Fate of 15-N-labelled amendments in physical soil fractions two years after application

MSc thesis

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Max Fuchs (Reg. Nr. 950606-248-020)

Supervisors: Dr. Gabriel Moinet[1] & Dr. Else Bünemann-König[2]

[1]Soil Biology Group, Wageningen University & Research (WUR), Wageningen, the Netherlands

[2]Department of Soil Sciences, Research Institute of Organic Agriculture (FiBL), Frick, Switzerland

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Abstract

Although fertilisation with animal manure is important for closing nutrient cycles on-farm, the fate of nitrogen (N) from such fertilisation remains poorly understood due to the challenge of considering both short-term and residual N effects. The focus of this research was to investigate the fate of ^{15}N-labelled amendments (mineral fertiliser (Min) and cattle slurry (Slu)) in soil organic matter (SOM) physical fractions as well as their residual effect on plant growth two years after application. Two experiments were carried out to: (1) investigate the fate of ^{15}N in SOM physical fractions using two SOM fractionation methods, (2) examine the uptake of ^{15}N by ryegrass plants (*Lolium multiflorum*) from the previously applied fertilisers as influenced by *Trichoderma asperellum* fungi in a pot experiment. With the first fractionation method, free particulate organic matter (POM), occluded POM, sand fraction (2000-63 µm), coarse silt fraction (63-20 µm) and fine silt/clay fraction (<20 µm) were isolated. With the second method, POM (>20 µm) and mineral-associated organic matter (MAOM, <20 µm) were isolated. All fractions were analysed for ^{15}N abundance.

Most ^{15}N was found in fractions <20 µm, and the ^{15}N recovery, in percent of the originally applied quantity of ^{15}N as fertiliser, tended to be higher for Slu (fine silt/clay fraction: 19.8% , MAOM: 25.1%) than for Min (fine silt/clay: 12%, MAOM: 16.8%) in these fractions. Although the differences were not statistically significant for the fine fractions, the ^{15}N recovery was significantly higher for Slu than for Min in the bulk soil. As compared to the control treatment (without fertiliser), fertilisers applied two years ago had little effect on aboveground plant biomass. In contrast, a slight but significant increase in belowground biomass for Slu was observed. A possible explanation is the larger proportion of total N in the coarse silt fraction observed for Slu, where N might be more available to plants. *Trichoderma asperellum* had no effect, neither on plant growth nor on ^{15}N uptake, probably due to the low N availability as shown by the low N concentration of the plants (N%: 0.75±0.06). Finally, most ^{15}N in plants seemed to derive from MAOM. This suggests that the availability of the N stored in MAOM could be larger than previously thought.

Keywords: ^{15}N-labelled amendments, soil organic matter physical fractions, ^{15}N bioavailability, beneficial fungi

Table of contents

1 Introduction

Global population growth is putting pressure on the agricultural sector to increase food production. This has resulted in more agricultural-related negative environmental impacts since the green revolution (Tilman et al., 2011). Since crop growth depends to a large extent on soil nitrogen (N) supply (Li et al., 2014), the applied quantity of inorganic and organic N fertilisers has increased by 943 % between 1961 and 2018 (FAOSTAT, 2018). This increase has supported increasing plant production worldwide but came with large environmental side-effects, in the form of GHG emissions associated with the production and transport of fertilisers, as well as large N losses to the environment, particularly atmosphere and water bodies.

Nitrogen losses from farms to the broader ecosystems are caused by three main processes: nitrate (NO_3^-) leaching, ammonia (NH_3) volatilisation and nitrous oxide (N_2O) emissions (Figure 1). Nitrate leaching is a natural process where nitrates leave the soil in water drainage. Nitrate is a soluble and mobile form of N and is not adsorbed to soil particles. This negatively affects drinking water quality in groundwater and thus has detrimental effects on human health and particularly on infants (Hansen et al., 2017). It also causes eutrophication when NO_3^- accumulates in lakes and estuaries (Nieder and Benbi, 2008). Eutrophication causes the appearance of harmful algal blooms, inducing oxygen depletion and thereby negatively impacting aquatic biodiversity (Smith and Schindler, 2009). Ammonia volatilisation leads to atmospheric deposition in natural N limited areas, where it causes undesirable community changes in vegetation (Fenn et al., 2003). Finally, nitrous oxide is an extremely potent greenhouse gas that has a 300 times higher global warming potential than carbon dioxide (CO_2) (Griffis et al., 2017).

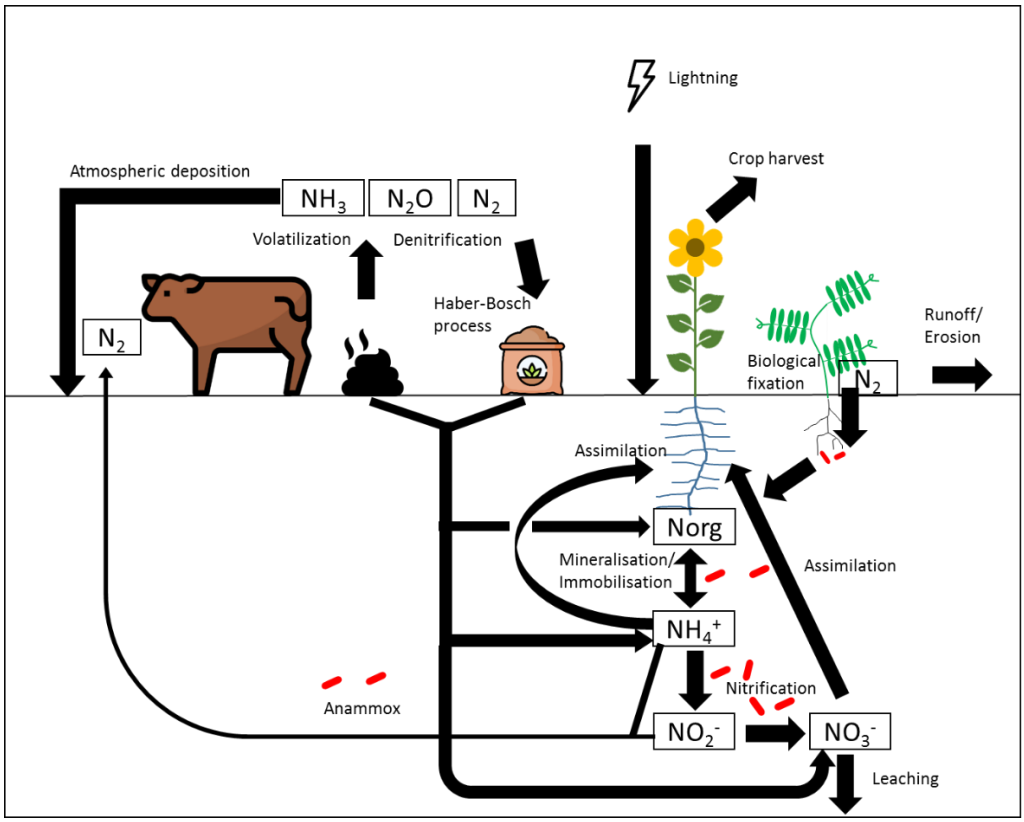

Figure 1: The nitrogen cycle.

More sustainable farming systems, also sometimes referred to as circular farming (Toop et al., 2017), aim to minimise these negative environmental impacts by fostering nutrient recycling and improving fertiliser N uptake efficiency. Mixed farming systems combining crop and livestock production can help to reduce environmental externalities by closing the loop in nutrient cycles without declines in profitability or yields (Garrett et al., 2020). For example, animals are able to convert by-products from the food industry and grass into valuable food and farmyard manure (Van Zanten et al., 2019). In this context, fertilisation with animal manure plays an important role. However, the fate of N from such fertilisation remains poorly understood due to the challenge of considering both short-term and residual N effects (Nannen et al., 2011).

Nitrate leaching from agricultural production negatively affects drinking water quality. In Switzerland, the Gäu-Olten region has nitrate concentrations above national average in its groundwater on account of both its valley-shaped aquifer and its agricultural production. Therefore, a project for reducing nitrate leaching in groundwater in this region was launched in 2017 and included a field experiment (established in 2018) comparing nitrate leaching from three fertiliser treatments (ON-Control (Con), ^{15}N-labelled cattle slurry (Slu) and ^{15}N-labelled ammonium nitrate fertiliser (Min)). My research was conducted in 2020 and aimed at studying the fate of ^{15}N in different SOM fractions two years after fertiliser application using two fractionation methods (Steffens et al., 2009; Cotrufo et al., 2019). Furthermore, I aimed to assess whether N is retained in SOM fractions after two years. In addition, I wanted to investigate from which SOM fractions ^{15}N is taken up by ryegrass plants in a pot experiment to gain insight on the role of different fractions on productivity. Finally, I wanted to assess the effect of *Trichoderma asperellum* fungi inoculation on ^{15}N uptake by ryegrass plants. This soil-borne filamentous fungus is broadly used as biofertiliser for its supposedly enhancing effect on plant nutrient acquisition.

1.1 The link between soil organic matter and nitrogen

Since soil organic matter (SOM) is the main N reservoir in soils, studying the fate of N in soils is combined with the study of SOM. Soil organic matter is essential to soil functioning as it sustains several ecosystem services such as growth of natural vegetation and agricultural production by providing nutrients to plants through mineralisation. It also filters and holds water and stores carbon (C). For these reasons, maintaining SOM levels in soils is crucial to preserve ecosystem services. The formation of SOM is a slow process that not only requires C (its main component) but also other nutrient such as N, phosphorus (P) and sulphur (S) (Kirkby et al., 2011; Tipping et al., 2016). These stoichiometric relationships demonstrate the intimate link between SOM and N.

Therefore, studying the mechanisms regulating the protection of SOM in soils is essential for understanding how N is retained in soils. In the past, SOM was thought to be mainly protected from degradation on the long term by its inherent chemical recalcitrance (Kleber and Johnson, 2010). However, new evidence shows that this mechanism plays a minor role in SOM protection and that physical protection through occlusion within aggregates and chemical protection within organo-mineral associations are more important (Schmidt et al., 2011). This new view has changed the study of SOM dynamics. It has led to the development of SOM fractionation methods, which aim to quantify the amount of SOM present in pools stabilized by different mechanisms (Duddigan et al., 2019). According to Duddigan et al. (2019), these fractionation methods typically distinguish three main fractions:

(i) SOM contained within aggregates and therefore physically protected through occlusion;

(ii) SOM complexed within organo-mineral associations and thereby chemically protected;

(iii) Unprotected (free) SOM accessible to microorganisms.

Different SOM fractions are obtained by physical and chemical fractionation methods, or a combination of both (von Lützow et al., 2007). On the one hand, physical methods are based on the principle that association of particles and their spatial arrangement play a key role in SOM dynamics. They involve various disaggregating treatments such as dry and wet sieving, slaking, dispersion by sonication, density separation and sedimentation. On the other hand, chemical methods are based on the extraction of SOM in aqueous solutions or on the hydrolysability of SOM in organic solvents or on the resistance of SOM to oxidation (von Lützow et al., 2007).

Despite the similarities of the methods, no consensus has been reached, making comparisons between studies difficult. In a comparison of 20 fractionation methods, Poeplau et al. (2018) concluded that a combination of physical (density, size) and chemical (oxidation, extraction) fractionation yields the best result for fractions with distinct turnover times. However, some of these methods are labour-intensive. With the intention of standardising and simplifying the scientific approach, Lavallee et al. (2020) suggested distinguishing only two fractions, namely particulate organic matter (POM) and mineral-associated organic matter (MAOM).

1.2 The fate of nitrogen fertilisers

The difficulty to accurately analyse soil N forms in the soil makes the study of its turnover time challenging. In soils, over 90% of N is found in organic forms while the remaining fraction is inorganic (Pansu and Gautheyrou, 2006). Soil organic N can be divided in two broad categories, namely N from organic residues (plant and animal) and N from SOM (Kelley and Stevenson, 1995). In the past, it was generally assumed that organic N is largely unavailable to growing plants and microorganisms because it must first be mineralised to NH_4^+. However, there is evidence that organic N can also directly be taken up by plants and microorganisms (Näsholm et al., 2009; Geisseler et al., 2010). Mineralisation of organic N and immobilisation of mineral N are two processes taking place simultaneously in soils. In the old view on N-mineralisation, the limiting factor for mineralisation was the conversion of organic N to NH_4^+ by microbes. Nonetheless, the new paradigm is that N-mineralisation is driven by the depolymerisation of N-containing polymers by microbial extracellular enzymes (Schimel and Bennett, 2004). The monomers (amino acids, amino sugars, nucleic acids, etc.) resulting from the depolymerisation are bioavailable to either plants or microorganisms. Indeed, plants and microorganisms compete at least partly for the same N sources. It remains not fully elucidated how much organic N can be taken up by plants in agroecosystems because soil N forms are transient and therefore difficult to measure.

As an example, Moran-Zuloaga et al. (2015) calculated that a maximum of 5%.of total N taken up by plants can be expected to be derived from direct uptake of amino acids. This could at least partly explain why results from laboratory experiments on agricultural soils have shown that SOM mineralisation is weakly correlated with plant N uptake and yield (Jilling et al., 2018). Consequently, gaining a better understanding of the mechanisms through which organic N is retained in soils is critical.

According to Weitzman and Kaye (2016), nitrogen retention in soils occurs through five different mechanisms: (1) crop N uptake, (2) microbial immobilisation, (3) reactions between inorganic N and SOM, (4) interactions between simple organic compounds and charged mineral surfaces and (5) physical protection into soil aggregates. These different processes occur on different timescales. In general, abiotic reactions take place within seconds to minutes while microbial turnover and translocation into aggregates can last days to weeks (Weitzman and Kaye, 2016). This is in line with several studies which showed direct transfer of N from amendments to MAOM (Bimüller et al., 2013; Jilling et al., 2020; Samson et al., 2020). As a result, the N stabilised through these rapid processes can remain in the soil for decades to centuries (Weitzman and Kaye, 2016). This not only depends on management practices such as the use of tillage and the amount of N

inputs, but also on environmental variables such as temperature and precipitation. In addition, the type of N fertiliser applied can affect N retention in soils. Indeed, it has been shown in field experiments that less N is taken up by plants from animal manure than from mineral fertiliser during the first year after application (Sørensen, 2004; Frick et al., in preparation). Consequently, a larger amount of residual N remains in the soil from animal manure than from mineral fertiliser. This residual N might either contribute to a build-up of soil N or be largely lost to the environment.

