millet:  $P = 0.01$ , soil  $\times$  N: not significant; millet  $\times$  N: not significant



**Fig. 4** Discrimination function analysis (DFA) of catabolic response of soil communities to 17 substrates plus H<sub>2</sub>O for **a** millet discrimination and **b** fertilizer discrimination

fungal vacuoles (Klionsky et al. 1990; Khan and Joergensen 2019). However, the uptake of mineral N was low and did not change from day 7 to 42; an exception was the N50 treatment of the Luvisol, indicating low microbial immobilisation rates of inorganic N. Microbial uptake rates for manure N were higher, at 10% and 5% after 2 and 6 weeks, respectively (Bohlen et al. 1999). N addition did not cause a shift in functional diversity according to the MSIR approach.

### Litter-induced priming of SOM mineralisation

The current increase in  $\Sigma\text{CO}_2\text{C}_3$  evolution derived from autochthonous SOM mineralisation after millet litter addition is a strong true positive priming effect (Kuzyakov et al. 2000). This was apparently not caused by N mining, as N addition did not reduce the priming response. However, the possibility cannot be excluded that in other cases N mining might be an important reason for priming effects (Dijkstra et al. 2013; Mason-Jones et al. 2018; Tian et al. 2019). The significant

soil  $\times$  N interaction for  $\Sigma\text{CO}_2\text{C}_3$  at day 7 in the Luvisol points to the possibility of N mining even in the current experiment. However, the absolute priming effect was similar in both soils, i.e. it was not related to different MBC and SOC levels but to the identical millet litter addition rate.

A large part of litter mineralisation is usually carried out by surface colonizing microorganisms (Flessa et al. 2002; Potthoff et al. 2005). In the current study, the extracellular enzymes produced by millet litter decomposing saprotrophic fungi were most likely also able to break down SOC as a co-substrate (Scheller and Joergensen 2008; Maynard et al. 2017; Finley et al. 2018). This means that priming mechanisms strongly differ between simple amendments, such as glucose or complex plant residues (Wu et al. 1993; Finley et al. 2018; Hicks et al. 2019). This might explain why true priming effects have rarely been observed under field conditions.

### CUE calculations and constraints

A mean CUE of 0.63, considering MRC, indicates the strong ability of soil microbial communities to use the organic substrates that enter soil nearly as efficiently as glucose (Joergensen and Wichern 2018). This mean CUE value is in line with those reviewed by Joergensen and Wichern (2018), which were obtained by much longer incubation times of between 56 (Muhammad et al. 2006; Zareitalabad et al. 2010) and 57 days (Rottmann et al. 2010). The absence of a significant difference in CUE values of millet litter between days 7 and 42 is remarkable. Between these two sampling dates, MBC slightly increased, whereas MRC exponentially increased with time. As the classical approach does not consider this formation of MRC, the  $\text{CUE}_{\text{MB}}$  values decline with time, indicating serious limitations (Joergensen and Wichern 2018). This exclusion of MRC from CUE calculations may cause the effects of nutrient supply (Fang et al. 2018), substrate quality (Öquist et al. 2017), temperature (Li et al. 2019; Qiao et al. 2019; Ye et al. 2019), and clay content or soil pH (Fang et al. 2018; Jones et al. 2019; Zheng et al. 2019) on CUE values.

However, other incubation periods than the two used in the current study might be even more appropriate for determining CUE values of plant residues, due to minor methodological constraints of the current approach. An incubation period of 7 days might be too short for soils with low turnover rates or for substrates that need more time to be colonised by decomposers (Eck et al. 2015). In this case, it is possible that not enough POMC was lost within 7 days to obtain a significant decline in comparison with initial values. Another problem is that only small MRC values might be formed within 7 days, as in the current Nitisol. The assumption that all POM recovered is non-decomposed substrate is not fully true, as all plant litter is colonised by microorganisms, which account for up to 2% of organic matter (Potthoff et al. 2008; Scheller and Joergensen 2008; Rottmann et al. 2011). This percentage is



roughly counterbalanced by the negligible substrate losses during sieving, as indicated by the high day-0 recovery of 95% and more.

The current results contradict the view that the CUE declines with increasing ratio of substrate to MBC (Bardgett and Sagar 1994; Witter and Kanal 1998; Chander and Joergensen 2001). This view is probably less important for plant litter than low-molecular-weight substances such as glucose (Bremer and Kuikman 1994).

### Differences in functional diversity between the soils

In the N0 treatment, the  $\Sigma\text{CO}_2\text{C}_4$  evolution did not differ between the two soils (Table 5), contrasting the results of Delgado-Baquerizo et al. (2015), who observed strong soil type effects on leaf litter decomposition. However, less millet litter added was ultimately mineralised to  $\text{CO}_2$  in the Nitisol than in the Luvisol, averaging all N treatments (Table 1). This observation might be explained by the negative effects of N addition on the functional diversity expressed as the Shannon index (Fig. 3).

The Luvisol exhibited a higher functional diversity in combination with a higher respiratory response to most of the substrates added by the MSIR approach (Fig. 2). The temperate Luvisol with its higher MBC and SOC levels seems to buffer millet litter and N addition effects without further changes in functional diversity, which is in line with the study of van der Boma et al. (2018). However, the lower buffering capacity of the Nitisol led to a more variable response to the experimental treatments. This means that the functional diversity of the tropical Nitisol responded positively to the millet litter addition but negatively to N addition. Consequently, the Nitisol might also react rapidly to a continuous higher supply of organic matter.

### Conclusions

Nitrogen addition did not generally affect millet litter decomposition but reduced its mineralisation to  $\text{CO}_2$  in the tropical Nitisol. This reduction is in line with the negative N effects on the functional diversity of the Nitisol. The strong priming effect on autochthonous SOC mineralisation was apparently not caused by N mining but using SOC as a co-substrate after adding easily available millet litter. In contrast to N addition, millet litter increased the functional diversity of the Nitisol but not that of the Luvisol. The functional diversity of the temperate Luvisol was generally higher than that of the Nitisol and was not affected by millet litter or by N addition. This suggests that improved soil fertility is not only expressed by SOC and MBC but also by a higher functional diversity, all increasing the buffering capacity of a soil to environmental changes. Without N addition, similar percentages of millet leaf litter

were mineralised to  $\text{CO}_2$ , recovered as POM, and incorporated into MBC. Consequently, also the microbial CUE values of millet litter, including microbial residue C, did not differ between the two soils. They varied around 0.63, regardless of whether the incubation period was 7 or 42 days. However, the optimum incubation time within this period still needs further evaluation. Future research should also test the effects of plant residues, differing in N and lignin concentrations, on CUE values in a larger range of soils, especially also under field conditions.

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