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Residual nitrogen from slurry and mineral fertiliser two years after application: Fractionation and plant availability

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ABSTRACT

Fertilisation with animal manure has a long-standing tradition as a way to close nutrient cycles on farms. However, the fate of nitrogen (N) from manure in the soil beyond the year of application remains poorly understood. The aim of this research was to understand the residual N fertiliser value of mineral fertiliser (Min) and cattle slurry (Slu) in relation to the partitioning of N from the fertilisers in different soil physical fractions. To this end, we characterised the fate of ^{15}N-labelled Min and Slu in soil organic matter (SOM) physical fractions two years after field application and related it to plant uptake of residual N. A secondary objective was to compare two fractionation methods with respect to accuracy and easiness, namely a combined density and particle size fractionation, separating five fractions, and a simple particle size fractionation, separating two fractions. All fractions were analysed for ^{15}N abundance. The residual N fertiliser value was determined as the uptake of ^{15}N by ryegrass (*Lolium multiflorum*) during 6 weeks. Furthermore, we deduced the source of ^{15}N taken up by the plants from changes in the percentage of N derived from labelled fertilisers in each of the SOM physical fractions obtained by simple size fractionation before and after the pot experiment. Two years after application, most ^{15}N was found in the fractions <20 μm, for both fractionation methods, i.e. in the mineral-associated organic matter (MAOM). The ^{15}N recovery in these fractions in percent of the quantity of ^{15}N originally applied as fertiliser tended to be higher for Slu (density-size fractionation: 19.8 ± 6.6%; simple size fractionation: 25.1 ± 6.6%) than for Min (density-size fractionation: 12.0 ± 2.1%; simple size fractionation: 16.8 ± 2.6%). Irrespective of the fertiliser type, about 2% of applied ^{15}N was available to ryegrass plants two years after application. Out of these 2%, most ^{15}N in the plants originated from MAOM, suggesting that the availability of the N stored in MAOM could be larger than previously thought. This finding is in line with the emerging view that MAOM is a dynamic fraction that plays an important role in the N cycle.

1. Introduction

Animal manure is a valuable alternative to mineral fertilisers as it can help closing nutrient cycles on-farm while increasing soil fertility (Rayne and Aula, 2020). Nevertheless, N from animal manure has been shown to have a consistently low N use efficiency (NUE) (Smith and Chalk, 2018). N losses typically occur through nitrate (NO_3^-) leaching, ammonia (NH_3) volatilisation, nitrous oxide (N_2O) emissions, or dinitrogen (N_2) emissions from full denitrification, causing harm to the environment and resulting in a loss of productivity. Mineral fertilisers contain solely inorganic N forms, while animal manure contains 30–75% of N in organic forms (Webb et al., 2013). As a result of numerous interacting biotic and abiotic processes controlling N transformations in soils, the fate of N from animal manure is difficult to predict in the short-term as well as in the long-term. Gaining a better understanding of the fate of applied N fertilisers in the soil may help to minimise N losses by preventing asynchrony between crop N requirements and N release (Crews and Peoples, 2005).

Since SOM is the main N reservoir in soils, understanding SOM formation and interactions of SOM and fertilisers is essential for optimising N fertilisation practices. In the past, SOM was thought to be protected from degradation mainly by its inherent chemical recalcitrance (Kleber and Johnson, 2010). However, new evidence shows that physical protection through occlusion within aggregates and chemical protection

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within organo-mineral associations are more important (Dungait et al., 2012). Besides, there is growing evidence that up to half of newly formed SOM derives from microbial necromass (Liang et al., 2019) consisting of cell wall envelopes of fungi and bacteria (Miltner et al., 2012). In soils under grassland and arable crops, microbial compounds often predominate in newly formed SOM, while plant compounds are more abundant in forest soils (Angst et al., 2021). Considering the newly formed necromass as an important factor of SOM formation could help to better understand N retention after application of inorganic and organic fertilisers. Indeed, increased abundance of microorganisms and functional diversity are observed after substitution of mineral fertiliser by animal manure (Luan et al., 2020), potentially due to the addition of labile carbon (C). This might lead to increased immobilisation of N in microbial biomass and in turn more N retention in SOM. In this context, it is not well understood if mineral fertiliser and animal manure differ with respect to mechanisms of N protection and incorporation into SOM.

Various SOM fractionation methodologies were developed with the aim of defining functionally distinct pools for C dynamics. As soil C and N are highly intertwined (Castellano et al., 2012), these SOM fractionation schemes are also used to study N dynamics. Multiple methods are in use, but in a recent comparison of 20 fractionation methods, Poeplau et al. (2018) showed that a combination of physical (density, particle size) and chemical (oxidation, extraction) fractionation is best suited to yield C fractions with distinct turnover times. However, these methods are often costly and laborious and therefore not widely implementable. With the intention of standardising and simplifying the approach, Lavallee et al. (2020) suggested distinguishing only two fractions, namely particulate organic matter (POM) and mineral-associated organic matter (MAOM). While POM consists of plant- and fungal-derived compounds that are readily available to microbes but with high activation energies (Williams et al., 2018), MAOM is made of low molecular weight compounds of microbial and plant origin, which may be assimilated by plants and microbes after dissociation from mineral complexes (Jilling et al., 2018).

The physical fractionation of SOM has been used recently to gain a better understanding of the fate of organic amendments in soil. For example, increased N contents in MAOM fractions (<1.7 g cm^{-3}) were observed after nine years of continuous animal manure application (Samson et al., 2020). Similarly, Jilling et al. (2020) reported increased N content in coarse silt (>20 μm) and MAOM (<20 μm) fractions three years after inclusion of cover crops into the rotation. Bosshard et al. (2008) used ^{15}N isotope techniques to trace the incorporation of ^{15}N-labelled sheep faeces, urine and mineral fertiliser in SOM fractions. About four months after application, most ^{15}N was found in the mineral-associated fraction (<50 μm), irrespective of the applied fertilisers. Across studies, fractionation methods and amendment types vary but are rarely compared. Most importantly, although these studies provide insights into the fate of organic and inorganic fertilisers in SOM physical fractions, the residual N fertiliser value has not been studied in relation to SOM physical fractions. In our view, unravelling the relation of SOM physical fractions to plant N uptake is essential for improving our understanding of residual fertiliser effects.

MAOM is generally considered as the primary sink for C and N in soils because sorption to primary particles <20 μm (fine silt and clay) regulates the amounts of C and N residing in the soil (Hassink, 1997). Although POM is generally considered an important source of labile N, its relevance as a meaningful fraction has been debated (Curtin et al., 2019). To gain a better understanding of the relative contribution of POM and MAOM fractions to supply N to plants and microbes, Daly et al. (2021) recently proposed a conceptual framework where the nutritional role of POM and MAOM fractions depends on the ratio between N mobilised from POM and the mineral sorption potential. They argue that the mineral sorption potential is governed by soil colloid properties, soil texture, and the chemistry and quantity of MAOM and N in the soil solution. They describe three main scenarios: i) most N is immobilised when the supply of POM-derived N is smaller than the mineral sorption potential, ii) MAOM-derived N becomes the main source of bioavailable N when supply of POM-derived N and mineral sorption potential are balanced, and iii) POM is the main source of bioavailable N when the supply of POM-derived N is larger than the mineral sorption potential. This however, has not been empirically tested.