To gain a better understanding of the fate of N after fertiliser application, Bosshard et al. (2008) studied the incorporation of ^{15}N-enriched sheep faeces, urine and mineral fertiliser in soil aggregates (macro-, microaggregates and microstructures) of conventional and organic farming systems. Aggregates were further separated in three SOM fractions (free light fraction (LF), intra-aggregate particulate organic matter (iPOM) and mineral-associated fraction (MF)) by density fractionation. One hundred and twelve days after application, most ^{15}N was found in small macroaggregates and MF was the most important ^{15}N sink. Moreover, the ^{15}N recovery was higher for urine-derived ^{15}N than faeces or mineral fertiliser. They concluded that this higher recovery for urine-derived ^{15}N can be attributed to the form and amount of N coupled with the carbon addition with slurry. They hypothesised that C addition stimulated microbial activity and might have accelerated microbial immobilisation of N. Overall, 37-55% of ^{15}N contained in non-fractionated soil was lost during the soil dispersion procedure. This sheds light on the potential limitation of such fractionation methods.

1.3 Trichoderma spp.

Jilling et al. (2018) suggest that a significant amount of N can be mobilised by plants from MAOM by means of destabilisation pathways such as the exudation of low-molecular-weight root exudates. Similarly, microorganisms such as fungi or bacteria could play an important role to help plants mobilising nutrients from MAOM. Since a substantial amount of total N in cultivated soils is present in MAOM and presumably not bioavailable to plants, investigating pathways through which microorganisms could improve N uptake by plants is essential. *Trichoderma* is a soil-borne filamentous fungus known for its plant beneficial effects. It is broadly used as biofungicide and biofertiliser (Kubicek et al., 2019). *Trichoderma* species have been shown to have an antagonistic effect against pathogens both in greenhouse and field conditions (Mehetre and Mukherjee, 2015). More interestingly in the context of this research, *Trichoderma* species promote plant growth and trigger an increased root growth. However, the exact mechanisms remain poorly understood. A suggested mechanism is an increase in nutrient transfer from soil to root, which is supported by the ability of *Trichoderma* species to colonise the interior of roots. Furthermore, increased N use efficiency has been reported with *Trichoderma harzianum* inoculation for vanilla and sugarcane (Mehetre and Mukherjee, 2015). For this reason, one of the aims of this study was to investigate the effect of *Trichoderma asperellum* on ^{15}N uptake by ryegrass plants.

1.4 Research questions and hypotheses

My research questions and hypotheses were:

Is there a difference of ^{15}N distribution among the different fractions of SOM between cattle slurry and mineral fertiliser?

[1] Hypothesis: The ^{15}N content of the mineral-associated fractions is higher for cattle slurry than mineral fertiliser because the N applied two years ago with the mineral fertiliser was rapidly taken up by plants after application and less prone to microbial immobilisation.

Does *Trichoderma asperellum* enhance the ^{15}N uptake by ryegrass plants?

[2] Hypothesis: The ^{15}N uptake by ryegrass plants is higher with *Trichoderma asperellum* due to improved mobilisation of N from MAOM due to a larger root system.

From which SOM fractions is the ^{15}N taken up by ryegrass plants?

[3] Hypothesis: The ^{15}N present in the POM fraction is more accessible to plants than the ^{15}N in the MAOM fraction.

2 Material and methods

2.1 Background - the nitrate project ("Gäu-Olten")

In arable regions of Switzerland, drinking water quality has deteriorated due to increased nitrate concentration in the last decades. Consequently, the Swiss Government has set a nitrate threshold value of 25 µg l^{-1} for drinking water and has launched several "nitrate projects" in the 2000s. Despite these measures, the nitrate threshold value is still often exceeded on the Swiss plateau mainly because of agricultural production (Reinhardt et al., 2019). As a result, the NitroGäu project was established by the Department of Environment of the canton of Solothurn in Switzerland in 2017 in collaboration with local farmers. This study is a follow-up experiment of an on-farm ^{15}N microplot study in the Gäu-region carried out by Hanna Frick. The aims of this on-farm experiment were:

(1) To quantify the N leaching losses from cattle slurry in the field;

(2) To investigate which proportion of N fertiliser (slurry or mineral fertiliser) is retained in the soil and in which forms (mineral, microbial, soil organic N);

(3) To assess how cattle slurry can be managed to reduce N leaching losses.

A microplot experiment was established in 2018 comparing three fertiliser treatments (Con, Slu and Min) in terms of N-uptake, nitrate leaching and residual N over a period of 2.5 years. The ^{15}N-labelled cattle slurry used in this study was produced according to Frick et al. (in preparation): In short, a young heifer was fed with ^{15}N-labelled ryegrass for 9 days. Faeces and urine were sampled separately and frozen on a daily basis. Later, faces and urine fractions with the highest 15N-label were recombined and diluted 1:1 with demineralised H_2O in order to achieve a representative slurry.

The experiment is finished but not all results have been fully evaluated. The first results evaluated by Hanna Frick indicate that the ^{15}N recovery in plants is higher for Min than Slu in the first year. Conversely, the ^{15}N recovery in the following year is higher for Slu than for Min, albeit the difference is small between the two treatments. In addition, soil analyses indicate that most of the ^{15}N from cattle slurry is still in the soil after 2 years and that most of it is present in the 0-30 cm soil layer.

2.2 Field site description and soil material

The microplot experiment from which soil samples were collected was installed in April 2018 in Rickenbach in the canton of Solothurn, Switzerland (1'242'514 N, 2'632'054 E). The area has a mean annual temperature of 10.6°C and a mean annual precipitation of 1016 mm ("Agroscope - Agrometeo," 2020). The soil is a loamy (22 % clay, 43 % silt, 35 % sand) Cambisol and the initial soil pH (H_2O) was 6.7. The field was managed conventionally by the farmer before the experiment, following the proof of ecological performance (PEP) requirements. The PEP are ecological measures that Swiss farmers have to implement in order to receive direct payments from the Swiss government. The experimental design consisted of 12 microplots (1.5 m x 2 m) placed 5 m apart from each other on a 3 m-wide strip (Figure 2). Three fertiliser treatments (Con, Slu and Min) were compared in a randomised complete block design with four replicates.

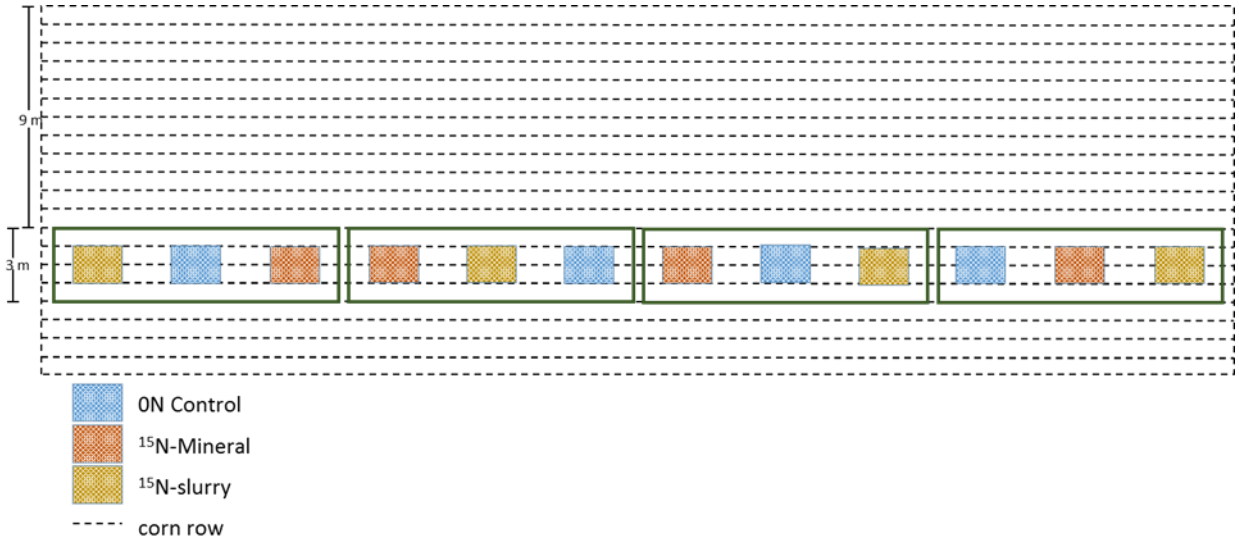

Figure 2: Experimental design of the field experiment carried out by Hanna Frick from 2018 to 2020 comparing three fertiliser treatments (Con, Min and Slu) with 4 blocks each containing each treatment once (Frick, 2020).

The crop rotation during the experiment was as follows: grass-clover mixture (2018/19) – silage maize (2019) - winter wheat (2019/20). The different ^{15}N fertilisers were only applied in 2018 while common fertiliser application was done by the farmer in the following years with urea and cattle slurry. In 2018, 30 t ha^{-1} of ^{15}N-labelled cattle slurry was applied each time after cutting the grass-clover (in total four times). At each slurry application, the same amount of available N (based on ammonium-N content of the slurry) was applied for the mineral fertiliser treatment. Meanwhile, triple superphosphate and potassium sulphate were applied to ensure equal availability of P and K in all treatments. Exact amounts of N applied in Min and Slu treatments and the ^{15}N abundance of the amendments in atom% are given in Table 1. The increase in ^{15}N recovery observed from year 1 to year 2 is probably due to the turnover of root material and a low ^{15}N uptake by the plants in year 2.

Table 1: Abundance of ^{15}N fertiliser, application rate and percentage of initial ^{15}N addition in the bulk soil after 1, 2 and 3 years of the experiment carried out by Hanna Frick from 2018 to 2020 (Frick, 2020).

Parameter	**Unit**	**Slurry**	**Mineral fertiliser**
^{15}N abundance	atom%	7.89	8
Application rate (4x)	kg N ha^{-1}	60(x4=**240**)	36.8(x4=**147.2**)
	kg NH_4^+/NO_3^- ha^{-1}	36.8 (x4=147.2)	36.8 (x4=147.2)
^{15}N recovery 1st year (0-30 cm)	%	~55	~32
^{15}N recovery 2nd year (0-30 cm)	%	~60	~34
^{15}N recovery 3rd year (0-30 cm)	%	*pending*	*pending*

Soil samples (0-30 cm depth) for this study were collected from the field on 22/07/2020. Following drying, the samples were sieved at 2 mm.

2.3 SOM fractions

To test hypotheses [1] and [3], a combination of density and size fractionation was used in this study. Two fractionation methods were compared to gain a better understanding of ^{15}N losses during the fractionation process. The first method (Steffens et al., 2009) distinguishes six SOM fractions whereas the second one (Cotrufo et al., 2019) distinguishes only two fractions. The

second method was chosen with the aim of simplifying fractionation schemes because it is cheaper and widely implementable.

2.3.1 Sonication intensity pre-test

According to Griepentrog and Schmidt (2013), until now most studies that performed density fractionation schemes with ultrasonic dispersion used a specific dispersion energy without giving the reasons of their choice. As dispersion energy has a significant effect on the SOM fractions obtained, the authors recommend testing and reporting dispersion energy for making studies more comparable in the future. The dispersion energy should be adjusted based on the texture and the C content of the samples. They suggest testing different amounts of dispersion energy and calculating the mass recovery as well as the C contents of the occluded POM fraction. The target dispersion energy should have the maximum C content in oPOM fraction to avoid contamination with minerals and organic material from heavy fractions. Therefore, I performed pre-tests with different dispersion energy ranging from 25 to 500 J ml^{-1} (25, 50, 75, 100, 150, 200, 300, 400 and 500 J ml^{-1}) with the aim of choosing the optimal dispersion energy for the soil used. The maximum C recovery in oPOM fraction was obtained with a dispersion energy of 100 and 150 J ml^{-1}. As a result, a dispersion energy of 100 J ml^{-1} was used during the fractionation process. The results of the pre-tests can be consulted in Appendix 1.

2.3.2 Fractionation methods

The method developed by Steffens et al. (2009) (referred to as Steffens) was slightly adapted by excluding the last step which further separates the fractions <20 µm. I chose not to include this step because sorption of SOM to minerals is the dominant mechanism within the entire fraction. Furthermore, equipment for processing this fraction was not available at the Research Institute of Organic Agriculture (FiBL). Therefore, the fractionation scheme used consisted of 5 fractions as depicted in Figure 3.

Briefly, 30 g of 2 mm oven-dried soil was weighed in 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 extracted with a vacuum pump. To obtain the POM occluded (oPOM) in aggregates, the subsequent heavy fraction was treated by ultrasound (Sonopuls HD 2200.2 homogeniser, Bandelin Electronic, Berlin, Germany). The dispersion energy was set at 100 J ml^{-1} based on pre-tests as previously explained. The tip of the Sonotrode (Model VS 70T) was immersed 30 mm into the soil suspension for continuous circulation of the sediments. During sonication, the soil suspension was cooled with ice to avoid overheating. With a subsequent density fractionation, the oPOM floating on the suspension was extracted with a vacuum pump. The residues of the density fractionation procedure were wet-sieved with bi-distilled water through a 63 µm (Retsch GmbH, Haan, Germany) and a 20 µm sieve (ATECHNIK GmbH, Leinburg, Germany) until the liquid that passed the sieves was clear. The three following fractions were obtained: sand (2000-63 µm), coarse silt (cSilt; 63-20 µm) and fine silt/clay fractions (fSilt-c; <20 µm). Care was taken to keep the total amount of water used for the wet-sieving procedure below 2 dm^3. All particle size fractions were washed with bi-distilled water. After that, the organic fractions were lyophilised with a freeze dryer (Alpha 1-4 LSC, Martin Christ Gefriertrocknungsanlagen 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 complete protocol can be found in Appendix 2.

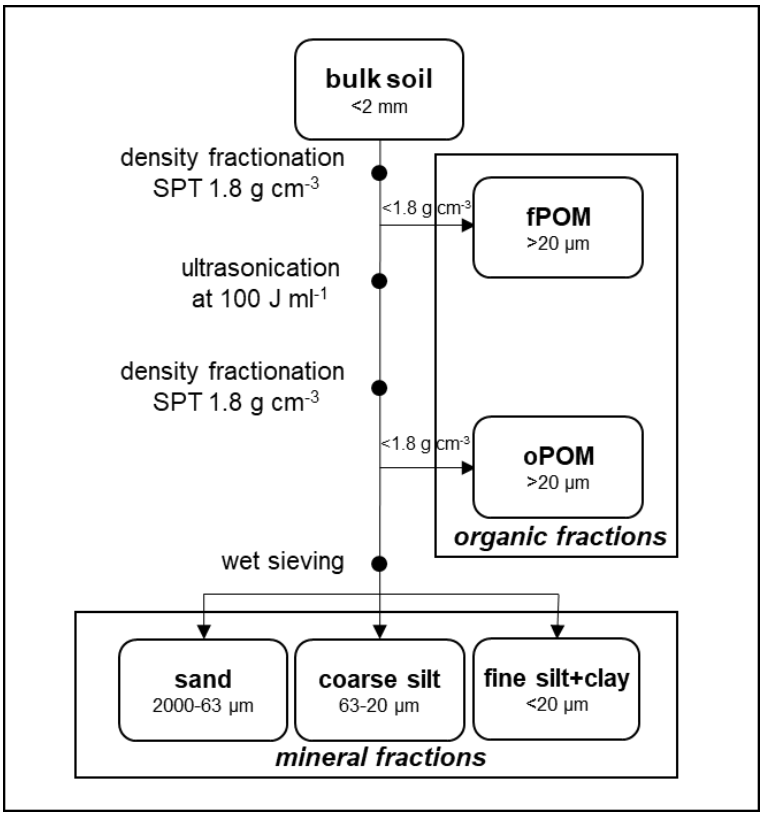

Figure 3: Fractionation scheme adapted from Steffens et al. (2009) resulting in five fractions. SPT, sodium polytungstate; fPOM, free particulate organic matter; oPOM, occluded particulate organic matter.

