



Biogas residues in substitution for chemical fertilizers: A comparative study on a grassland in the Walloon Region☆☆☆

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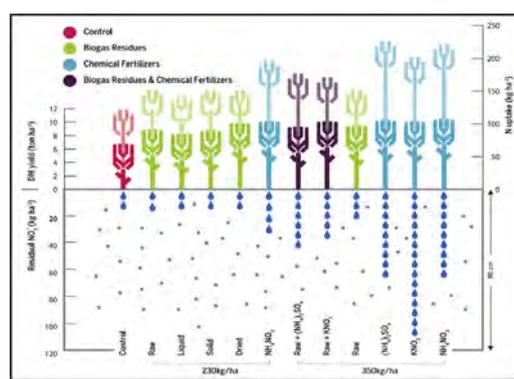
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HIGHLIGHTS

- Use of biogas residues as biofertilizers mitigates nitrate leaching risk.
- Biomass yield and N uptake are similar for biogas residues and chemical fertilizers.
- Partial substitution of synthetic N by biogas residues has environmental benefits.
- Higher organic and synthetic N rate does not lead to higher agronomic performance.
- Annual rainfall can affect the utilization and losses of plant available nitrogen.

GRAPHICAL ABSTRACT



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ABSTRACT

To provide sufficient quantities of food and feed, farming systems have to overcome limiting factors such as the nutrient depletion of arable soils. Nitrogen being the main mineral element required for plant growth, has led to the extensive use of chemical fertilizers causing nitrogen pollution of the ecosystems. This field study investigates the use of biogas residues (BRs) as biofertilizers and their contribution to the mitigation of nitrate leaching in agricultural soils, while also demonstrating the polluting nature of chemical fertilizers. Nine different fertilization treatments classified in three schemes and two nitrogen doses were tested for three consecutive years on a grassland in the Walloon Region of Belgium. Residual soil mineral nitrogen, percentage contribution of treatments in residual nitrate and agronomic performance were assessed for each fertilization treatment. The results obtained showed significant differences on treatment and scheme level regarding nitrate accumulation in the soil, with chemical fertilizers posing the highest nitrate leaching risk. BRs did not cause nitrate accumulation in the soil, and were N rate and rainfall independent, while the chemical treatments indicated a cumulative tendency under high N rate and low precipitation. Forage yield did not demonstrate statistical differences on treatment and scheme level but varied with changing precipitation, while the maximum application rate suggested a

☆ BRs: Biogas Residues.

☆☆ FCNA: Fertilizer Contribution to Nitrate Accumulation.

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plateau. Aboveground nitrogen content was significantly higher after the application of chemical fertilizers only in the first year, while all the chemical treatments indicated a dilution effect under elevated annual rainfall. Finally, the partial substitution of chemical fertilizers by raw digestate reduced the concentration of NO_3^- in the soil without having a negative impact on the yield and N content of the biomass. These results strongly advocate for the environmental benefits of BRs over chemical fertilizers and underline their suitability as biofertilizers and substitutes for chemical fertilizers in similar agricultural systems.

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1. Introduction

As the human population is constantly growing, agriculture aims to increase the yield of crops to provide sufficient quantities of food and feed. To meet this challenge, farming systems have to overcome limiting factors such as the nutrient depletion of arable soils. Nitrogen is an essential mineral element required for plant growth, functioning and formation (Hawkesford and Howarth, 2011; Wienhold et al., 1995). This has led to its industrial transformation (220 Tg N yr^{-1}) (Fowler et al., 2015) causing a nitrogen cascade through the environmental media (Galloway et al., 2003). The environmental cost of all N losses in Europe is calculated at 70–320 billion euros per year, which outweighs the economic benefits of N in agriculture (Sutton et al., 2011). Different aspects of reactive nitrogen (NH_3 , NO_x , N_2O , NO_3^-) pollution have led to the establishment of treaties, commissions and legal instruments in Europe putting constraints on the use of fertilizers on agricultural lands. The contamination of drinking water was, *inter alia*, the main driver for the enactment of the European Nitrates Directive 91/676/EEC, which aims to protect ground and surface waters from agricultural nitrate leaching, setting the threshold of $50 \text{ mg NO}_3^- \text{ L}^{-1}$ (EEC Council Directive, 1991), mainly in countries such as Belgium, France and Germany, where N input exceeds $300 \text{ kg N ha}^{-1} \text{ yr}^{-1}$ (Velthof et al., 2014). However, to date, specific measures within the Nitrates Action Programmes for the Walloon Region in Belgium have established the total quantity of nitrogen (organic and mineral) applicable to $350 \text{ kg N ha}^{-1} \text{ yr}^{-1}$, restricting livestock manure application to $230 \text{ kg N ha}^{-1} \text{ yr}^{-1}$ for the grasslands, and to 170 kg N ha^{-1} for the nitrate vulnerable zones (Grant et al., 2011). These measures are considered impractical by farmers and eventually lead to excessive mineral fertilization and the consequent increase of nitrate loads in the soil and groundwater. To achieve the sustainable management of nitrogen in regions with extensive agricultural activity, either a reduction in inputs, an enhanced recovery in products or an increased storage capacity of nitrogen within the agricultural system is required (Cuttle and Scholefield, 1995).