The overall objective of this study was to understand the residual N fertiliser value of mineral fertiliser and cattle slurry in relation to the partitioning of N from the different amendments in different soil physical fractions. To this end, we characterised the fate of ^{15}N-labelled amendments (mineral fertiliser (Min) and cattle slurry (Slu)) in SOM physical fractions two years after field application. Then, we assessed the uptake of residual N by ryegrass plants grown in these soils in a pot experiment in relation to its distribution in POM and MAOM. A secondary objective was to compare the results from two fractionation methods in determining the fate of N into different fractions. We formulated three hypotheses: (1) for both Min and Slu, we expected to find the majority of residual ^{15}N in the mineral-associated fraction, and that this proportion would be greater for Slu than for Min because of increased microbial immobilisation after application of an organic fertiliser; (2) we expected that most ^{15}N taken up by plants would originate from POM, which is assumed to be very dynamic and often considered the primary source of plant available N; (3) as a result of the first two hypotheses, plants would recover more residual ^{15}N from Min than from Slu.

2. Material and methods

2.1. Experimental design

The soil for the present study was collected from a field site in Rickenbach in the canton of Solothurn, Switzerland. The region has a mean annual temperature of 9.0 °C and a mean annual precipitation of 1129 mm (1981–2010). The soil at the experimental site is a loamy Cambisol (WRB, 2015) (22% clay, 43% silt, 35% sand) and the initial soil pH (H_2O) was 6.7. The 12 microplots (1.5 m × 2 m each) from which soil samples were collected had been installed in April 2018 in the frame of a field experiment on nitrate leaching from animal manure, using ^{15}N-labelled fertilisers (Frick et al., 2022a). Three fertiliser treatments (0N- Control (Con), ^{15}N-labelled cattle slurry (Slu) and ^{15}N-labelled ammonium nitrate fertiliser (Min)) were compared in a randomised complete block design with four replicates. The crop rotation of the field experiment was as follows: grass-clover ley (2018/19) – silage maize (2019) – winter wheat (2019/20).

In 2018, four experimental fertiliser applications with ^{15}N-labelled fertilisers were performed each time after cutting the grass-clover. Since the aim was to apply the same amount of mineral N with both fertiliser treatments, cumulative amounts of total N applied were lower in Min (147 kg N ha^{-1}) than in Slu (240 kg N ha^{-1}) (SI, Table S1). The ^{15}N abundance of the amendments were 8.00 and 7.89 atom% for Min and Slu, respectively. The ^{15}N-labelled fertilisers were applied in 2018 only, while the whole field, including microplots and irrespective of fertiliser treatment, was fertilised with unlabelled fertilisers according to the farmer's practice in the following years. At the end of the experiment (July 2020, 26 months after the first application of labelled amendments), the share of ^{15}N from the initially applied quantities of labelled fertilisers recovered in the bulk soil (0–30 cm layer) was 20% for Min and 44% for Slu (Frick et al., 2022b).

Soil samples (0–30 cm depth) were collected with a shovel from the centre of each microplot on 22/07/2020, subsequently sieved to 2 mm and air-dried.

2.2. Soil organic matter fractionation

Two fractionation methods were compared to gain a better understanding of ^{15}N losses during the fractionation process. The first method, adapted from Steffens et al. (2009), is a combination of density and

particle size fractionation, which distinguishes five SOM fractions. The second one, adapted from Cotrufo et al. (2019), is a particle size fractionation that distinguishes only two fractions. The second method was chosen to investigate the possibility to simplify fractionation schemes, given that it is cheaper and can be widely implemented.

The method developed by Steffens et al. (2009) was slightly adapted by excluding the last step which further separates the fraction <20 μm. We chose not to include this step because sorption of SOM to minerals is the dominant mechanism within the entire fraction (Hassink, 1997). Therefore, the fractionation scheme, referred to as density-size fractionation, consisted of five fractions as depicted in Fig. 1.

Briefly, 30 g of 2 mm sieved, air-dried soil was weighed into a crystallising dish. The free POM (fPOM) fraction was separated using a sodium polytungstate (SPT, $Na_6H_2W_{12}O_{40}$, low N content) solution with a density of 1.8 g cm^{-3} as heavy liquid for separation. The particles floating at the surface of the SPT solution (referred to as fPOM) were aspirated with a vacuum pump. To obtain the POM occluded in aggregates (oPOM), the remaining heavy fraction was treated with ultrasound for aggregate dispersion (Sonopuls HD 2200.2 homogeniser, Bandelin Electronic, Berlin, Germany). We performed pre-tests with a range of different dispersion energies (25, 50, 75, 100, 150, 200, 300, 400 and 500 J ml^{-1}) to identify the optimal dispersion energy for the soil used. According to Griepentrog and Schmidt (2013), the dispersion energy has a significant effect on the SOM fractions obtained and should be adjusted to the texture and the C content of the samples. The target dispersion energy should yield the maximum C content in the oPOM fraction, indicating minimal contamination with minerals and organic material from heavy fractions. In our pre-tests, the maximum amount of oPOM-C was retrieved with a dispersion energy of 100 and 150 J ml^{-1}, while lower and higher energies decreased C retrievals (SI, Fig. S1). Based on the pre-tests, the dispersion energy was set at 100 J ml^{-1}, equivalent to an ultrasonic treatment duration of 5 min and 44 s. The tip of the Sonotrode (Model VS 70T) was immersed 30 mm into the soil suspension to ensure complete turbation of sample material. During sonication, the soil suspension was cooled with ice to avoid overheating which could alter chemical composition. After sonication, samples were centrifuged at 8500 rpm for 10 min and the density fractionation was repeated as described above in order to extract the oPOM. fPOM, oPOM and the remaining mineral fractions were rinsed with bi-distilled water to avoid contamination with nutrients from the SPT solution. The remaining mineral soil was wet-sieved with bi-distilled water through a 63 μm and a 20 μm sieve until the liquid that passed the sieves was clear. The following three fractions were obtained: sand (2000-63 μm), coarse silt (cSilt; 63-20 μm) and fine silt/clay (fSilt-c; <20 μm). To ensure water evaporation from the mineral fractions within a reasonable timespan, care was taken to keep the total amount of water used for the wet-sieving procedure below 2 L. The organic fractions were lyophilised with a freeze dryer (Alpha 1–4 LSC, Martin Christ GmbH, Osterode am Harz, Germany), and the mineral fractions were oven-dried at 60 °C. Finally, all fractions were weighed and ground for ^{15}N analysis.

The method developed by Cotrufo et al. (2019) (referred to as simple size fractionation) was adapted by lowering the limit between the two fractions (POM and MAOM) from 53 μm to 20 μm (Fig. 1). This was done to allow for a better comparison of the two methods and is justified by the fact that the mineral fraction <20 μm was shown to be a good indicator of the potential for stable SOC sequestration (Hassink, 1997), which was subsequently used as an estimate for maximum C storage capacity in different soils (Wiesmeier et al., 2014, 2015). Briefly, 5 g of 2 mm oven-dried soil was shaken in a 0.5% sodium polyphosphate $(NaPO_3)_n$ solution with 12 glass beads (⌀ 5 mm) for 18 h at 125 rpm to disperse the soil. After that, the dispersed soil was rinsed onto a 20 μm sieve until the liquid that passed the sieve was clear. The fraction passing through (<20 μm) was considered as MAOM whereas the fraction remaining on the sieve was collected as POM. Both fractions were then dried at 60 °C, weighed and ground for ^{15}N analysis as described above.