The method developed by Cotrufo et al. (2019) (referred to as Cotrufo) was adapted by lowering the limit between the two fractions (POM and MAOM) from 53 µm to 20 µm (Figure 4). This was done to make the two methods more comparable. 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 (Ø 5 mm) for 18 h at 125 rpm to disperse the soil. After that, the dispersed soil was rinsed onto a 20 µm sieve (ATECHNIK) 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 60 °C oven-dried and ground for ^{15}N analysis. The complete protocol can be found in Appendix 3.

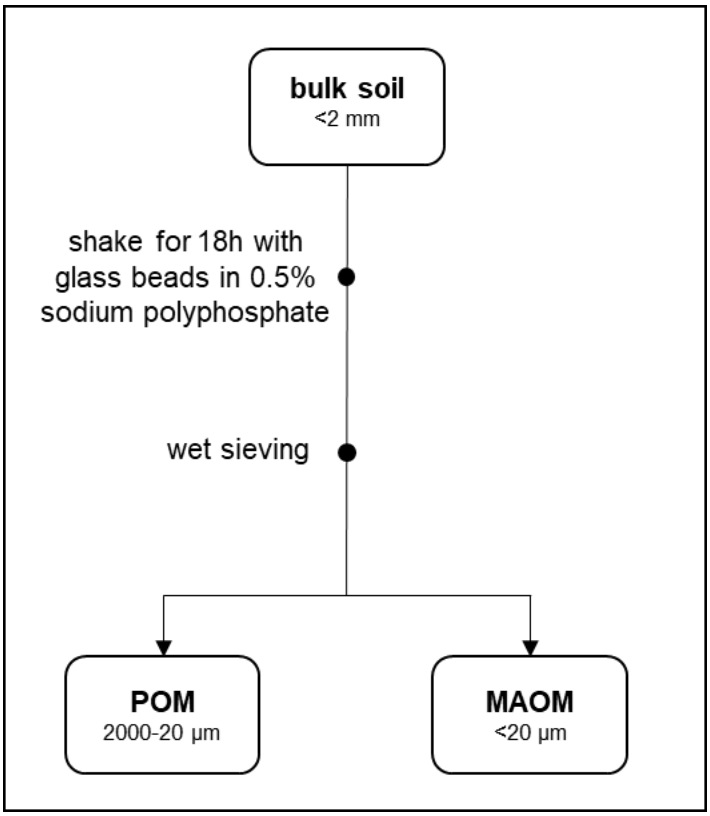

Figure 4: Fractionation scheme adapted from Cotrufo et al. (2019) resulting in two fractions. POM, particulate organic matter; MAOM, mineral-associated organic matter.

2.4 Pot experiment

To test hypotheses [2] and [3], a pot experiment was carried out in a greenhouse at FiBL under light- and temperature-controlled conditions (14 h light per day, heating temperature D/N 15 °C/13 °C, and heating ventilation threshold at 20 °C) for six weeks. The soil samples were pre-incubated in plastic bags for 1 week at room temperature at a gravimetric water content of 15 % (15 g H_2O per 100 g dry soil). Maximum water holding capacity (mWHC) was determined following the FiBL protocol (Appendix 4). Before filling the pots, a N free nutrient solution was added and thoroughly mixed to the soil to ensure no other limiting factors than N. The following amounts of nutrients were added (in mg kg^{-1} soil dry matter): 50 P, 250 K, 102 Ca, 48 Mg, Cu 2, Mn 2, Zn 1, B 1, Fe 1, Mo 0.1, Co 0.1. These nutrients were added in the chemical forms indicated in Appendix 5. After that, forty-eight (4 pots x 12 microplots) cylindrical pots (10.5 cm in diameter, 7.5 cm deep, 400 ml) were filled with the soil from the field site described in chapter 2.1 at a bulk density of 1.1 g cm^{-3}. The soil sample from each microplot was divided in four 400 ml pots. Before being filled with soil, each pot was equipped with a plastic bag inside to avoid leaching. Ryegrass seeds (*Lolium multiflorum*, variety Pulse) were sown at a density of 30 g m^{-2}, and after that, two out of four pots per microplot were inoculated with Trichoderma asperellum (T-Gro©, Andermatt Biocontrol, $2x10^9$ spores/g) at a concentration of 40 g inoculum/kg seeds and the other two were mock-inoculated (autoclaved solution) to avoid influence of the spore carrier. To enable optimal germination, seeds were covered with a fine layer of soil and pots covered with a plastic film for 2-3 days for keeping moisture during germination. All pots were maintained daily at 60 % mWHC by weighing the pots. The pots were divided in two blocks (with all treatments in each) on the growth table 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. Following dry weight measurements, samples were finely ground with a ball mill (MM200, Retsch GmbH, Haan, Germany) prior to ^{15}N analysis. To attempt assessing from which SOM fractions the ^{15}N was taken up by ryegrass plants, the simple fractionation method described in chapter 2.3.2 was repeated on root-free soil collected at the end of the pot experiment, and POM, MAOM and bulk soil were analysed for ^{15}N abundance.

2.5 Nitrogen and carbon analyses

Following drying, 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 an Isotopic Ratio Mass Spectrometer (Isoprime 100, Elementar GmbH, Langenselbold, Germany) in a continuous flow.

2.5.1 ^{15}N atom% excess

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 where no ^{15}N was applied were used as reference samples (Table 2).

2.5.2 N derived from the labelled fertiliser

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

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

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 ($Ndff_{mass}$) in the plant or soil samples, the following formula was used:

$$[2]\ Ndff_{mass}\ (mg\ N\ kg^{-1}soil) = \left(\frac{^{15}Nex_{sample}}{^{15}Nex_{lf}}\right) \times total\ \ N\ in\ the\ sample\ per\ kg\ soil\ (mg\ N\ kg^{-1}soil)$$

where total N in the plant sample per kg soil was calculated by dividing the total N in the plant 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.

2.5.3 Recovery of ^{15}N

The ^{15}N recovery, which refers to the percentage of initial N applied remaining in the plant or soil samples after a given time, was calculated as follows:

$$[3]\ ^{15}N\ recovery\ (\%) = \left(\frac{Ndff_{mass}\ (mg\ N\ kg^{-1}soil)}{Nf\ (mg\ N\ kg^{-1}soil)}\right) \times 100$$

where *Nf* is the amount of N applied in the field in mg kg^{-1} soil. *Nf* was calculated by multiplying the soil volume of 1 ha by the soil bulk density. The soil bulk density for the 0-30 cm layer was

1.45 g/cm^3. This resulted in 4350 t soil dry matter per ha. Subsequently, the total amount of N applied in the field was divided by the dry weight. This resulted in 33.84 mg N kg^{-1} soil for the mineral fertiliser and 55.17 mg N kg^{-1} soil for the slurry.

2.6 Statistical analyses

Statistical analyses were conducted using RStudio version 1.4.1103 (RStudio Team, 2020) coupled to R version 4.0.3. The following packages were used: lme4, lmerTest, emmeans, lattice, car and ggplot2. One-way ANOVA was used with fertiliser treatment as fixed effect to compare the following parameters: mass fraction, total N and C:N ratio. In case of significant effects, separation of means was tested using Tukey's HSD post-hoc test with a significance level of $p < 0.05$ or with Kruskal-Wallis test when normality of residuals was violated. Student's t-test was used for comparing Min and Slu for $Ndff_{\%}$ and ^{15}N recovery in SOM physical fractions.

Linear mixed effect model with random intercept was used to compare the different treatments for dry weight parameters, ^{15}N recovery and $Ndff_{\%}$ in the plants. Fertiliser treatments and Trichoderma inoculation were analysed as fixed effects whereas the field block and the greenhouse block were treated as random effects. In case of significant effects, Tukey's HSD post-hoc test was used for multiple comparisons with a significant level of $p<0.05$. Furthermore, linear mixed effect model with random intercept and slope was used for assessing the relationship between ^{15}N uptake by plants and the ^{15}N content of the different SOM fractions before the pot experiment. The ^{15}N content of the different SOM fractions was treated as fixed effect and the field block and the greenhouse block as random effects. Correlation analysis was used to investigate the relation between total N in mg in POM and MAOM fractions. To estimate from which SOM fractions ^{15}N was taken up by ryegrass plants, the difference in $Ndff_{mass}$ (mg N kg^{-1} soil) in the fractions was calculated by subtracting the $Ndff_{mass}$ (mg N kg^{-1} soil) after the pot experiment from the $Ndff_{mass}$ (mg N kg^{-1} soil) before the pot experiment. A linear mixed effect model with random intercept was used for the analysis. The difference in $Ndff_{mass}$ was treated as fixed effect and the field block and the greenhouse block as random effects. For all statistical analyses, normality and homoscedasticity of residuals were assessed both visually and using Shapiro test and Levene test.

3 Results

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

3.1.1 Mass fraction (%), nitrogen concentration and ^{15}N atom%

The mass fraction represents the percentage of soil recovered in each fraction. After the fractionation according to Steffens, total mass fraction was on average 95% for the three fertiliser treatments. For Cotrufo, however, Con (100.84%±0.94) had a significantly higher total mass fraction than Min (98.59%±1.14) and Slu (97.61%±1.21), $F(2,9)=9.02$, $p=0.007$. Min and Slu total mass fractions were not significantly different. All fraction properties were calculated with the total recovered mass and means of the three fertiliser treatments are presented when no statistical differences were found.

Mass fraction for the two fractionation methods was dominated by <20 µm fractions (>40% of total recovered mass for both methods; Table 2). With Steffens, fSilt-c mass fraction was 43% while sand and cSilt accounted for 26% and 25% of the recovered mass, respectively. Free POM and oPOM constituted a much smaller proportion with on average 0.24% and 0.15%, respectively. There were no significant differences between fertiliser treatments for all Steffens fractions. For Cotrufo, POM mass fraction accounted on average for 50%, without significant differences between fertiliser treatments. Contrastingly, Con (51.16%±0.48) had a slight but significantly higher MAOM mass fraction than Min (48.09%±1.44) and Slu (46.95%±2.23), $F(2,9)=7.85$, $p=0.011$. Mass fractions in Min and Slu were not significantly different.

The N concentration and the ^{15}N atom% of all SOM physical fractions can be consulted in Table 2. No statistical analyses were carried out to compare the different fertiliser treatments for the ^{15}N atom% because this parameter is considered and analysed in the next sections.

Table 2: Mass fraction (%), nitrogen (N) concentration and ^{15}N atom% in 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 ± standard deviation in parentheses. For the mass fraction and N concentration, different letters indicate significant differences between fertiliser treatments ($P < 0.05$) by Tukey's HSD test.

		Mass fraction%			**N%**			**^{15}N atom%**		
		Fertiliser treatment (n=4)								
Fractionation method	**Fraction**	**Con**	**Min**	**Slu**	**Con**	**Min**	**Slu**	**Con**	**Min**	**Slu**
Steffens	**fPOM**	0.26 (0.06)	0.23 (0.01)	0.24 (0.05)	0.596 (0.060)	0.604 (0.060)	0.651 (0.108)	0.3760 (0.0002)	0.5113 (0.0338)	0.5801 (0.0493)
	oPOM	0.14 (0.03)	0.15 (0.02)	0.15 (0.02)	1.923 (0.148)	1.798 (0.077)	1.862 (0.082)	0.3752 (0.0003)	0.4734 (0.0129)	0.4907 (0.0215)
	sand (2000-63 µm)	25.48 (0.41)	26.70 (0.58)	26.75 (2.00)	0.015 **ab** (0.001)	0.013 **a** (0.001)	0.017 **b** (0.002)	0.3753 (0.0006)	0.4589 (0.0235)	0.5250 (0.0461)
	cSilt (63-20 µm)	24.84 (0.51)	25.47 (0.77)	25.82 (0.55)	0.044 **ab** (0.003)	0.042 **a** (0.005)	0.051 **b** (0.004)	0.3749 (0.0001)	0.4349 (0.0115)	0.4750 (0.0273)
	fSilt-c (<20 µm)	44.23 (0.92)	42.40 (0.50)	42.26 (1.46)	0.304 (0.010)	0.306 (0.014)	0.309 (0.007)	0.3756 (0.0002)	0.3996 (0.0047)	0.4382 (0.0194)
	total	94.96 (0.62)	94.95 (0.28)	95.22 (0.54)						
Cotrufo	**POM (2000-20 µm)**	49.67 (0.67)	50.51 (0.46)	50.66 (1.86)	0.044 (0.003)	0.038 (0.004)	0.044 (0.006)	0.3686 (0.0003)	0.4557 (0.0047)	0.5000 (0.0194)
	MAOM (<20 µm)	51.16 **b** (0.48)	48.09 **a** (1.44)	46.95 **a** (2.23)	0.342 **a** (0.010)	0.347 **ab** (0.012)	0.364 **b** (0.009)	0.3751 (0.0001)	0.4007 (0.0033)	0.4347 (0.0155)
	total	100.84 **b** (0.94)	98.59 **a** (1.14)	97.61 **a** (1.21)						

Bulk soil	0.195 **b** (0.010)	0.176 **a** (0.007)	0.188 **ab** (0.008)	0.3760 (0.0003)	0.4085 (0.0048)	0.4516 (0.0194)

3.1.2 Losses of ^{15}N during the fractionation procedure

To assess N losses occurring during the fractionation procedure for the two methods, the distribution of ^{15}N was calculated as a proportion of the amount of ^{15}N present in the bulk soil before the fractionation step. Higher losses were observed for Steffens than Cotrufo (Figure 5). For both methods, more N was lost from POM fractions in Slu than Min. Occluded POM showed a significantly higher proportion of ^{15}N after the fractionation for Min (4.77%±0.76) than Slu (2.35%±0.37), t(6)=5.73, p=0.001. Likewise, there was a higher proportion of ^{15}N after the fractionation in POM for Min (29.89%±5.14) than for Slu (20.90±1.67), t(6)=3.33, p=0.016.