To prevent further acceleration of environmental pollution caused by chemical fertilizer production and use in agriculture, the recycling of anaerobically digested organic wastes from various sources such as agriculture, food/feed industries, households and more, back to arable lands, seems to be a promising approach. Anaerobic digestion (AD) is an environmentally sound multipurpose process (with the smallest ecological footprint) (Anton and Steinicke, 2012) that generates both cost competitive bioenergy in the form of biogas and biofertilizer as digestate (biogas residues, BRs) (Lukehurst et al., 2010). The biogas residues resulting from the anaerobic digestion are not waste but a valuable co-product, which contains all the nutrients initially present in the substrate (Corré and Conijn, 2016). Therefore, they can be used as a biofertilizer, recycling nutrients such as nitrogen, phosphorus and potassium back to agricultural soils (Abubaker, 2012) while improving soil quality (Nkoa, 2014). Furthermore, developing digestate treatment technologies have the potential to provide concentrated biofertilizers of high-value while allowing the recovery of the water contained in the liquid fraction of biogas residues and the reduction of transportation costs (Adam et al., 2018). Moreover, the on-farm anaerobic digestion of readily available organic resources can cover the demands of the farm in heat and power, while implementation on a global scale has the

potential to reduce gross greenhouse gas emissions by 10% (Tsachidou et al., 2018). The number of biogas plant reactors is drastically increasing (in Europe from 6227 installations in 2009 to 17,662 in 2016, 12,496 of which run on agricultural substrates) (EBA, 2017) as a response to the worldwide energy demands, high fossil fuel prices, climate change, the need for treatment and disposal of organic wastes, among others. This growth produces large amounts of biogas residues (128 million tonnes per year in Europe – EBA, 2017) that require immediate handling (Angelidaki et al., 2003).

Even though the residues from the anaerobic digestion process are a relatively new type of fertilizer, there are a number of research publications that highlight their benefits for soil and plants (Chiew et al., 2015; Koszel and Lorencowicz, 2015; Liu et al., 2009; Odlare et al., 2008). However, owing to the wide range of organic materials used in the process of anaerobic digestion, the residues generated create concerns due to their potential content of heavy metals, organic pollutants, pesticides and pathogens. Nevertheless, biogas residues are known to be particularly rich in ammonium nitrogen (Möller, 2015; Losak et al., 2014) and organic matter, which induce soil biological activity (Alburquerque et al., 2012).