2.3. Greenhouse pot experiment

A pot experiment was carried out in a greenhouse at the Research Institute of Organic Agriculture (FiBL, Switzerland) under light- and

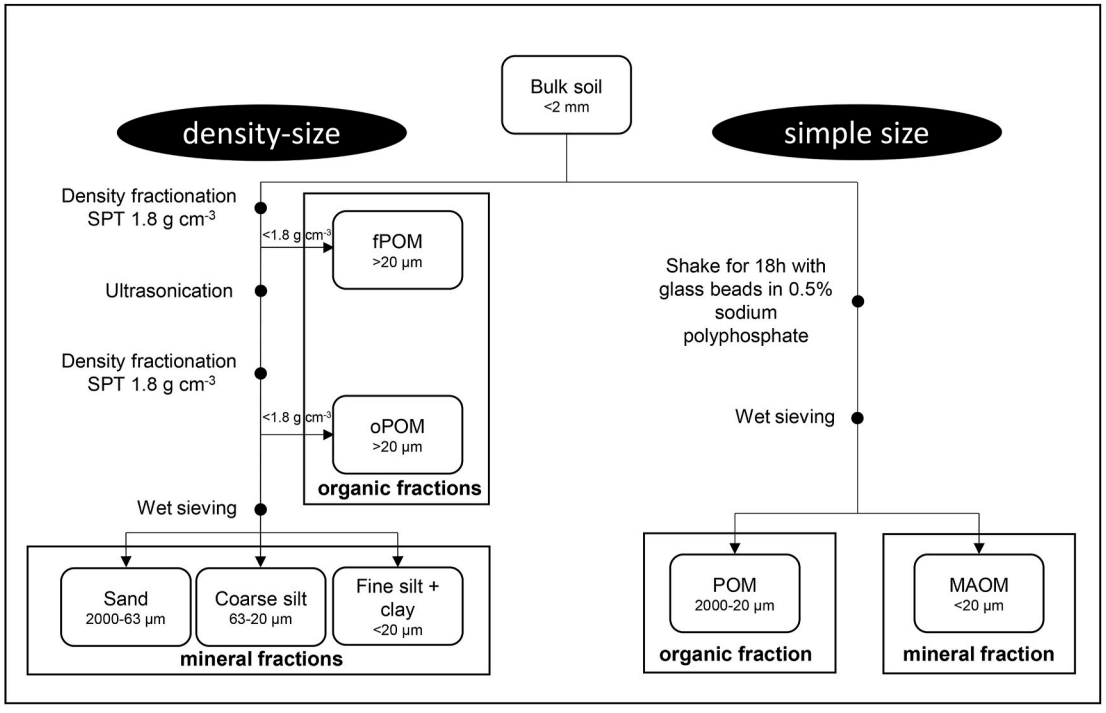

Fig. 1. Density-size fractionation scheme adapted from Steffens et al. (2009) resulting in five fractions (left side) and simple size fractionation scheme adapted from Cotrufo et al. (2019) resulting in two fractions (right side). SPT, sodium polytungstate; fPOM, free particulate organic matter; oPOM, occluded particulate organic matter; POM, particulate organic matter; MAOM, mineral-associated organic matter.

temperature-controlled conditions for six weeks (14 h light per day, heating temperature D/N 15 °C/13 °C, and heating ventilation threshold at 20 °C). The soil samples collected at the end of the microplot experiment were pre-incubated in plastic bags for 1 week at room temperature at a gravimetric water content of 15 g H_2O per 100 g dry soil, equivalent to 30% of maximum water holding capacity (WHC). Before filling the pots, an N free nutrient solution (modified Hoagland solution; SI, Table S2) was added and thoroughly mixed into the soil to ensure no other limiting factors than N. The following amounts of nutrients were added (in mg kg^{-1} dry soil): 50 P, 250 K, 102 Ca, 48 Mg, 2 Cu, 2 Mn, 1 Zn, 1 B, 1 Fe, 0.1 Mo, 0.1 Co. Four cylindrical pots (10.5 cm in diameter, 7.5 cm deep, 400 ml, with a plastic bag inside to avoid leaching) were filled with soil from each of the 12 microplots at a bulk density of 1.1 g cm^{-3}, resulting in 48 pots. Ryegrass seeds (*Lolium multiflorum*, variety Pulse) were sown at a density of 30 g m^{-2}. To enable optimal germination, seeds were covered with a thin layer of soil and the pots were covered with a plastic film for 2–3 days to maintain moisture during germination. All pots were weighed daily to keep them at 60% of maximum WHC. The pots were arranged in two blocks to account for any difference in growing conditions in the greenhouse. Within each block, pots were randomly shuffled each week.

After six weeks, the pots were harvested, and shoot (including stubble) and root dry weight were measured. To collect root-free soil and roots, pots were turned upside down above an aluminium tray, roots were gently shaken and non-adhering soil was sampled. Subsequently, roots were washed with water on a 1 mm-sieve and oven-dried. Shoot and root samples were finely ground with a ball mill (MM200, Retsch GmbH, Haan, Germany) prior to ^{15}N analysis. To assess from which SOM fractions ^{15}N was taken up by the ryegrass plants, the simple fractionation was repeated on root-free soil collected at the end of the pot experiment, and resulting POM, MAOM and bulk soil were analysed for ^{15}N abundance.

2.4. Analyses and calculations

Plant and soil samples were analysed for total C, total N and ^{15}N abundance on a Thermal Conversion Elemental Analyzer (Vario Pyro Cube, Elementar GmbH, Langenselbold, Germany) coupled to a continuous flow Isotopic Ratio Mass Spectrometer (Isoprime 100, Elementar GmbH, Langenselbold, Germany).

The ^{15}N atom% excess represents the ^{15}N abundance of the sample minus the natural abundance of the reference samples. In this study, samples from the Con treatment to which no ^{15}N had been applied were used as reference samples.

The proportion of N derived from the ^{15}N-labelled fertiliser ($Ndff_{\%}$, expressed as a percentage) for Min and for Slu was used as a proxy of the proportion of total N within each plant or soil sample originating from the fertiliser. It was calculated according to isotope pool dilution principles (Hauck and Bremner, 1976):

$$Ndff_{\%} = \left(\frac{^{15}Nex_{sample}}{^{15}Nex_{lf}}\right) \times 100 \quad (1)$$

where $^{15}Nex_{sample}$ represents the atom% ^{15}N excess of the plant or soil sample, and $^{15}Nex_{lf}$ represents the atom% ^{15}N excess of the labelled fertiliser.

To calculate the absolute quantity of N derived from the labelled fertiliser per kg soil ($Ndff_{mass}$, expressed in mg N kg^{-1} soil) in the plant or soil samples, the following formula was used:

$$Ndff_{mass} = \left(\frac{^{15}Nex_{sample}}{^{15}Nex_{lf}}\right) \times TN \quad (2)$$

where TN (expressed in mg N kg^{-1} soil) was calculated by dividing the total N in the sample by the amount of dry soil per pot (0.375 kg dry soil). For the soil fractions, the total N in the fraction was divided by the quantity of dry soil weighed before the fractionation procedure.

The ^{15}N recovery ($^{15}N_{recov}$), which is expressed as a percentage and refers to the proportion of initially applied ^{15}N recovered in the plant or soil samples at a given time, was calculated as follows:

$$^{15}N_{recov} = \left(\frac{Ndff_{mass}}{Nf}\right) \times 100 \quad (3)$$

where *Nf* is the amount of initially applied N in the field in mg kg^{-1} soil. It was calculated by dividing the total amount of N applied per ha by the mass of soil per ha in the 0–30 cm layer (soil volume multiplied by bulk density), resulting in a N dose of 33.4 mg N kg^{-1} soil for the mineral fertiliser and 55.2 mg N kg^{-1} soil for the cattle slurry.