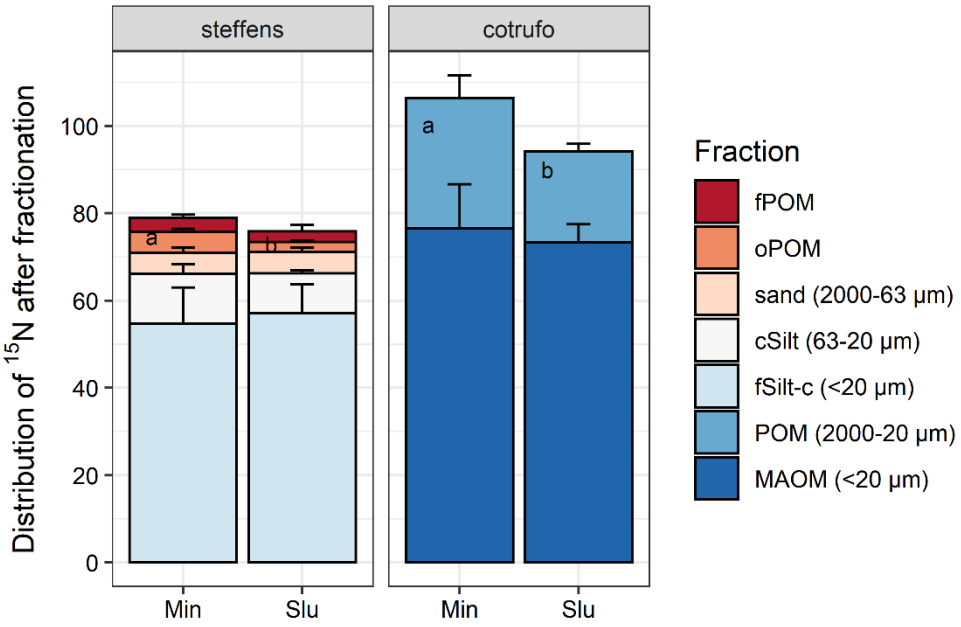

Figure 5: Distribution of ^{15}N after the fractionation procedure based on bulk soil ^{15}N content in SOM physical fractions from the two fractionation methods of the mineral fertilised (Min) and slurry fertilised (Slu) treatments. Error bars represent standard deviation (n=4) and different letters indicate significant differences between fertiliser treatments in each fraction (P < 0.05) by Student's t-test.

3.1.3 Total N distribution and C:N ratio

Most N out of the recovered N was found in <20 µm fractions for both fractionation methods (Figure 6). For Steffens, Slu (85.5%) had a significantly lower proportion of N in fSilt-c than Con and Min (both 87.7%), F(2,9)=7.10, p =0.01. Control and Min were not significantly different. Conversely, the proportion of N in cSilt was larger for Slu (8.6%) than Con (7.1%) and Min (7.2%), F(2,9)=13.83, p =0.002. Control and Min were not significantly different. For all other SOM fractions, no significant differences were detected between fertiliser treatments. Free POM, oPOM and sand accounted on average for 1%, 1.8% and 2.6% of the recovered N, respectively. For Cotrufo, POM constituted 10.9% of the recovered N while MAOM stored 89.1%.

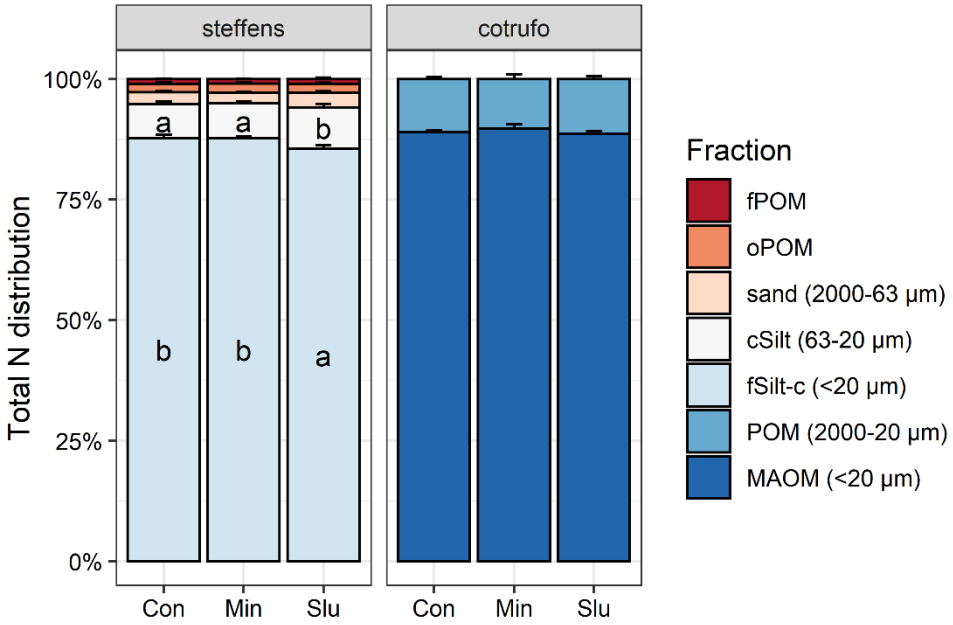

Figure 6: Distribution of total nitrogen (N) out of the recovered N across SOM physical fractions of the two fractionation methods from the control (Con), mineral fertilised (Min) and slurry fertilised (Slu) treatments. Error bars represent standard deviation (n=4), and for a given fraction, different letters indicate significant differences between fertiliser treatments (P < 0.05) by Tukey's HSD test.

C:N ratios of fPOM and oPOM were higher than those of the mineral fractions (Figure 7). Likewise, the C:N ratio of POM was higher than that of MAOM. For Steffens, no significant differences were detected between fertiliser treatments across SOM physical fractions, and therefore means of the three fertiliser treatments are presented. The C:N ratio was highest for fPOM (19.9±0.7), declining with decreasing particle size for oPOM (19.1±0.5), sand (12±1.2), cSilt (11.6±0.3) and fSilt-c (8.4±0.2). For POM, the C:N ratio was lower for Con (12.5±0.4) than for Min (13.3±0.4) and Slu (13.8±0.2), F(2,9)=16.41, p<0.001. Min and Slu were not significantly different. The C:N ratio for MAOM was on average 7.6 and not significantly different between fertiliser treatments. Similarly to POM, the C:N ratio of the bulk soil was significantly lower for Con (8.4±0.2) than Min (9.2±0.1) and Slu (9.4±0.4), F(2,9)=15.33, p=0.001. Min and Slu were not significantly different.

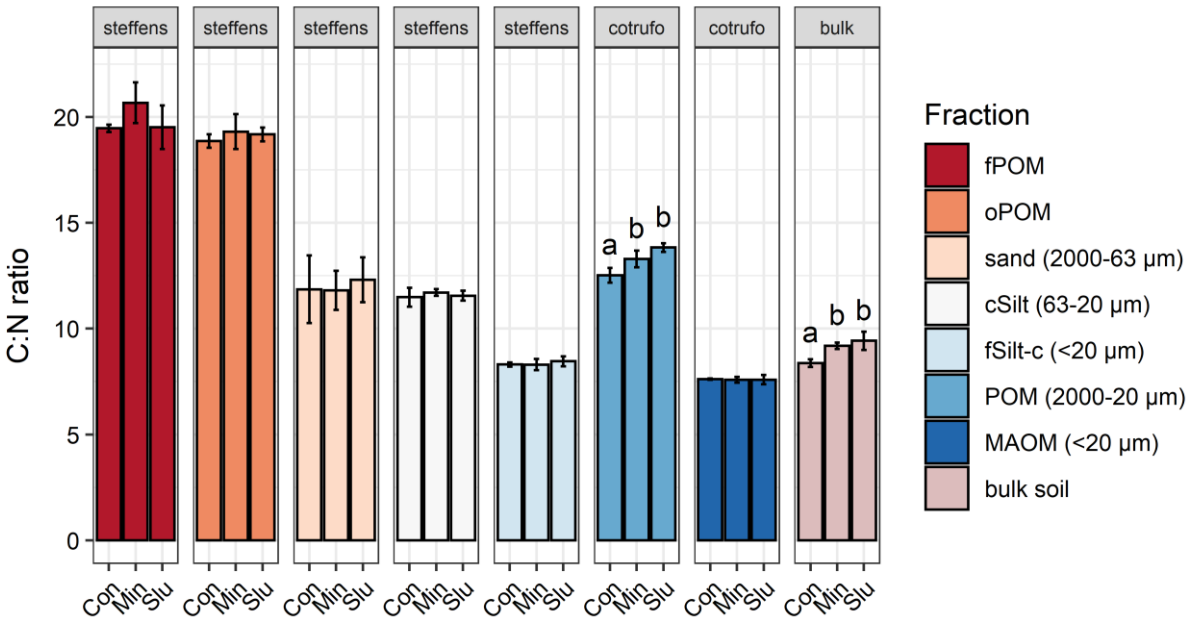

Figure 7: C:N ratios across all SOM physical fractions of the two fractionation methods and bulk soil from the control (Con), mineral fertilised (Min) and slurry fertilised (Slu) treatments. Error bars represent standard deviation (n=4) and different letters indicate significant differences between fertiliser treatments ($P < 0.05$) by Tukey's HSD test.

3.1.4 Nitrogen derived from the labelled fertilisers and recovery of ^{15}N from the applied labelled fertilisers in SOM physical fractions

The proportion of N derived from the labelled fertiliser across all SOM physical fractions ranged between 0.31%±0.06 in fSilt-c and 2.72%±0.66 in fPOM. For both fractionation methods, the $Ndff_{\%}$ was higher for the POM fractions than for the mineral-associated fractions (Figure 8), indicating a greater proportion of N from the labelled fertiliser out of the total recovered N in the coarser fractions. The organic fractions fPOM and oPOM did not show significant differences between Min and Slu, while all other fractions and the bulk soil had significantly higher proportions of labelled N for Slu than for Min.

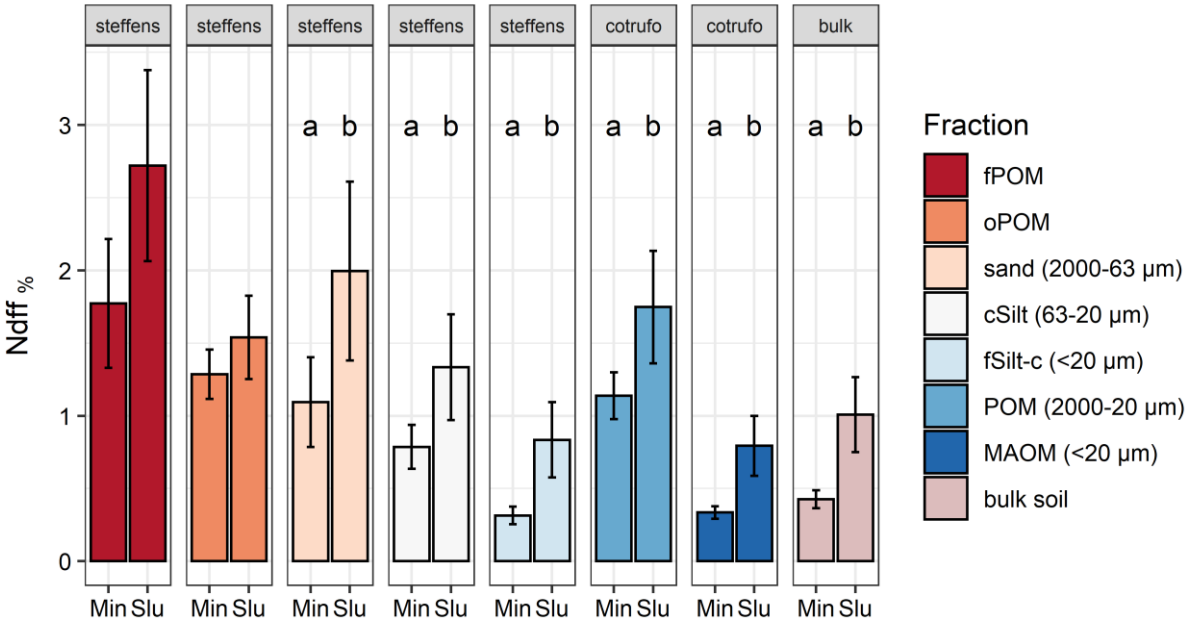

Figure 8: Proportion of nitrogen (N) derived from the labelled fertilisers in SOM physical fractions from the two fractionation methods and bulk soil of the mineral fertilised (Min) and slurry fertilised (Slu) treatments. Error bars represent standard deviation (n=4) and different letters indicate significant differences between fertiliser treatments ($P < 0.05$) by Student's t-test.

The results obtained for the two fractionation methods were comparable, with most ^{15}N recovered in the fractions <20 µm (Figure 9). Furthermore, the ^{15}N recovery was significantly higher for Slu (34.11%±7.31) than Min (22.09%±3.11) in the bulk soil, t(6)=-3.02, p=0.023. A similar pattern was observed in fSilt-c and MAOM, although not significant. The other fractions did not show significant differences between fertiliser treatments except for oPOM, which had a higher ^{15}N recovery in Min (1.05%±0.21) than Slu (0.78%±0.07), t(6)=2.48, p=0.048.