The plant uptake of residual ^{15}N in the bulk soil ($^{15}N_{resid}$) represents the proportion of plant N derived from the fertiliser N present in the bulk soil at the beginning of the pot experiment. It was calculated as follows:

$$^{15}N_{resid} = \left(\frac{Ndff_{mass}\ plant}{Ndff_{mass}\ bulk\ soil}\right) \times 100 \quad (4)$$

where $Ndff_{mass}$ plant (mg N kg^{-1} soil) represents the quantity of N derived from the fertiliser in the plant sample (shoots or roots) and $Ndff_{mass}$ bulk soil (mg N kg^{-1} soil) represents the quantity of N derived from the fertiliser in the bulk soil at the beginning of the pot experiment.

2.5. Statistical analyses

Statistical analyses were conducted using RStudio version 1.4.1103 (RStudio Team, 2020) coupled to R version 4.2.2. The following packages were used: lme4 (Bates et al., 2015), lmerTest (Kuznetsova et al., 2017), emmeans (Lenth, 2022), lattice (Sarkar, 2008), car (Fox and Weisberg, 2019), MuMIn (Bartoń, 2022) and ggplot2 (Wickham, 2016). A significance of $p < 0.05$ was used throughout all statistical analyses. Two-way ANOVA was used to compare the effect of fertiliser treatment and fraction on the following parameters: N concentration, mass distribution, total retrieved N and C:N ratio. In case of significant effects, separation of means was tested using Tukey's HSD post-hoc test or with Kruskal-Wallis test when residuals were not normally distributed. Student's t-test was used for comparing Min and Slu for $Ndff_{\%}$ and $^{15}N_{recov}$ in SOM physical fractions.

Linear mixed effect models with random intercept were used to assess the effect of different fertiliser treatments on dry weight, $^{15}N_{recov}$, $Ndff_{\%}$ in the plants and $^{15}N_{resid}$. Fertiliser treatment was analysed as fixed effect whereas the field block was treated as random effect. In case of significant effects, Tukey's HSD post-hoc test was used for multiple comparisons. Furthermore, a linear mixed effect model with random intercept was used for assessing the relationship between ^{15}N uptake by plants and the ^{15}N content of the different SOM fractions at the beginning of the pot experiment. The ^{15}N content of the different SOM fractions was treated as fixed effect and the field block as random effect. Marginal and conditional R^2 were calculated according to Nakagawa and Schielzeth (2013) with the r.squaredGLMM function from the MuMIn package. To estimate from which SOM fractions ^{15}N was taken up by ryegrass plants, the difference in $Ndff_{mass}$ in the fractions was calculated by subtracting the $Ndff_{mass}$ after the pot experiment from the $Ndff_{mass}$ before the pot experiment. A linear mixed effect model with the difference in $Ndff_{mass}$ as fixed effect and field block as random effect was used to analyse differences between the Min and Slu treatments. For all statistical analyses, normality and homoscedasticity of residuals were assessed both visually and using Shapiro-Wilk test and Levene's test.

3. Results

3.1. Fate of ^{15}N in SOM physical fractions

With both fractionation methods, most of the originally applied ^{15}N ($^{15}N_{recov}$) was recovered in the fraction <20 μm (Fig. 2). Furthermore,

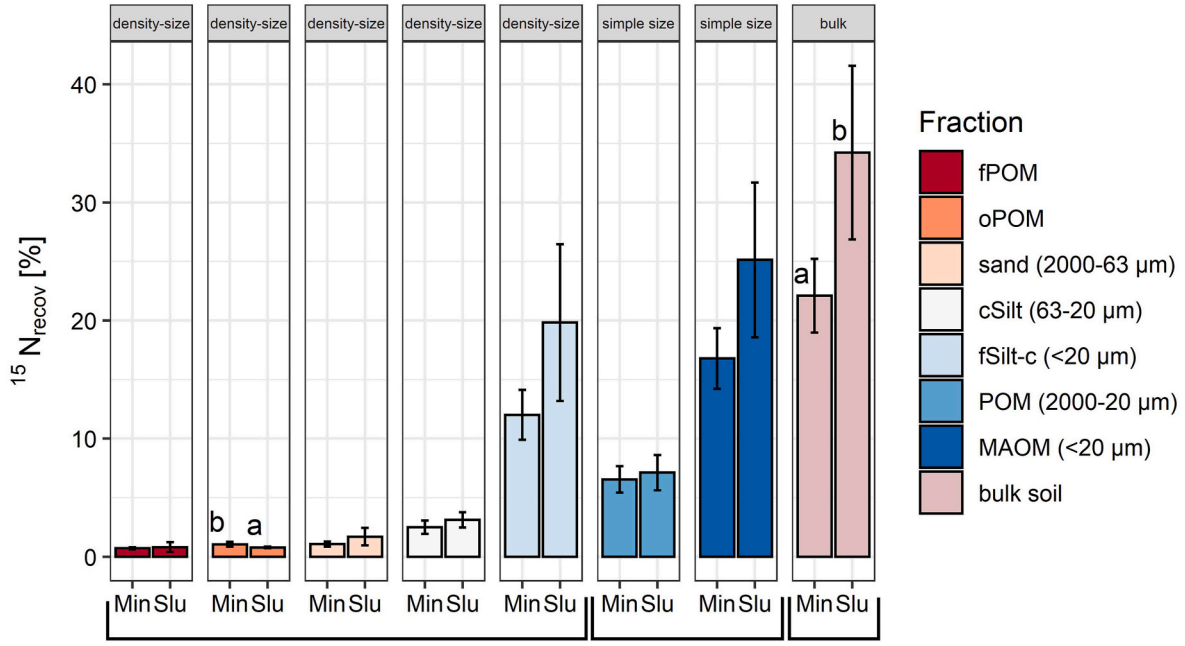

Fig. 2. Recovery of ^{15}N in percent of the original quantity of ^{15}N applied with mineral fertiliser (Min) or slurry (Slu) in SOM physical fractions obtained by density-size and simple size fractionation methods and in bulk soil. Error bars represent standard deviation (n = 4) and different letters indicate significant differences between fertiliser treatments ($p < 0.05$) by Student's t-test.

the $^{15}N_{recov}$ in the bulk soil was significantly greater for Slu (34.1 ± 7.3%) than for Min (22.1 ± 3.1%) ($p = 0.023$). A similar pattern was observed in fSilt-c and MAOM, although the difference between Slu and Min was not significant (fSilt-c: $p = 0.066$, MAOM: $p = 0.056$). Among the other fractions, only oPOM showed significant differences between fertiliser treatments, with higher $^{15}N_{recov}$ in Min (1.1 ± 0.2%) than Slu (0.8 ± 0.1%) ($p = 0.048$).

3.2. Availability of N from SOM

Shoot dry weight was similar in all fertiliser treatments, while root dry weight was significantly greater for Slu than for Con and Min ($p < 0.001$; Table 1). Approximately two thirds of total N in the plant was stored in the shoots. Shoot N content was similar in all fertiliser treatments, while root N content was greater for Slu than for Con and Min.

The $Ndff_{\%}$ in total plant biomass was small, but significantly greater for plants grown in Slu (2.2 ± 0.6%) than those grown in Min (1.1 ± 0.2%) ($p = 0.016$), with similar values found for shoots and roots. The recovery of ^{15}N based on the quantities of ^{15}N fertiliser applied two years before tended to be greater for Slu than for Min both in the shoots and in the roots, but the difference was not significant, even for total biomass ($p = 0.085$).

A clear positive relationship was observed between the quantity of N derived from the fertilisers ($Ndff_{mass}$) in the plants and the $Ndff_{mass}$ in SOM physical fractions before the pot experiment (Fig. 3). In particular, $Ndff_{mass}$ in the plants was positively correlated to the $Ndff_{mass}$ in the bulk soil as well as in the individual fractions, except fPOM and oPOM.

To further assess from which fractions the ^{15}N was taken up by the ryegrass, the absolute difference in $Ndff_{mass}$ in POM and MAOM before and after the pot experiment was calculated (Fig. 4). $Ndff_{mass}$ in both POM and MAOM fractions was significantly lower after the pot experiment for both Min and Slu ($p = 0.001$ and $p = 0.007$ for POM and MAOM, respectively), but in absolute terms the change was greater for MAOM than for POM. For the bulk soil, no significant changes in $Ndff_{mass}$ before and after the pot experiment could be detected.

3.3. Method comparison

The mass distribution after fractionation, i.e. the proportion of soil retrieved in each fraction, summed up to an average of 95% when using

Table 1
Dry weight, total N content, N derived from the fertiliser ($Ndff_{\%}$), ^{15}N recovery ($^{15}N_{recov}$) in percent of initially applied quantity and plant uptake of ^{15}N in percent of bulk soil ^{15}N content before the pot experiment ($^{15}N_{resid}$) of ryegrass shoots and roots grown in soil from control (Con), mineral fertilised (Min) and slurry fertilised (Slu) treatments. Mean ± *standard deviation* (n = 4). Different letters indicate significant differences between fertiliser treatments within a row ($p < 0.05$) by Tukey's HSD test.

		Unit	Fertiliser treatment		
			Con	**Min**	**Slu**
Shoots	Dry weight	g pot^{-1}	2.05 ± *0.02*	2.08 ± *0.10*	2.04 ± *0.04*
	N content	mg pot^{-1}	16.47 ± *0.57*	17.02 ± *1.63*	16.67 ± *0.44*
	$Ndff_{\%}$	%	–	1.07 ± *0.18* **a**	2.25 ± *0.59* **b**
	$^{15}N_{recov}$	% of initially applied quantity	–	1.43 ± *0.26*	1.81 ± *0.45*
	$^{15}N_{resid}$	% of residual ^{15}N in bulk soil	–	6.56 ± *1.25*	5.12 ± *0.37*
Roots	Dry weight	g pot^{-1}	1.35 ± *0.10* **a**	1.41 ± *0.07* **a**	1.63 ± *0.07* **b**
	N content	mg pot^{-1}	9.08 ± *0.58* **a**	9.19 ± *0.56* **a**	10.62 ± *0.37* **b**
	$Ndff_{\%}$	%	–	1.16 ± *0.19* **a**	2.32 ± *0.64* **b**
	$^{15}N_{recov}$	% of initially applied quantity	–	0.84 ± *0.16*	1.19 ± *0.33*
	$^{15}N_{resid}$	% of residual ^{15}N in bulk soil	–	3.84 ± *0.67*	3.45 ± *0.24*

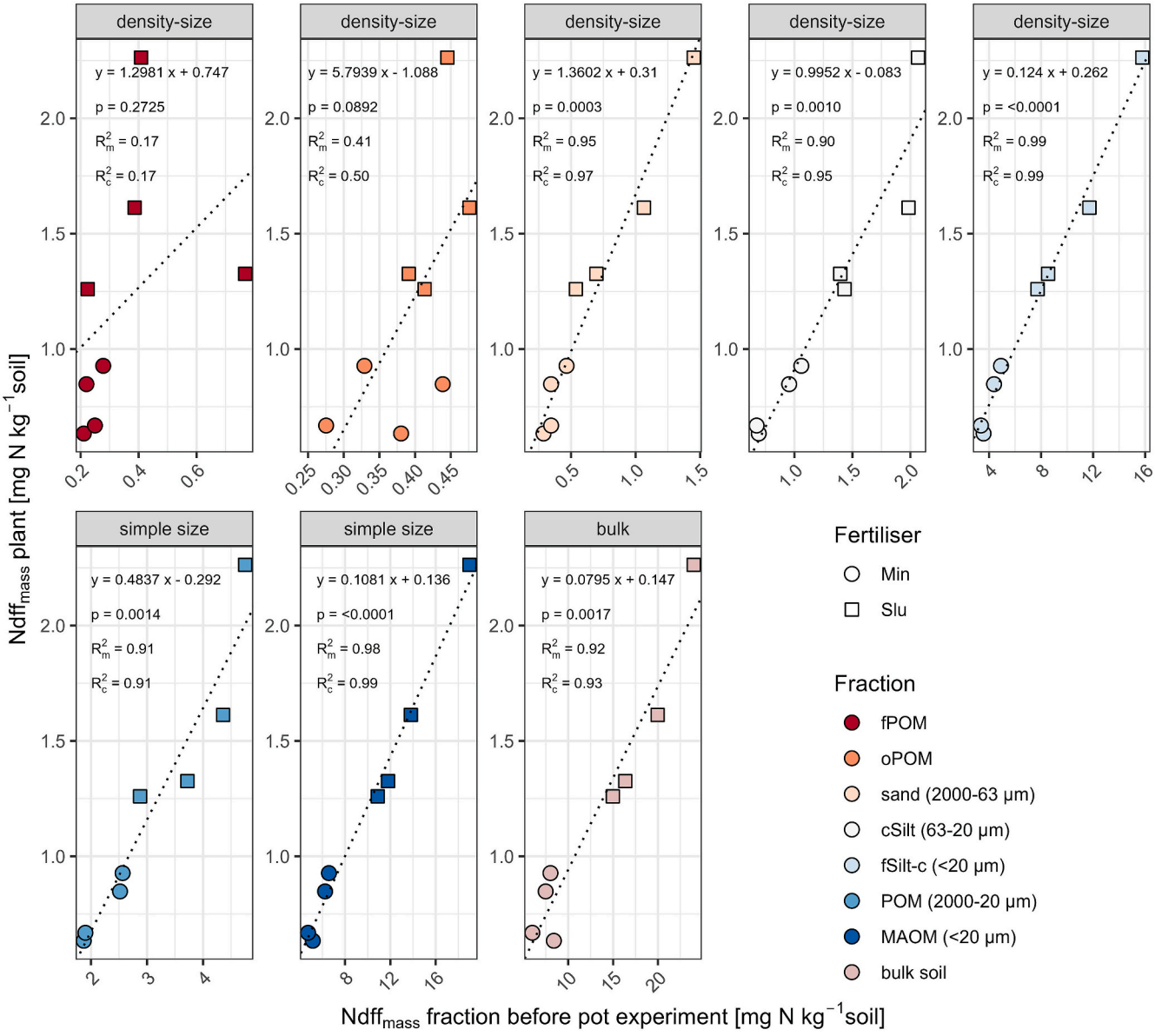

Fig. 3. Linear relationship between $Ndff_{mass}$ in the plant and $Ndff_{mass}$ in SOM physical fractions before the pot experiment for both investigated fractionation methods and for the bulk soil (n = 8), R^2_m and R^2_c refer to marginal and conditional R^2 according to Nakagawa and Schielzeth (2013).

the density-size fractionation (SI, Table S3). For the simple size fractionation, however, a significantly higher total mass was retrieved from Con (100.8%) than Min (98.6%) and Slu (97.6%). For both fractionation methods, more than 40% of soil mass was retrieved in the <20 µm fraction.