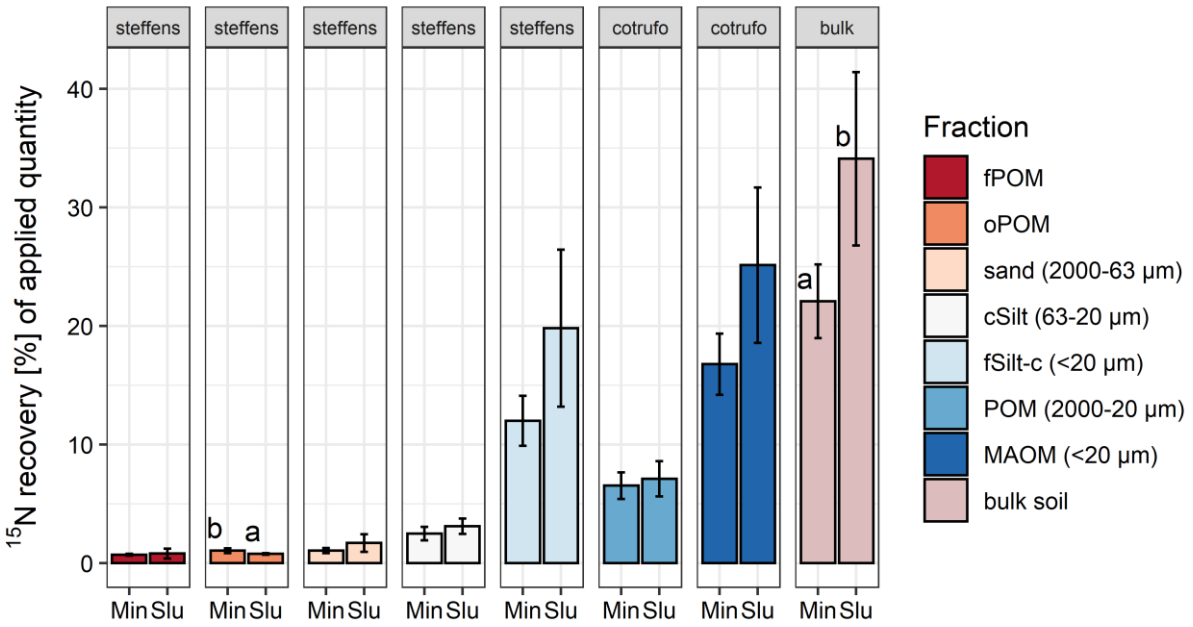

Figure 9: Recovery of ^{15}N in percent of the originally applied quantity of ^{15}N in SOM physical fractions from the two fractionation methods and bulk soil of the mineral fertilised (Min) and slurry fertilised (Slu) treatments. Error bars represent standard deviation (n=4) and different letters indicate significant differences between fertiliser treatments ($P < 0.05$) by Student's t-test.

3.2 Availability of previously applied ^{15}N to ryegrass plants

3.2.1 Plant dry matter

The root dry weight for Slu (1.62 g pot^{-1}±0.18) was significantly greater than for Con (1.35g pot^{-1} ±0.19) and Min (1.35 g pot^{-1}±0.15), F(2,41)=17.24, p<0.001. Control and Min, however, were not significantly different (Figure 10). Regarding shoot dry weight, no significant differences were observed between fertiliser treatments and no effect of *Trichoderma asperellum* inoculation on plant growth was detected.

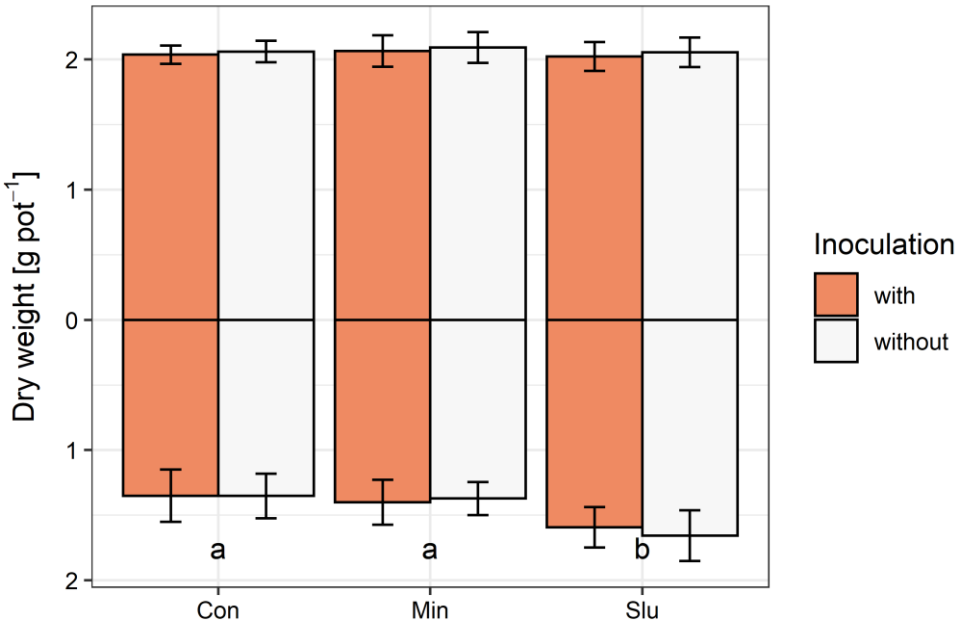

Figure 10: Shoot (upper bars) and root (lower bars) dry weight of ryegrass grown in soil from the control (Con), mineral fertilised (Min) and slurry fertilised (Slu) treatments with and without *Trichoderma asperellum* inoculation. Error bars represent standard deviation (n=8) and different letters indicate significant differences between fertiliser treatments (P < 0.05) by Tukey's HSD test.

Approximately two third of the total N in the plant was stored in the shoots while one third in the roots (Table 3). As *Trichoderma asperellum* did not show any effect on N concentration and N content, the average value per fertiliser treatment is shown. For the N concentration and N content in the shoots, no differences were observed between fertiliser treatments. Likewise, the N concentration in the roots was similar for the three fertiliser treatments. The N content in the roots, however, was significantly higher for Slu than for Con and Min, F(2,41)=15.12, p<0.001. Control and Min were not significantly different. Regarding the C:N ratio of the shoots, no differences between fertiliser treatments were observed. Contrastingly, a significantly lower C:N ratio was observed in the roots for plants treated with Slu than Min, F(2,38)=4.28, p=0.02. The control treatment was not significantly different from Min and Slu.

Table 3: Total nitrogen (N) concentration and total N content of the shoots and the roots per pot from the control (Con), mineral fertilised (Min) and slurry fertilised (Slu) treatments. As *Trichoderma asperellum* inoculation was not significant, all parameters are averaged for each fertiliser treatment, n=16 ± standard deviation in parentheses. Different letters indicate significant differences between fertiliser treatments within a row ($P < 0.05$) by Tukey's HSD test.

		Fertiliser treatment (n=16)		
		Con	**Min**	**Slu**
Shoots	**N%**	0.81 (0.59)	0.82 (0.06)	0.82 (0.05)
	N [mg pot^{-1}]	16.47 (0.85)	17.02 (1.58)	16.67 (0.55)
	C:N ratio	50.4 (3.7)	49.2 (3.8)	49.0 (3.5)
Roots	**N%**	0.67 (0.04)	0.67 (0.03)	0.66 (0.07)
	N [mg pot^{-1}]	9.08 (0.88) **a**	9.19 (0.68) **a**	10.62 (1.04) **b**
	C:N ratio	60.8 (2.5) **ab**	61.3 (2.1) **b**	59.1 (2.9) **a**

3.2.2 Nitrogen derived from the labelled fertiliser in the plant

To assess the proportion of N from Min and Slu in the plant, Ndff$_{\%}$ was calculated. Plants grown in Slu (2.22%±0.58) recovered a substantially higher proportion of N in the total biomass from the labelled fertiliser than those grown in Min (1.10%±0.16), $F(1,25)=119.90$, $p<0.001$. Similar patterns were found for shoots and roots (Figure 11). Nevertheless, similarly as for dry weight parameters, no effect of *Trichoderma asperellum* on Ndff$_{\%}$ were observed.

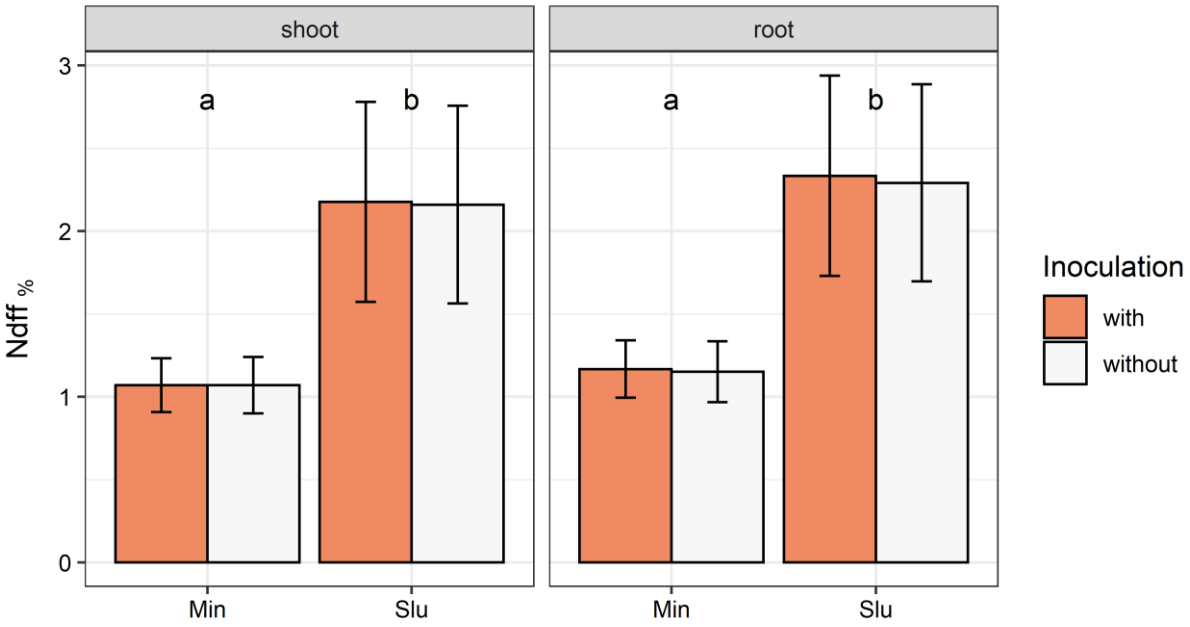

Figure 11: Proportion of nitrogen derived from the labelled fertilisers by ryegrass grown in soil from the mineral fertilised (Min) and slurry fertilised (Slu) treatments with and without *Trichoderma asperellum* inoculation. Error bars represent standard deviation (n=8) and different letters indicate significant differences between fertiliser treatments ($P < 0.05$) by Tukey's HSD test.

3.2.3 Recovery of ^{15}N from the applied labelled fertilisers in the plant

The recovery of ^{15}N based on the quantities of fertiliser applied two years ago was higher for Slu than for Min both in the shoots (Slu: 1.74%±0.46, Min: 1.43%±0.24; $F(1,25)=8.32$, $p=0.008$) and in the roots (Slu: 1.19%±0.33, Min: 0.84%±0.15, $F(1,25)=18.55$, $p<0.001$). No significant effect of Trichoderma asperellum on ^{15}N recovery was detected (Figure 12).

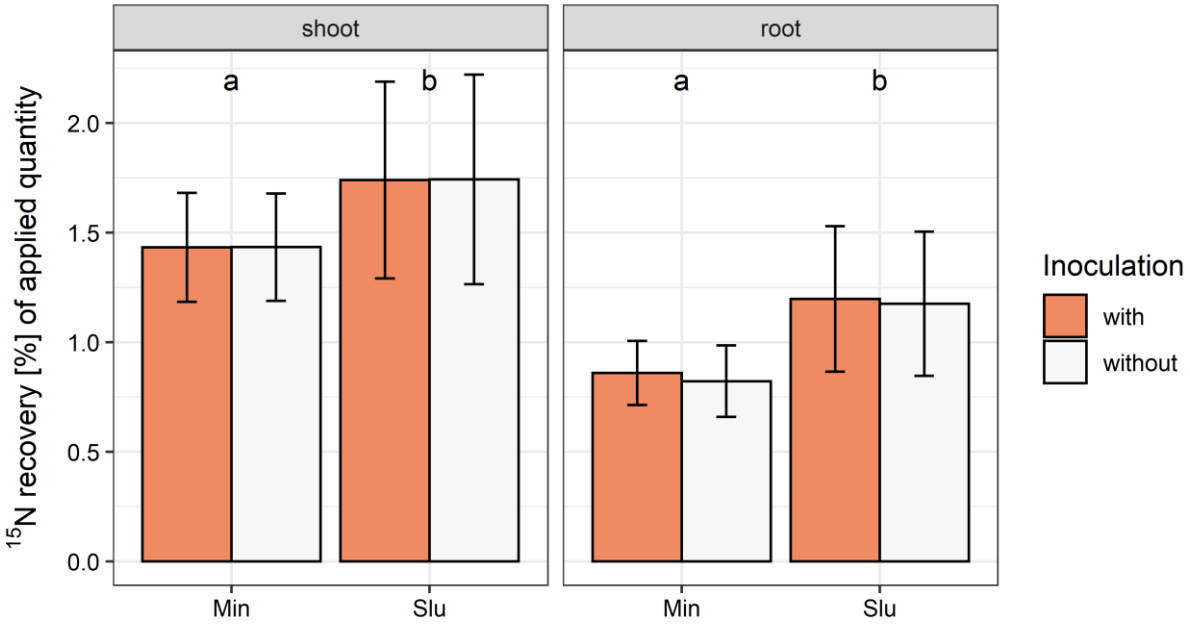

Figure 12: Recovery of ^{15}N in percent of the originally applied quantity of ^{15}N in the ryegrass plant from the mineral fertilised (Min) and slurry fertilised (Slu) treatments with and without *Trichoderma apserellum* inoculation. Error bars represent standard deviation (n=8) and different letters indicate significant differences between fertiliser treatments ($P < 0.05$) by Tukey's HSD test.

3.3 Relationship between the nitrogen derived from the fertiliser in the plants and the nitrogen derived from the fertiliser in different SOM fractions

A clear positive relationship between the $Ndff_{mass}$ in the plants and the $Ndff_{mass}$ in SOM physical fractions before the pot experiment as determined with the method of Cotrufo was observed (Figure 13). Since the $Ndff_{mass}$ of POM and MAOM was correlated to the $Ndff_{mass}$ of the plants, the correlation between total POM-N and total MAOM-N was calculated. The calculation of the correlation showed a moderate correlation(R^2_{adj}=0.539, p=0.004, Appendix 6). Likewise, sand, cSilt and fSilt-c showed strong positive relationships between the $Ndff_{mass}$ in the plants and the $Ndff_{mass}$ in SOM physical fractions before the pot experiment as determined with the method of Steffens (data not shown). Free POM and oPOM, however, did not show a significant linear relationship (data not shown).

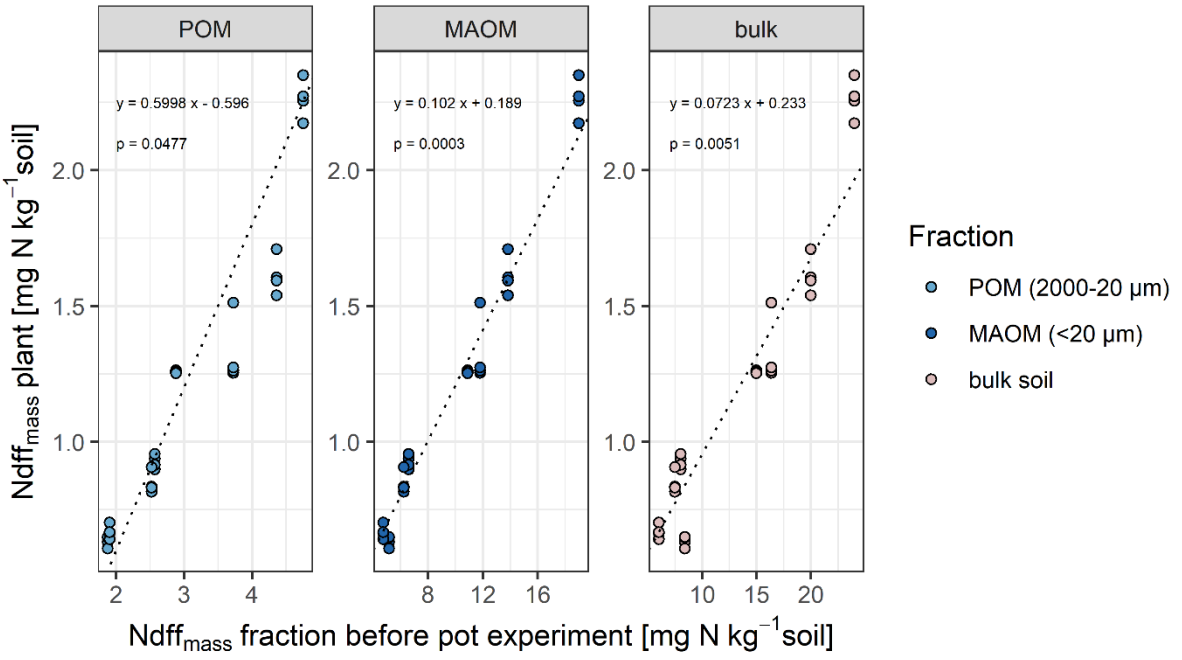

Figure 13: Linear relationship between $Ndff_{mass}$ in the plant and $Ndff_{mass}$ in SOM physical fractions for Cotrufo before the pot experiment.

In addition, the difference in $Ndff_{mass}$ in POM and MAOM before and after the pot experiment was calculated (Figure 14). The use of a linear mixed-effects model showed significantly lower $Ndff_{mass}$ in MAOM after the pot experiment for both Min and Slu, t(5.06)=-6.91, p=0.001. However, no significant differences between Min and Slu were found. For POM and the bulk soil, the changes were not significantly different from 0 for Min, indicating no significant changes in $Ndff_{mass}$ before

and after the pot experiment. Nonetheless, for Slu significant differences from Min were observed for both fractions (POM: t(26)=-2.55, p=0.017; bulk: t(27)=-2.90, p=0.007).

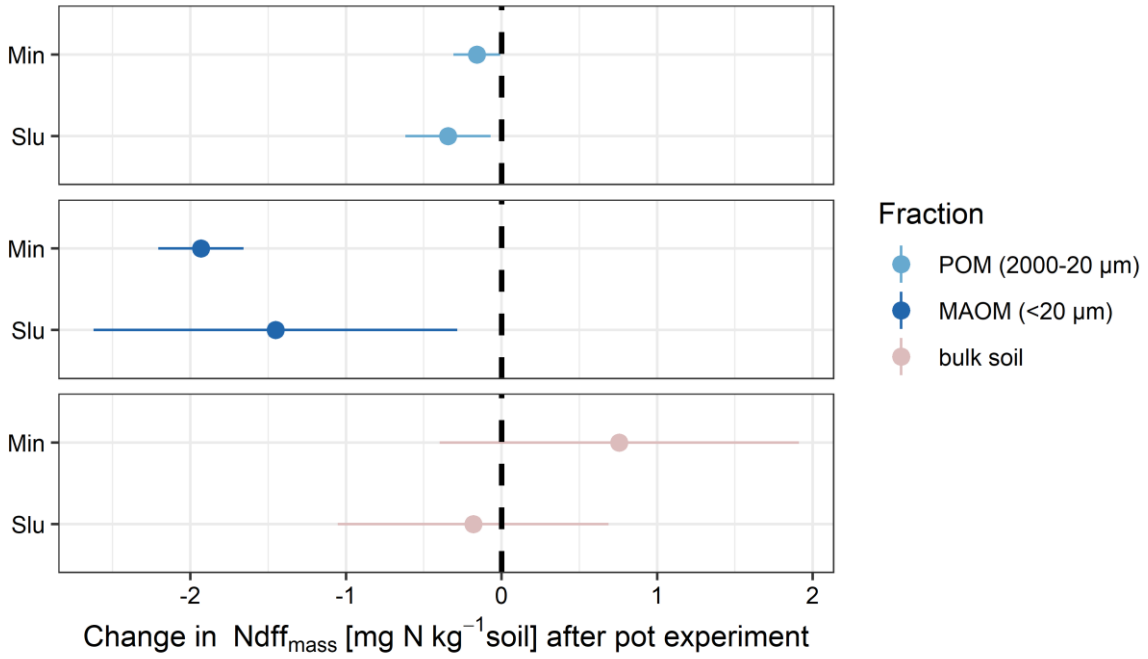

Figure 14: Change in content of nitrogen derived from the labelled fertilisers (after minus before) in the soil from the pot experiment for POM, MAOM and bulk soil. Error bars represent standard deviation (n=16).

4 Discussion

The aim of my study was to investigate the fate of ^{15}N from cattle slurry (Slu) and mineral fertiliser (Min) two years after application both in SOM physical fractions and as a source of N for plants. Significantly more ^{15}N was found in the bulk soil two years after fertiliser application in the plots treated with Slu (34%) compared to those treated with Min (22%) in line with previous studies (Sørensen, 2004; Smith and Chalk, 2018). The opposite pattern was observed for plants in the year of application where more ^{15}N was recovered in the plants treated with Min than with Slu (Frick et al., in preparation). For both fractionation methods, most of the remaining ^{15}N was found in SOM fractions <20 µm. Although plants grown in Slu had a significantly higher root dry weight than those grown in Min and the control treatment (Con), no effect on shoot dry weight was detected. In addition, inoculation with the fungus *Trichoderma asperellum* did not show any effect on plant dry weight nor on ^{15}N uptake. Finally, the analysis of the difference in $Ndff_{mass}$ of POM and MAOM fractions before and after the pot experiment revealed that plants took up ^{15}N mainly from MAOM. This sheds light on the potentially dynamic nature of mineral-organic associations, thereby possibly providing N to plants as suggested by different authors (Whalen et al., 2000; Jilling et al., 2018).

4.1 Comparison of the fractionation methodologies

The total mass fractions (Table 2) for Steffens (95%) and Cotrufo (99%), which represent the total recovered soil material after the fractionation procedure, were similar to values found in the literature (van Wesemael et al., 2019; Jilling et al., 2020). Contrary to expectations, significant differences in total and MAOM mass fractions were found between fertiliser treatments for Cotrufo. Despite being significant, these differences were minor (total mass fraction: Con=100.84%±0.94, Min=98.59%±1.14, Slu=97.61%±1.21) and therefore probably do not have further implications in this study.

The comparison of ^{15}N losses during the fractionation procedure for the two fractionation methods (Figure 5) revealed more ^{15}N lost with Steffens than with Cotrufo. This can be explained by the different methodologies. Indeed, Steffens includes a rinsing step of the mineral fractions to avoid contamination with nutrients from the SPT solution. Although the samples are centrifuged, a substantial amount of minerals is lost when siphoning and thereby also part of the ^{15}N. On the contrary, the protocol of Cotrufo only includes a wet sieving step where no major losses can occur. The losses of ^{15}N during the fractionation procedure were higher for Slu than for Min in oPOM (Steffens) and POM (Cotrufo), which may indicate that the ^{15}N present in these fractions is less stable and thereby more prone to be either leached or taken up by the plants. Nonetheless, this is only a hypothesis because the processes occurring during the fractionation procedure and the ones taking place in soil profiles are probably not analogous. Intriguingly, the proportion of ^{15}N recovered after the fractionation procedure was higher than 100% (out of residual ^{15}N in the bulk soil) for Min with Cotrufo. This may be attributed to the high total mass fraction obtained and to the variation between samples for the ^{15}N abundance in the bulk soil.

There is no consensus on whether the size limit between POM and MAOM fractions should be at approximately 50 µm (Cotrufo et al., 2019) or at 20 µm (van Wesemael et al., 2019). Therefore, an interesting outcome of this study is that the sand fraction and the cSilt fraction had similar C:N ratios for Steffens, while the C:N ratio in the fSilt-c fraction was distinct (Figure 7). In addition, similar N isotopic composition were found for both fractions (Table 2), suggesting analogous mechanisms for N retention and/or mineralisation. This could be an argument in favour of a 20-µm limit. Therefore, it would be interesting to see if similar patterns are observed in other soil types. Nonetheless, 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).

4.2 Fate of N from cattle slurry and mineral fertiliser in SOM physical fractions

To account for the higher initial amount of total N applied for Slu (240 kg N ha^{-1}) than Min (147.2 kg N ha^{-1}) in the field, the ^{15}N recovery was calculated based on the initial ^{15}N quantities applied. My first hypothesis [1] that more ^{15}N from Slu than Min would be in the mineral-associated fractions had to be rejected. Indeed, although there was a clear trend for a higher ^{15}N recovery from Slu than Min in this fraction for both fractionation methods, the difference was not significant due to the relatively large variation within treatment (Figure 9). Interestingly, a considerable amount of ^{15}N was still present in the mineral-associated fractions of Min two years after application. This indicates that, after application, ^{15}N from Min could have been immobilised by soil microorganisms (Jacquin et al., 1992), directly transferred to mineral-associated fractions (Castellano et al., 2012) or taken up by roots and be derived from the root turnover. Similar to my experiment, Sørensen (2004) reported more ^{15}N recovered from cattle slurry than mineral fertiliser 2.5 years after application due to three main reasons. First, there was a higher ^{15}N recovery in the plants for mineral fertiliser in the year of application. Second, more ^{15}N was immobilised in the microbial biomass for slurry. Third, there was a lower remineralisation rate for slurry. Chantigny et al. (2004) observed a rapid (after 1 day) clay fixation of ^{15}N pig slurry and this fixation was higher in a clay soil than in a sandy loam, demonstrating this direct transfer of ^{15}N to the mineral-associated fraction.

The proportion of total N originating from the fertilisers was small in all SOM fractions ($Ndff_{\%}$: 0.3-2.7%, Figure 11) but it was, nonetheless, significantly higher for Slu than for Min in most fractions (except fPOM and oPOM). These two SOM fractions appeared to be less responsive to fertiliser applications on the mid-term. Comparably, Jilling et al. (2020) reported low responsiveness of fPOM and oPOM to conservation tillage and cover crops after three years, while the mineral-associated fraction showed a rapid increase in N content. The higher $Ndff_{\%}$ for Slu than for Min might be the result of the experimental design. Indeed, more ^{15}N was applied for Slu (240 kg N ha^{-1}) than Min (147.2 kg N ha^{-1}) because the applied amounts were calculated based on the ammonium-N content of the slurry. An interesting outcome is that the $Ndff_{\%}$ tended to decrease with decreasing particle size of the SOM fractions. Since a larger proportion of total N was found in fractions with small particle size (Figure 6), a possible explanation is that more ^{15}N in absolute quantity was necessary to make up a similar percentage (as compared to coarser fractions). Another explanation is that both fertilisers may have entered the mineral-associated fractions partly through plant debris. However, the data does not allow for separating the incorporation of ^{15}N through microbial or abiotic processes (sorption of NH_4^+). Bosshard et al. (2008) also found a decreasing $Ndff_{\%}$ with decreasing particle size for mineral fertiliser and urine three months after application. In contrast, they observed a higher $Ndff_{\%}$ from faeces in the 250-2000 µm fraction than in the other fractions (fPOM and fractions <250 µm) after 3 months, suggesting that particulate faeces N compounds may have been incorporated into aggregates.

Regarding the distribution of total N across SOM physical fractions, most N was stored in the mineral-associated fractions (fSilt-c: 87% and MAOM: 89%) for both fractionation methods (Figure 6). These values are in the same range as in other studies (Jilling et al., 2018, 2020) and depend to a large extent on the soil clay content (Chantigny et al., 2004; Chivenge et al., 2011). Moreover, there was a significantly higher proportion of N in cSilt for Slu than for Con and Min. Conversely, a significantly lower proportion of N was observed in fSilt-c for Slu than for Con and Min. This could be explained by the greater C content in Slu, which might have stimulated microbial activity (Ma et al., 2020) and as a result more N was incorporated in cSilt.

The C:N ratios across all SOM physical fractions decreased with increasing density and decreasing particle size (Figure 7). This is in line with other studies (Sollins et al., 2006; Kirkby et al., 2011; Samson et al., 2020) and a possible explanation is more sorption of N-containing compounds to mineral surfaces with increasing density or decreasing particle size (Sollins et al., 2006). Also, a lower C:N ratio suggests a higher degree of decomposition with decreasing particle size (Bosshard et al., 2008). Significantly higher C:N ratios were found for Min and Slu as compared to Con in POM and in the bulk soil. Nevertheless, the differences are minor and only observed with the method of Cotrufo.

4.3 Fate of ^{15}N from SOM physical fractions to plants

4.3.1 Relation between plant growth and N fertilisation

The measurements of dry weight parameters and ^{15}N content in plants did not show any effects of *Trichoderma asperellum* inoculation on plant growth. Therefore, hypothesis [2] had to be rejected. A possible explanation is the very low N availability during this pot experiment due to the absence of fresh N fertiliser application, which could have led to competition between the fungi and the plants. Indeed, the N concentration of the shoots (0.8%) and the roots (0.7%) of ryegrass plants in this research were rather low as compared to values (0.6-6.26%) found in other studies (Gislum et al., 2004; Jiang et al., 2016). As a result, plants may have outcompeted the fungi. Additionally, the small size of the pots used in this study could have further limited the ability of *Trichoderma aperellum* to establish. Similar to these findings, Ortega-García et al. (2015) reported no effect of *Trichoderma asperellum* on onion growth at low N-P-K fertiliser rate. Conversely, onion growth was significantly greater with *Trichoderma asperellum* inoculation at a fertiliser rate above 50% of the recommended dose. This suggests that *Trichoderma asperellum* requires a minimum amount of nutrients for having this nutrient solubilising effect.