Likewise, most N was found in the <20 µm fractions (Table 2). With the density-size fractionation, 74 ± 4% of bulk soil N was stored in fSilt-c, while the corresponding fraction (MAOM) in the simple size fractionation contained 93 ± 4% of bulk soil N. Treatments did not affect N retrieval in the different fractions. Total N retrieval was greater with the simple size method (104 ± 5%) than with the density-size method (86 ± 5%), indicating that with the density-size scheme greater N losses occurred during fractionation.

The C:N ratios declined with decreasing particle size (Table 2). C:N ratios of fPOM and oPOM fractions were higher than those of the mineral fractions ($p < 0.001$) (SI, Table S4). Likewise, the C:N ratio of POM was higher than that of MAOM ($p < 0.001$) (SI, Table S5).

The sum of ^{15}N in the different fractions as a proportion of ^{15}N present in bulk soil ($^{15}N_{resid}$) also indicated higher losses for the density-size fractionation than the simple size fractionation (Fig. 5). Despite the similar size range of fSilt-c and MAOM fractions, a smaller proportion of ^{15}N was recovered in the <20 µm fraction for the density-size fractionation than the simple size fractionation. Thus, with the density-size fractionation scheme, most ^{15}N was lost from fSilt-c, in accordance with losses in soil mass (SI, Table S3). With respect to treatment differences, oPOM contained a significantly greater proportion of ^{15}N for Min (4.8 ± 0.8%) than Slu (2.4 ± 0.4%). Likewise, there was a significantly greater proportion of ^{15}N in POM for Min (29.9 ± 5.1%) than for Slu (20.9 ± 1.7%).

4. Discussion

4.1. Fate of N from ^{15}N-labelled cattle slurry and mineral fertiliser in SOM physical fractions two years after application

The majority of residual ^{15}N ($^{15}N_{recov}$) from Min and Slu was present in the mineral-associated fractions two years after application (Fig. 2), confirming our first hypothesis. This suggests that ^{15}N remaining in the

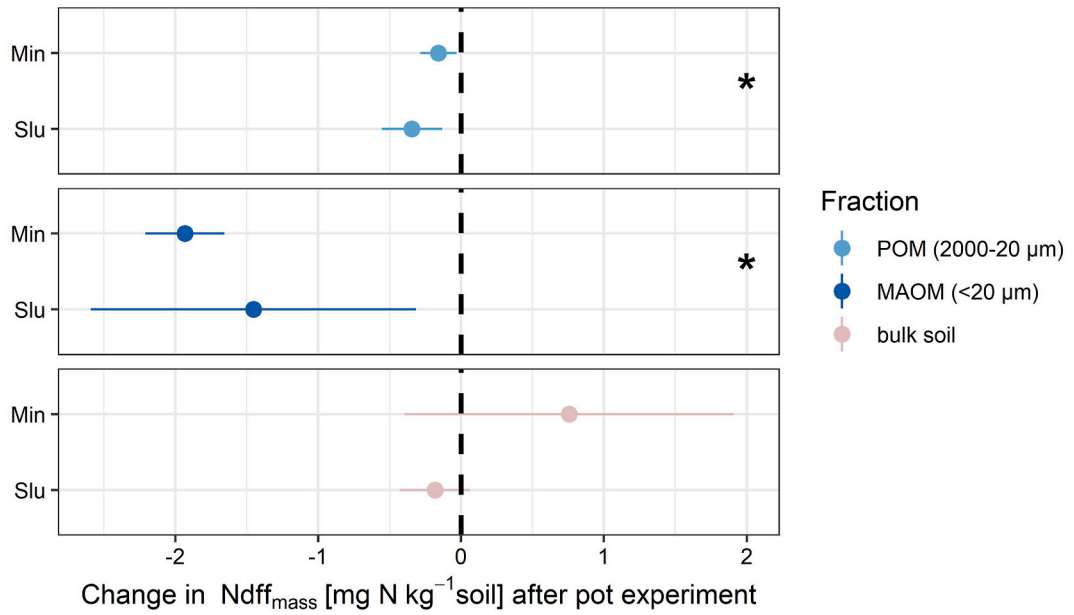

Fig. 4. Change in content of nitrogen derived from the labelled fertilisers (after minus before) in the soil from the pot experiment for POM, MAOM and bulk soil. Error bars represent standard deviation (n = 4), asterisk indicates significant difference of the intercept from 0 ($p < 0.05$) by linear mixed effect model with random intercept.

Table 2
Nitrogen (N) concentration, retrieved N (% of bulk soil N) and C:N ratio of the bulk soil and all SOM physical fractions isolated in this study from the control (Con), mineral fertilised (Min) and slurry fertilised (Slu) treatments. All parameters are averaged across treatment, n = 4, mean ± *standard deviation*. Different letters indicate significant differences between fertiliser treatments ($p < 0.05$) by Tukey's HSD test.

Fractionation method	Fraction	N concentration (mg N kg^{-1} fraction)			Retrieved N (% of bulk soil N)			C:N		
		Con	Min	Slu	Con	Min	Slu	Con	Min	Slu
Density-size	fPOM	596 ± *60*	604 ± *60*	651 ± *108*	0.8 ± *0.1*	0.8 ± *0.1*	0.9 ± *0.3*	19.5 ± *0.2*	20.7 ± *1.0*	19.5 ± *1.0*
	oPOM	1923 ± *148*	1798 ± *77*	1862 ± *82*	1.4 ± *0.2*	1.7 ± *0.2*	1.6 ± *0.1*	18.9 ± *0.3*	19.3 ± *0.8*	19.2 ± *0.3*
	sand (2000–63 µm)	15 ± *1* **ab**	13 ± *1* **a**	17 ± *2* **b**	2.0 ± *0.2*	2.0 ± *0.3*	2.6 ± *0.5*	11.9 ± *1.6*	11.8 ± *0.9*	12.3 ± *1.1*
	cSilt (63–20 µm)	44 ± *3* **ab**	42 ± *5* **a**	51 ± *4* **b**	5.9 ± *0.6*	6.4 ± *0.4*	7.3 ± *0.5*	11.5 ± *0.4*	11.7 ± *0.2*	11.6 ± *0.2*
	fSilt-c (<20 µm)	304 ± *10*	306 ± *14*	309 ± *7*	72.5 ± *1.9*	77.9 ± *4.7*	73.0 ± *5.5*	8.3 ± *0.1*	8.3 ± *0.3*	8.5 ± *0.2*
	total				82.6 ± *1.7*	88.8 ± *4.3*	85.3 ± *5.2*			
Simple size	POM (2000–20 µm)	44 ± *3*	38 ± *4*	44 ± *6*	11.0 ± *0.4*	11.1 ± *0.9*	12.0 ± *1.0*	12.5 ± *0.4* **a**	13.3 ± *0.4* **b**	13.8 ± *0.2* **b**
	MAOM (<20 µm)	342 ± *10* **a**	347 ± *12* **ab**	364 ± *9* **b**	89.0 ± *1.6*	96.3 ± *3.2*	93.0 ± *5.3*	7.6 ± *0.0*	7.6 ± *0.1*	7.6 ± *0.2*
	total				100.1 ± *1.5*	107.4 ± *2.6*	105.0 ± *5.3*			
Bulk soil		195 ± *10* **b**	176 ± *7* **a**	188 ± *8* **ab**				8.4 ± *0.2* **a**	9.2 ± *0.1* **b**	9.4 ± *0.4* **b**

soil (i.e. not taken up by plants or lost from soil) was either immobilised by soil microorganisms (Recous and Machet, 1999), still present in decomposing roots, or directly transferred to mineral-associated fractions (Castellano et al., 2012). Already in spring after application of the labelled fertilisers, Frick et al. (2022a) found most residual ^{15}N in soil as non-microbial organic N, emphasising that incorporation of fertiliser N into SOM was rapid and happened already in the year of application for both Min and Slu.

Most soil N, calculated as mean of the fertiliser treatments, was stored in the mineral-associated fractions (fSilt-c: 74% and MAOM: 93%) (Table 2). These values are in the same range as in other studies, as reviewed by Jilling et al. (2018). Soil N retention depends to a large extent on the soil clay content, since a high clay content offers more binding sites for N compounds (Chantigny et al., 2004; Chivenge et al., 2011). In our study, soil clay content was on average 21.6 ± 1.0%.

The $^{15}N_{recov}$ in the mineral-associated fractions based on the initially applied ^{15}N quantities tended to be higher for Slu than for Min (Fig. 2), in accordance with our hypothesis. Likewise, $^{15}N_{recov}$ in the bulk soil was significantly greater for Slu (34%) than for Min (22%), which reflects the higher application amount of Slu compared to Min. These findings are in line with those of Sørensen (2004), who reported more ^{15}N recovered from cattle slurry than mineral fertiliser in the bulk soil two and a half years after application. Bosshard et al. (2008) also found most ^{15}N in the mineral-associated fraction (<50 µm) 112 d after application of ^{15}N-labelled sheep manure and mineral fertiliser, but without differences between fertiliser treatments. In our study, the higher $^{15}N_{recov}$ in the mineral-associated fractions observed for Slu than for Min might be explained by several mechanisms: (1) direct incorporation of organic N added with Slu that was not yet mineralised (Bhogal et al., 2016); (2) sorption of NH_4^+ to clay particles (Nieder et al., 2011), or (3) incorporation into SOM via microbial immobilisation (Recous, 2005). Since less than 5% of total N in the slurry used in this experiment was undigested dietary N, only a small part might have been recalcitrant enough to remain unmineralised (Frick et al., 2022a). On the other hand, 75% of the slurry was water-soluble N and 60% was in the form of NH_4^+, indicating that a major share might have been either sorbed directly as NH_4^+ to clay particles or transformed into organic N through microbial immobilisation shortly after application. Indeed, there is growing evidence based on analysis of biomarker amino sugars that an important share of newly formed SOM derives from microbial necromass (Liang et al., 2020). Addition of labile C with the slurry might have stimulated microbial growth and in turn increased the amount of necromass in the

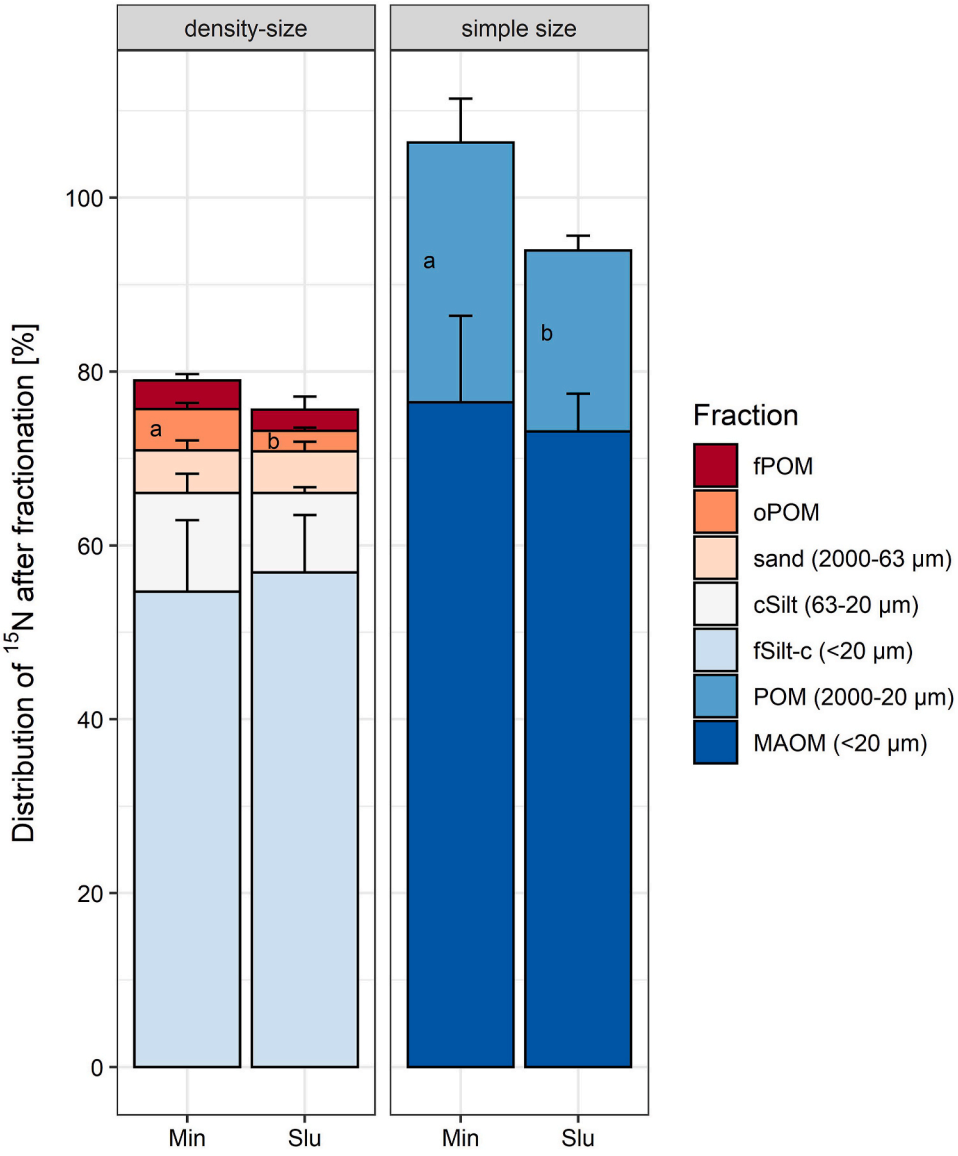

Fig. 5. Distribution of ^{15}N in percent of bulk soil ^{15}N content of the mineral fertilised (Min) and slurry fertilised (Slu) treatments in SOM physical fractions obtained by density-size and simple size fractionation methods. Error bars represent standard deviation (n = 4) and different letters indicate significant differences between fertiliser treatments in each fraction ($p < 0.05$) by Student's t-test.

soil (Wang et al., 2021), leading to more incorporation of ^{15}N in the mineral-associated fraction.

In the field experiment preceding this study, Frick et al. (2022b) traced ^{15}N-labelled mineral fertiliser and cattle slurry into crop biomass, soil and nitrate leaching. They reported an overall higher cumulated $^{15}N_{recov}$ for Min (52.6%) than for Slu (29.6%) in the biomass of three consecutive crops over a 2-year period, suggesting that a larger amount of N from Slu was either incorporated into SOM or lost via leaching, volatilisation or denitrification. This data coincides well with our results, which showed an overall higher $^{15}N_{recov}$ into the mineral-associated fraction for Slu than for Min.