The shoot dry weight of ryegrass plants was not influenced by the fertiliser treatments tested in this study, which indicates a limited residual N effect of mineral fertiliser and cattle slurry on aboveground biomass two years after application. This is also confirmed by the relatively low ^{15}N recovery observed in the shoots (Figure 12), although the ^{15}N recovery was significantly higher for Slu (1.7%) than Min (1.4%). Similarly, Sørensen (2004) found a ^{15}N recovery of 1% for mineral fertiliser and of 0.7-1.67% for cattle slurry in barley aboveground biomass two years after application. He also observed little residual N effect of cattle slurry on barley N uptake. As a result, he concluded that the release rate of immobilised N from cattle slurry is low and therefore mainly contributes to the long-term storage of N in the soil. According to literature, mineral fertiliser application is known to generally have little residual N effect on subsequent crops (Suarez-Tapia et al., 2018). Contrastingly, animal manure has been shown to have a substantial residual N effect in long-term experiments (Schröder et al., 2005; Riley, 2016). This significant residual N effect observed in long-term experiments may be attributed to the cumulative effect of repeated animal manure applications (Webb et al., 2013). This could explain the absence of residual N effect on aboveground biomass observed in my study.

The root dry weight was significantly higher for Slu than for Con and Min (Figure 10). A possible explanation is the higher proportion out of total N found in c-Silt fraction for Slu as compared to Con and Min (Figure 6). The N found in this fraction might have been more available to plants, thereby mostly promoting root growth due to the poor N conditions. Another result confirming this hypothesis is the difference in $Ndff_{mass}$ in the POM fraction (Figure 14), which showed that more ^{15}N was taken up from POM by ryegrass plants treated with Slu than with Min. Samson et al. (2020) investigated the effect of long-term cattle slurry application on the N content of different SOM fractions. They observed a build-up of total N in the coarse-MAOM fraction (>53 µm after removal of the light fraction <1.7 g cm^{-3}) after 9 years, showing a similar trend as in this study.

Although cattle slurry was only applied during one year in my experiment, the higher proportion of N in c-Silt could be a reason for the increased root dry weight. Plants are known to increase their root to shoot ratio when N is limiting following the functional equilibrium principle (Lambers, 1983) as observed in this study. This suggests that, out of total soil N, more N from slurry was available to ryegrass plants, thereby promoting root growth only in this treatment.

4.3.2 Source of N for plant growth

With regard to the source of N for plant growth, there was a clear positive relationship between the amount of ^{15}N in POM and MAOM fractions before the pot experiment and the ^{15}N uptake by the plants (Figure 13). Nonetheless, the regression analysis did not allow to draw relevant conclusions on the origin of the ^{15}N in the plants due to the covariation of N content in the fractions (Appendix 6). A previous study also showed a strong collinearity between N mineralisation and N isolated in physiochemical fractions (Jegajeevagan et al., 2013). The authors concluded that the different fractions explained a similar part of the variation in the N mineralisation rate.

Calculation of the difference $Ndff_{mass}$ before and after the pot experiment in POM, MAOM and bulk soil gave further insights. The MAOM fraction appeared to be the main source of ^{15}N for plants both in Min and Slu (Figure 14), whereas POM seemed to be a source of ^{15}N only for plants grown in Slu. Consequently, hypothesis [3] had to be rejected because only little ^{15}N in the plant seemed to derive from POM. This finding challenges the view that MAOM is inaccessible to microbial decomposers and that its nutrients are rather unavailable to plants. Jilling et al. (2018) suggest that plants can destabilise MAOM either directly or indirectly. On the one hand, the direct pathway includes the exudation of low molecular organic acids, which can decrease the pH or directly compete with MAOM for binding sites. On the other hand, root exudates might result in a priming effect by stimulating microbial activity and thereby fostering N mineralisation from MAOM. Li et al. (2021) tested the effect of different compounds of root exudates (oxalic acid, catechol and glucose) on the destabilisation of MAOM on minerals differing in reactivity (ferrihydrite, goethite, amorphous $Al(OH)_3$ and gibbsite). They demonstrated that simple exudates released by plants and microbes can destabilise MAOM via both direct and indirect pathways. Similarly, a previous study observed soil C mineralisation from MAOM by addition of oxalic acid (Keiluweit et al., 2015).

Since there was no significant difference in $Ndff_{mass}$ between Min and Slu in MAOM (Figure 14), it can be assumed that similar mineralisation processes took place for both fertilisers in this fraction. In contrast, plants took up more ^{15}N from POM in Slu than Min. This could explain that a higher root biomass was observed for Slu. Indeed, the additional nitrogen derived from the fertiliser taken up from POM could have triggered a greater root growth. Regarding the difference in $Ndff_{mass}$ in the bulk soil, the positive value for Min was unexpected as this would imply that more ^{15}N was present after the pot experiment than before. Despite being positive, the difference in $Ndff_{mass}$ was not statistically different from 0, meaning no statistical difference. The relative high variation in ^{15}N content across measurements in the bulk soil might at least partially explain this outcome. For Slu, the difference in $Ndff_{mass}$ was significantly lower than Min in the bulk soil, indicating a lower ^{15}N content after the pot experiment.

5 Conclusions

My findings show that most ^{15}N recovered two years after application was stored in the mineral-associated fractions both for Slu and Min. The hypothesis that a higher ^{15}N recovery would be found in <20 µm fractions for Slu than for Min had to be rejected, although this trend could be observed. Despite the difference in ^{15}N recovery not being significant between Min and Slu in < 20 µm fractions, a significantly higher ^{15}N recovery was observed in the bulk soil for Slu than for Min. Therefore, the trend for a higher ^{15}N recovery in the mineral-associated fractions might suggest real differences.

Although I observed very little effect of residual N from Min and Slu on plant aboveground biomass, I measured a slight but significantly higher root dry weight for ryegrass plants grown in Slu as compared to Con and Min. This might be because a higher proportion of total N was found in cSilt for Slu and possibly more bioavailable. From a financial perspective for the farmer, this result has probably no major implications as, most of the time, solely the aboveground biomass is harvested. However, more root biomass might increase C sequestration, water uptake and other nutrient acquisition at a later stage. This could be beneficial for increasing SOM content and improving crop performances. Overall, it is important to note that the residual N effect in this research was assessed based on fertiliser applications spread over only one growing season. However, repeated applications of slurry over many years could show an increased residual N effect on plants. Furthermore, *Trichoderma asperellum* fungi did not influence plant growth or ^{15}N uptake, possibly due to the low N availability in this study. Further research in N-rich conditions assessing the effect of this beneficial fungus would certainly be useful to unravel its potential for improving plant N uptake.

Finally, the comparison of the ^{15}N content of POM and MAOM fractions before and after the pot experiment suggested that most ^{15}N in the plants derived from MAOM. This finding supports growing evidence that MAOM is a dynamic N pool, which plays an important role in N cycling. It is however important to mention that N conditions were very poor in this study as shown by the low N concentration in ryegrass plants as compared to other studies. Next to that, the soil was disturbed due to sampling and sieving. Therefore, the ability of plants to access N from MAOM might be overestimated as compared to an agricultural setting. I would highly recommend carrying out a similar experiment in N-rich conditions to examine whether plants also take up a substantial amount of N from MAOM. Since solely the method of Cotrufo was used to assess the difference in $Ndff_{mass}$ before and after the pot experiment, I would also recommend carrying out a similar experiment using the fractionation method of Steffens after the pot experiment. This could give further insight on the role of different SOM physical fractions on plant growth, especially which coarse fractions are responsible for the higher ^{15}N uptake and higher root dry weight observed for Slu.

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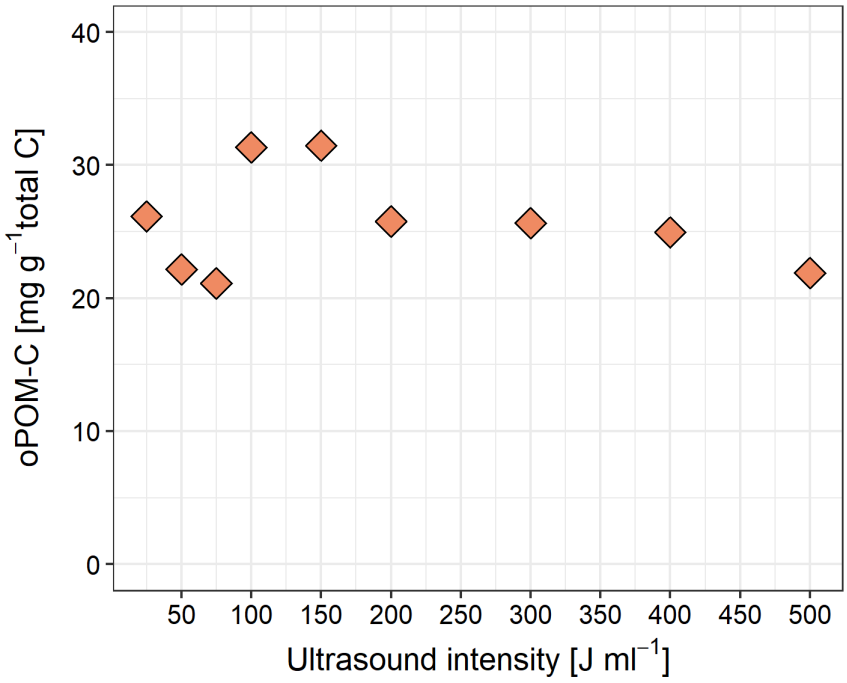
Appendices

Appendix 1: Amount of oPOM-C released depending on ultrasonic dispersion energy.



Appendix 2: Protocol density fractionation DynaCarb – Method Steffens et al. (2009)

Last modified: (20/05/2019, Marius Mayer)

1. Label all tubes

2. Mix the **SPT-solution** (under deduction): (*large spoon, SPT, large plastic beaker with handle, tall measuring beaker, bar magnet, magnetic stirrer, density spindle, 2l-laboratory bottle*)

- ➔ Solve 990 g SPT in approx. 500 ml demin. water and stir it until the salt is well dissolved
- ➔ Transfer it in measuring beaker + add bar magnet
- ➔ Put on the magnetic stirrer until water column is completely whirled through (help with large spoon)
- ➔ Target a density of 1.8g cm^3 with the density spindle → add demin. water slowly until it reaches the correct density (

3. Weigh + label: (*crystallising dish, spoon, scale, soil sample, tweezer, tube(s), tape, water resistant pen*)

- ➔ 30 g in crystallising dish (CD) → shake until the soil is evenly distributed
- ➔ Remove the large organic parts with the tweezer and transfer in tube (saves up liquid for lyophilisation)

4. Overflow the samples: (*250 ml SPT, measuring beaker, pasteur-pipette, aluminium foil*)

- ➔ Put 250 ml SPT in measuring beaker and pour the liquid at the edge of the CD until the liquid reaches the centre of the soil. Then slowly pour the rest of the SPT at the edge of the CD (try to avoid any turbulences → otherwise can destroy aggregates and oPOM is released)
- ➔ Shake gently the CD → releases the fPOM stuck under soil particles
- ➔ Cover the CD with an aluminium foil and let it rest overnight

5. Suck off + rinse the fPOM from salt solution: *(Vacuum pump + tube, suction bottle + plug, 20 µm-sieve, 2x 1l- glass beaker, conductivity meter, small tube, small pasteur pipette)*

- ➔ The fPOM can stick and dry out at the edge of CD due to shaking. (Suck off everything)
- ➔ Suck off fPOM with vacuum pump in suction bottle → also suck off as much SPT as possible (but no sediment)
- ➔ Check tube → large fPOM can stay stuck → rinse in suction bottle.
- ➔ Put 20 µm-sieve on first 1l-glass beaker and pour the solution from suction bottle on the sieve (keep SPT-solution aside in glass beaker)
- ➔ Put sieve on second glass beaker and rinse suction bottle with demin. water
- ➔ Rinse fPOM until conductivity <10 µS/cm → then salt-free!
- ➔ Rinse out fPOM with pipette from sieve in small tube (as little water as possible!) → freeze

6. Ultrasonication: *(600 ml beaker, CD, tape, «BOY», ice, scraper, 1l-glass beaker with trapped SPT, pipette)*

- ➔ Attach CD with tape auf «BOY», add approx. 1cm water
- ➔ Scrape remaining sediment from CD with scraper in 600 ml glass beaker→ rinse with SPT from 1l-Becherglas (pipette)
- ➔ Mix the remaining SPT + soil sediment in 600 ml glass beaker (rinse the edge with pipette)
- ➔ Put glass beaker in CD, then add ice between glass beaker and CD, then add some more water
- ➔ Write down the immersion depth (3 cm) on sonotrode and immerge at the appropriate depth (all sediment should flow around in the beaker)
- ➔ Put correct amount of time (5:44) and amplitude (60%), clean tip of sonotrode, check if eroded
- ➔ Continuously check temperature and if needed adjust so that the sonotrode is in the middle of the glass beaker.

7. Centrifuge + Suck off + rinse oPOM: *(2x 250 ml plastic centrifuging bottle, balance, funnel, spray bottle with demin. water, vacuum pump, suction bottle, 20 µm-sieve, 1 l-glass beaker, small tube)*

- ➔ Split ultrasonicated soil + SPT in 2 250 ml- plastic centrifuging bottles () and label both lid and bottle.
- ➔ Centrifuge: adapted rotor + set adapted rotor number (12254) → Time: 10 min, RPM: 8500 U/min
- ➔ Suck off floating oPOM + all SPT
- ➔ Put 20 µm-sieve on 1 l-glass beaker → Pour oPOM from suction bottle onto 20 µm-sieve+ rinse until salt-free (<10 µS/cm) + transfer in small tube and freeze (= **oPOM >20 µm**)

8. Centrifuge sediment till salt-free: *(balance, wide neck bottle, suction bottle, vacuum pump, 250 ml tube for centrifuging, spray bottle with demin. water)*

- ➔ Put demin. water in centrifuging bottle (difference of 1 g maximum) and put in centrifuge oppositely

- ➔ Centrifuge 10 min at 8500 U/min → Suck off water, measure conductivity, put new demin. water + mix well the bottles → repeat until conductivity <100 µS/cm (ca. 6x)
- ➔ Transfer to wide neck bottle + put in the fridge before wet sieving

9. Freeze-drying for organic fractions (fPOM + oPOM)

- ➔ Take out tray with cable from freeze-drier and put in freezer at -20 °C
- ➔ Closed the water-exit screw (left bottom) → if water in bucket, empty it
- ➔ Turn on machine
- ➔ Put lid on → nothing should be on the sealing ring (cable, etc…
- ➔ Manually: cool down (warm-up), then press RUN
- ➔ Let it run for approx. 30 minutes until -55 °C (warm-up mainly for pump → value is reached quicker)
- ➔ Value → Setpoint for manual
- ➔ Change set value→ Manually: Haupttrocknen (Start at -10 °C, 0.7 mbar, security value 1.51)
- ➔ Close hind valve for vacuum (long piece up)
- ➔ Remove lid
- ➔ Put samples (eventually put white sensor in the samples)
- ➔ Put lid back
- ➔ Manually → Haupttrocknen (!Ventil opens → CLICK-Geräusch!)
- ➔ Wait 1 h, the set value should be reached after few seconds
- ➔ After 1 h → Value → Change set value (0.5 mbar) lower
- ➔ Wait 1 h
- ➔ Set set value at 0°C and at 0.4 mbar (security value 1.0 approx. → leave it like that over night)
- ➔ Manually → Nachtrocknen, for approx 3 hours → it automatically goes 0.05 mbar down
- ➔ Press Standby
- ➔ Open carefully the valve for vacuum (approx. 2 min until no noise anymore)
- ➔ When ventilation finshed → Turn off machine
- ➔ Open water-exit screw + empty bucket if needed

10. Wet sieving of the mineral fractions through 63-µm and 20-µm sieves

11. Drying in the oven at 60°C of mineral fractions

Last modified: (5/17/2017 Haddix)

When using this protocol please cite:

Cotrufo, M.F., Ranalli, M.G., Haddix, M.L., Six, J., Lugato, E., 2019. Soil carbon storage informed by particulate and mineral-associated organic matter. Nature Geoscience 12(12), 989-994.