4.2. Availability of N from SOM physical fractions to plants in a pot experiment

The shoot dry weight was not influenced by the fertilisers applied two years earlier in the field, and $^{15}N_{recov}$ in the shoots was similarly low in both treatments (1.4–1.7%, Table 1). These residual values are in the same range as in other studies (Sørensen, 2004; Smith and Chalk, 2018) and indicate a slow mineralisation rate of fertiliser N stored in SOM fractions. A single application of mineral fertiliser or cattle slurry is known to generally have little residual N effect on subsequent crops (Webb et al., 2013; Smith and Chalk, 2018). In contrast, animal manure can have a substantial residual N effect in long-term experiments (Schröder et al., 2005; Riley, 2016), which may be attributed to the cumulative effect of repeated animal manure applications (Webb et al., 2013). The fact that, in our study, labelled fertilisers were applied only in one year can explain the observed small residual N effect on above-ground biomass. For root dry weight and consequently also N content, a slight but significant increase was observed for Slu as compared to Con and Min. Assuming that application of slurry stimulated microbial turnover and thereby reduced plant access to N, plants might have had more root biomass in field conditions. Hence, N derived from the root turnover of these plants might have been more available to ryegrass plants in the pot trial, but this would warrant further research.

We hypothesised that most ^{15}N in the plants would originate from POM because this fraction is very dynamic and often considered the primary source of plant available N (Gosling et al., 2013). The regression analysis between N derived from the fertiliser ($Ndff_{mass}$) in the plants and $Ndff_{mass}$ in SOM fractions before the pot experiment showed

contrasting results for the two fractionation methods (Fig. 3). With the density-size fractionation scheme, the $Ndff_{mass}$ in mineral fractions (sand, cSilt and fSilt-c) was positively correlated with the $Ndff_{mass}$ in plants. fPOM and oPOM fractions, however, did not show significant relationships. Contrary to our hypothesis, these two POM fractions did not seem to play an important role for supplying N to plants. With the simple size fractionation, however, both POM and MAOM showed a significant positive relationship between the $Ndff_{mass}$ in the plant and the $Ndff_{mass}$ in the fraction. A possible explanation for the different results obtained with the two fractionation methods might be that the POM fraction in the simple size method also contains the entire cSilt and sand fraction, which were related to plant N uptake in the density-size method. This could be an argument for using density-size fractionation schemes that distinguish more than two fractions and allow a more detailed understanding of soil processes. Nonetheless, it is important to keep in mind that the regression analysis is not a proof of causality and that transfer of ^{15}N between SOM fractions during the pot experiment cannot be excluded.

Calculation of the difference between $Ndff_{mass}$ before and after the pot experiment in POM, MAOM and bulk soil gave further insights. The MAOM fraction where most residual ^{15}N was found (Fig. 2) appeared to be the main source of N derived from the fertilisers for plants both in Min and Slu (Fig. 4). This finding is in line with the emerging view that plants can access nutrients from MAOM through direct (root exudates) and indirect (microbes) pathways (Jilling et al., 2018), particularly when supply of POM-derived N is equal to or smaller than the mineral sorption potential, and little fertiliser is applied (Daly et al., 2021). Our research showed that this greater role of MAOM as compared to POM is particularly true for residual N. This is most likely because there was no inorganic N left from the fertilisers two years after application. It is nevertheless important to keep in mind that calculating the change in $Ndff_{mass}$ is an indirect measurement and can therefore only be used as a proxy of the origin of N in plants.

As we found that most N in the plants derived from MAOM rather than from POM, we had to reject our third hypothesis stating that plants would recover more residual ^{15}N from Min than from Slu. This was confirmed by the similar percentage of residual ^{15}N taken up by the plants for Min and Slu both in the shoots and in the roots (Table 1). Likely, it also relates to the fact that most residual ^{15}N was recovered in the MAOM fraction.

The measured values of $Ndff_{mass}$ in the plants (Fig. 3) and the calculation of the difference in $Ndff_{mass}$ in the fraction before and after the pot experiment (Fig. 4) showed values in a similar range (0–2 mg N kg^{-1} dry soil). This could be an indication that the use of ^{15}N enables to monitor both the rate of incorporation of ^{15}N in SOM and the residual uptake of immobilised ^{15}N. Hence, it contradicts the idea that dilution of ^{15}N by the unlabelled mineralisable soil organic N pool compromises the use of ^{15}N tracer techniques to explore residual uptake of immobilised ^{15}N as argued by Smith and Chalk (2018).

4.3. Methodological considerations on SOM fractionation procedures

In spite of the similar size range of fSilt-c and MAOM fractions, a smaller proportion of ^{15}N ($^{15}N_{resid}$) was recovered in the <20 μm fraction for the density-size fractionation than for the simple size fractionation (Fig. 5). The density-size fractionation includes a rinsing step of the mineral fractions to avoid contamination with nutrients from the sodium polytungstate solution. Although the samples are centrifuged, a substantial amount of minerals and thereby also part of the ^{15}N is lost when siphoning. In contrast, the simple size fractionation scheme only includes a wet sieving step where no major losses can occur. Since more ^{15}N than total N was lost upon fractionation, this may indicate that the rather recently added ^{15}N was still less stabilised than the vast majority of SOM and thereby more prone to be either leached or taken up by the plants. However, the processes occurring during the fractionation procedure and those taking place in soil profiles are probably not analogous.

The size limit between POM and MAOM is operationally defined and there is no consensus on whether this limit should be at approximately 50 μm (Cotrufo et al., 2019) or at 20 μm (van Wesemael et al., 2019). Related to that, an interesting outcome of our study is that the sand fraction and the cSilt fraction had similar C:N ratios, while the C:N ratio in the fSilt-c fraction was distinct (Table 2). A possible explanation is that POM material was still able to pass through the sieves during wet-sieving, but could not pass the 20 μm sieve to reach the fSilt-c fraction. Also, the degradation in size of POM material might have been enhanced by ultrasonic dispersion. These methodological issues could be an argument in favour of a 20 μm limit because the probability of having (redistributed) POM material in the <20 μm fraction is lower than in the <50 μm fraction. Therefore, the <20 μm fraction better represents SOM bound to mineral surfaces via sorption to minerals. Nevertheless, it is important to mention that the C:N ratio is known to be suitable for predicting N mineralisation from labile SOM but that it often fails for more stable SOM such as mineral-associated SOM (Hoffland et al., 2020). The main issue when using the C:N ratio is the lack of any information about the biochemical composition of the analysed material (Bonanomi et al., 2019) and that the analysed material is mostly not uniform.

5. Conclusions

Two years after field application of labelled mineral or organic fertiliser, most of the remaining ^{15}N was stored in the mineral-associated fraction, regardless of the type of fertiliser, with a residual N fertiliser value of around 2%. Our study thus revealed the largely similar behaviour of N from slurry or mineral N fertiliser in the soil beyond the application year. Furthermore, most ^{15}N in plants originated from MAOM, challenging the idea that nutrients in MAOM are protected and thus inaccessible.

The simple size fractionation method was cheap, easy to implement and did not lead to major losses of ^{15}N. In contrast, the density-size fractionation method was time-consuming and led to significant ^{15}N losses during the rinsing step of the procedure. Although the density-size fractionation method showed several disadvantages, the distinction of fPOM and oPOM fractions by density fractionation and lack of correlation of N derived from fertilisers in these fractions and plants supported the idea that POM-derived N did not sustain plant N uptake to a large degree.

To gain a better understanding of N retention in soils, future studies should focus on continuously tracing ^{15}N in distinct SOM fractions after application of labelled fertilisers under field conditions. In addition, distinguishing between mineral and organic N forms in SOM fractions could help unravel the factors leading to rapid incorporation of N into SOM.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2022.108908.

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