Timeline & Protocol

Supplies

50 mL conical centrifuge tubes (1 per sample)
0.5 % Sodium Hexametaphosphate (5g per 1 L DI water)
Aluminum pans (large and small per sample)
Reciprocal shaker
2 mm sieve
53 μm sieve
Squirt bottle with DI water
Oil pan
2-place scale

Soil Prep:

1) Soils need to be 2mm sieved and roots and rocks greater than 2mm removed.
2) Soils should be air-dried (preferred) or oven dried.

Day 1:

1) A total of 20 samples can be done in a run
2) If not all samples can be fractionated in one set do fractionation runs by replicate
3) Make sure you have enough DRY CLEAN 50 mL centrifuge tubes with lids (1 per soil sample).
4) Label and weigh aluminum pans for POM and MAOM (2 per soil sample). (Small loaf pans for POM and large loaf pans for MAOM.)
5) Weigh and label centrifuge tubes on 2 place scale
6) Weight out 5.25-5.75g of air dry sample into a weighed centrifuge tube. You do not need to record the weight
7) Place samples in 60C oven overnight

Day 2:

1) Takes samples out of the oven and let cool in desiccator for ~30 minutes
2) Weigh samples on 2 place scale
3) Add 12 glass beads to each centrifuge tube
4) Fill each centrifuge tube to the 30mL line with 0.5% Sodium Hexametaphosphate
5) Place tubes on the sides on reciprocal shaker on low for 18 hours ± 15 minutes

Day 3:

1) Shake up sample and pour sample over a 2mm sieve on top of a 53µm sieve in a large pan.
2) Remove beads from the 2mm sieve and rinse the sieve clean on both sides over the 53µm sieve.
3) Rise the 53µm sieve thoroughly on both sides and make sure the rinse water runs clear.
4) Rinse POM+ sand from top of 53µm sieve and transfer into pre-weighed aluminum pans.
5) Place in oven at 60˚C ~2 days to dry.
6) Pour the MAOM into a pre-weighed large loaf pan make sure to rinse the large pan thoroughly
7) Dry the MAOM fraction in the large 60C oven for ~3 days, or until completely dry.
8) Weigh POM and MAOM on a two place scale
9) Once all fractions are dry and weighed, enter weights into spreadsheet and check recovery. If recovery is less than 95% or greater than 105% re-dry and reweigh samples to confirm weights. If weights do not change those samples need to be re-run.

Day 4:

1) Grind samples and transfer to vials

Appendix 4: FiBL protocol for determining maximum water holding capacity (mWHC)

Method	Determination of the maximum water holding capacity (mWHC)
Area	Soil investigation for site characterisation
Principle	Field-moist samples, sieved at max. ≤ 5 mm, are saturated with water. After the non-capillary bound water has run off, the maximum water holding capacity (mWHC) of the soil sample can be determined by weighing.
Literature	Referenzmethoden der Eidg. landwirtschaftlichen Forschungsanstalten Code B-WHK Revision 01.02.1998 FiBL-Methode WHK, A. Fliessbach, Karin Nowack, 1.4.1997
Intended use	The mWHC is used to adjust field-moist soils to a defined soil moisture level e.g. for soil microbiological measurements

Material	(A) Plastic cylinder (e.g. 50 ml centrifugation tubes), 5 cm inner diameter, 20 cm height, open at the top, closed at the bottom with a fine-mesh fabric (mesh size 60 µm). (B) Water case, at least 25 cm high, e.g. Plexiglas (aquarium) or 250 ml beakers (C) Sand bath filled with fine sand 2 mm sieved, pouring height approx. 2 cm, with drainage. (D) Balance (weighing range 1000 g, 0.1 g graduation). (E) Drying oven with ventilation

Instructions	
Preparation of the soil sample	Field-moist soil is filled up to a height of 12 cm into pre-tared cylinders (A), slightly compacted by tapping on a soft base and weighed (FWB).
Water saturation	The cylinders are placed in the water case (B) with 2 - 3 cm of water height. After the water has risen due to the capillary effect to the surface of the samples, the case outside the cylinders is filled with water

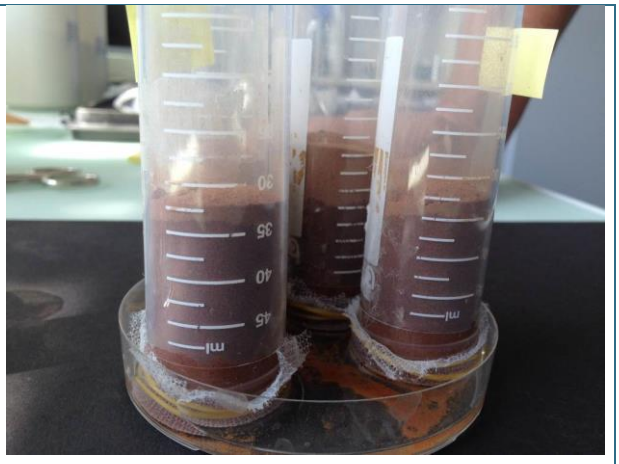
	up to 1 cm above the pouring height of the samples and then left for 1 h to fully saturate.
Comments	The soil should be filled loosely into the cylinders, but in a way that no large cavities are created. Never pour water into the cylinders.
Preparation of the sand bath	The sand bath is previously saturated with water. In order to ensure a constant water level, a water-filled volumetric flask is placed upside down on the surface of the sand bath by means of a stand and clamps. The water level is adjusted so that a film of water is just visible on the sand surface. Excess water is removed with a syringe.
Determination of capillary bound water	The cylinders are then placed on the sand bath for 4 h to allow non capillary bound water to drain. Cover the upper part with a foil to avoid evaporation. The excess water is regularly removed from the sand surface. After 4 h the samples are filled into tared drying dishes and the wet weight is determined. The samples are dried for at least 12 h at 105 °C. The dry weight is determined after cooling to room temperature.

Calculation	$$\boldsymbol{mWHC = \frac{AWB - TWB}{TWB - Tara}}$$ mWHC = maximum water holding capacity Tara = weight of the drying tray (g) AWB = gross weight of the soil sample at mWHC (g) TWB = gross weight of the dry soil sample (g)
Result	mWHC= g water/g soil dry matter, accuracy 0.001 g
Relative water holding capacity	First, the dry matter of field-moist soil (% DM) is calculated: **% DM = 100 * (TWB - Tara)/(FWB - Tara)** FWB = Gross weight of the field-moist soil sample (g) Second, the water content (WC) of naturally moist soil (g water per g dry matter) is calculated: **WC = (100 - % DM)/% DM** Then, the amount of water (y) that need to be added to field-moist soil per g soil dry matter to reach x % of mWHC is calculated: $$\boldsymbol{y = \left(mWHC * \frac{x}{100}\right) - (WC)}$$
Example	Field-moist soil with a %DM= 80%, and a mWHC = 0.600 g H_20/g soil dry matter: WC = (100 - 80)/80 = 0.25g H_20/g soil dry matter Goal: Amount of water (y) per 1g of soil dry matter that has to be added to field-moist soil to adjust to 50 % of the mWHC: $$\boldsymbol{y = \left(0.6 * \frac{50}{100}\right) - (0.25) \quad y = 0.05g}$$

FOTOSTORY:

Maximum Water Holding Capacity (mMWC)

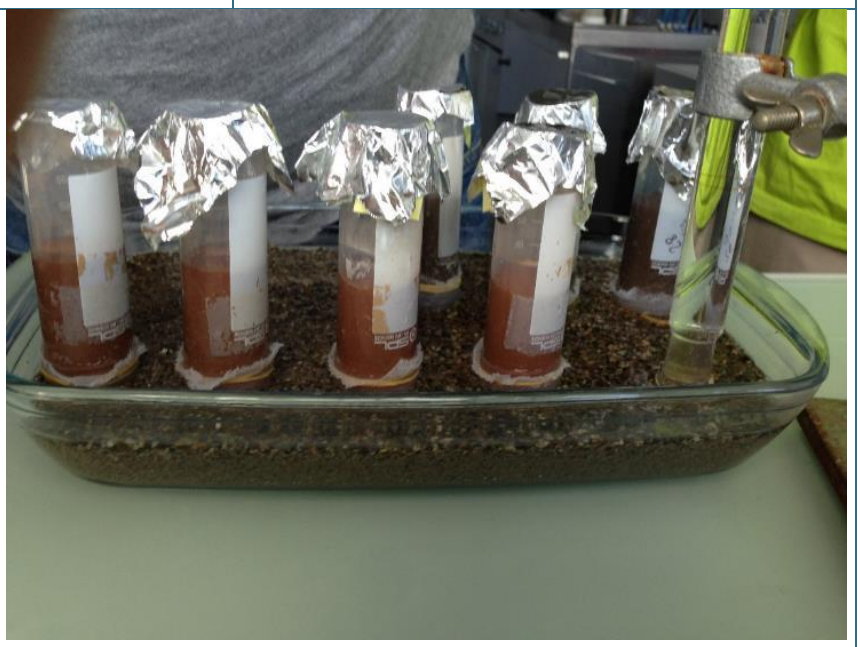
Close tubes with a fine fabric at the bottom. Field-moist soil is filled up to a height of 12 cm into pre-tared cylinders (A), slightly compacted by tapping on a soft base and weighed. (FWB).	
The cylinders are placed in a water case (B) or Petri dish.	

Fill the water case or Petri dish with water and wait for the capillary rise of the water. Leave the tubes standing with aluminium cover. Add more water if necessary Observe the soil surface. If wet and shiny, the process is at the end.	
	
Place the tubes into a beaker or keep it in the water case Fill water until the level passes the soil surface a few millimetres and leave standing for at least one hour for full saturation.	

Prepare a sand bath filled with water to the surface. Invert a fully filled bottle right to surface in such a way that water is flowing, when you remove water from the sand with a sponge or syringe.

Take the “oversaturated” tubes and put them on the prepared sand bath. Remove surplus water that comes from the soil from the surface of the sand Leave standing for four hours.

Remove surplus water with a sponge or syringe.



Empty the whole amount of soil into a suitable tray.

Weigh the tray (Tara). Weigh the wet soil (AWB). Put the wet soil into an oven at 105 °C overnight until weight is constant. Weigh the dry soil (TWB).

Determine the maximum water holding capacity (mWHC) in g water/g soil dry matter:

$$mWHC = \frac{AWB - TWB}{TWB - Tara}$$

Tara = Weight of the drying tray (g)
AWB = Gross weight of the soil sample at mWHC (g)
TWB = Gross weight of the dry soil sample (g)

Soil biological measures often need 40-60% of the mWHC. This can be calculated as follows:

First, the dry matter of field-moist soil (% DM) is calculated:

% DM = 100 * (TWB - Tara)/(FWB - Tara)

FWB = Gross weight of the field-moist soil sample (g)

Second, the water content (WC) of naturally moist soil (g water per g dry matter) is calculated:

WC = (100 - % DM)/% DM

Then, the amount of water (y) that need to be added to field-moist soil per g soil dry matter to reach x % of mWHC is calculated:

$$y = \left(mWHC * \frac{x}{100}\right) - (WC)$$

Appendix 5: N free nutrient solution added to the pot experiment. The solutions were composed in a manner that 2 ml per stock solution needed to be added per pot (containing 0.375 kg soil dry matter)

Stock	Element	Salt	Water solubility	salt molar mass	element mass	ratio element/salt	amount of element to be added	amount of salt to be added per kg soil	amount of dry soil per pot	amount of salt to be added per pot	volume of each solution to be added per pot	concentration salt	number of column	total volume needed	preparation mg salt	
			g/l	g/mol			mg/kg soil	mg/kg soil	kg	**mg**	ml/pot	mg/ml	-	ml	mg salt /150ml	g/ 150 ml
A	K	KCl	347	74.55	39.01	0.52	250	477.76	0.38	179.160792	2.00	89.58	48	96	13437	13.4
	P	Ca(H2PO4)2 x H2O	?	252.08	61.91	0.25	50	203.59	0.38	76	2.00	38.17	48	96	5726	5.7
	Ca	see above			40.1	0.16	32									
	Ca	CaCl2 . 2 H2O	740	147.02	40.1	0.27	70	256.64	0.38	96	2.00	48.12	48	96	7218	7.2
B	Mg	MgSO4 x 7H2O	710	246.47	24.31	0.10	48	486.65	0.38	182	2.00	91.25	48	96	13687	13.687
	Zn	ZnSO4 x 7H2O		287.54	65.39	0.23	1	4.40	0.38	2	2.00	0.82	48	96	124	0.124
	Mo	Na2MoO4 x 2H2O	650	241.95	95.94	0.40	0.10	0.25	0.38	0	2.00	0.05	48	96	7	0.007
C	Fe	Fe-Na-EDTA		421.1	56	0.13	1	7.69	0.38	3	2.00	1.44	48	96	216	0.216
	B	H3BO3	47.2	61.83	10.81	0.17	1	5.72	0.38	2	2.00	1.07	48	96	161	0.161
	Mn	MnSO4 x H2O	520	169.02	54.94	0.33	2	6.15	0.38	2	2.00	1.15	48	96	173	0.173
	Cu	CuSO4 x 5H2O	317	249.68	63.55	0.25	2	7.86	0.38	3	2.00	1.47	48	96	221	0.221
	Co	CoCl2 x 6H2O	529	237.93	58.93	0.25	0.1	0.40	0.38	0	2.00	0.08	48	96	11	0.011

Appendix 6: Correlation between total nitrogen (N) in POM and MAOM using a linear model.