An important physicochemical property that stresses the significance of the nitrogen form in biogas residues is the different ability of NH_4^+ and NO_3^- ions to be retained on the soil cation exchange complex. Since the surface of most soil particles has fixed negative charges, NH_4^+ adsorbs strongly to these sites and shows seasonal and spatial homogeneity, therefore providing plants with the required nitrogen in the long-term. On the other hand, NO_3^- , which is the main source of nitrogen in most chemical fertilizers, as an anion, does not bind to soil particles (Bloom, 2010) and consequently can move freely through the soil (Tinker and Nye, 2000), with the potential of invading the underground water reserves while depriving plants of available nitrogen (Marschner, 2012). Another considerable reason to distinguish the two ions is the difference between the energy required for the plants to acquire NH_4^+ and NO_3^- , which may have an impact on their growth (Bloom et al., 1993). In order for the plants to synthesize proteins and other essential nitrogenous compounds, nitrate absorbed from the soil must be first reduced to ammonia before entering the amino acid synthesis process (Jackson et al., 2008; Haynes, 1986). The reactions involved in this transformation are among the most energy intensive processes and have a great expenditure of 12ATPs per NO_3^- assimilated, consuming 25% of the total plant energy in shoots (Bloom et al., 1989) and roots (Bloom et al., 1992). According to Bloom et al. (1992), barley plants consumed less than one-fifth of the energy for root nitrogen acquisition under NH_4^+ nutrition compared to NO_3^- . By examining the kinetics of ammonium and nitrate uptake in maize root experiments, it was shown that exogenously supplied NH_4^+ was absorbed more than NO_3^- when both forms were present in the medium (Taylor and Bloom, 1998). Other studies on N fluxes in rice and maize seedlings showed that net NH_4^+ uptake was significantly greater than NO_3^- when both compounds were supplied (Colmer and Bloom, 1998). These findings indicate that biogas residues, being rich in NH_4^+ , have apparent advantages over chemical fertilizers and the potential to contribute towards more sustainable agricultural practices. Nevertheless, factors such as soil pH and texture, properties of biogas residues, season and methods of application should be considered as they may lead to N loss through NH_4^+ nitrification and NH_3 volatilization, as well as to insufficient NH_4^+

availability due to clay mineral fixation (Scherer et al., 2014), decreasing the utilization percentage and therefore the fertilizer value of BRs.

In contrast to most organic fertilizers, such as manure and composts, which have been extensively investigated, organic residues, such as BRs, are poorly documented. The long-term effect of biogas residues on soil properties remains an unexplored field of research (Nkoa, 2014), while references concerning potential nitrate leaching under fertilization with biogas residues are limited (Svoboda et al., 2013). Therefore, despite their potential, the use of BRs as biofertilizers is limited due to a lack of confidence in their quality and safety (Seadi et al., 2012). The overarching purpose of the current study is to investigate the contribution of different biogas residue fractions to the mitigation of nitrate leaching in the soil and their impact on the agronomic performance of the grasslands in the Walloon Region. Another aim is to establish scientific evidence on the polluting nature of chemical fertilizers, and the environmental benefits resulting from their substitution by biogas residues. Lastly, we seek to prove that providing the total admissible quantity of nitrogen through fertilization with biogas residues would not increase the risk of nitrate leaching as opposed to chemical fertilization.

The work described here is part of a larger research programme on the integration of the biogas sector in the circular bioeconomy, which is carried out through the consecutive INTERREG projects “OPTIBIOGAZ”, “ECOBIOGAZ” and “PERSEPHONE”.

2. Materials and methods

2.1. Site description

The current study is part of an ongoing long-term experiment established in early 2013 on a grassland located in the commune of Attert, near Arlon, a Walloon municipality of Belgium (49.745665 N; 5.812498 E). About 45% of the Walloon Region corresponds to utilized agricultural land – of which 50% is pastureland and 50% arable land – (Grant et al., 2011). The trial site has been a permanent meadow for over 20 years and had never been exposed to BRs or chemical fertilizers before the onset of the experiment in 2013. However, before the initiation of this experiment, the land was used for grazing. The vegetation growing on the grassland consists mainly of *Lolium perenne*, *Phleum pratense*, *Trifolium repens* and *Trifolium pratense*, with their roots reaching 40 to 80 cm deep. The soil profile of the experimental site is a light sandy loam with moderate to imperfect natural drainage. Prior to the trial, the chemical soil characteristics were evaluated and are summarized in Table 1. The Walloon Region has an Atlantic temperate climate with annual rainfall that amounts to 1014 mm yr⁻¹ (Grant et al., 2011) and an average temperature of 8.5 °C. The cumulative monthly precipitation and mean monthly temperature, recorded during the field trial, were obtained from the meteorological station of Useldange in Luxembourg, due to its proximity (Fig. 1). This paper demonstrates the results obtained in the first three consecutive years of the experiment, from 2013 to 2015.

Table 1
Soil characteristics of the grassland measured prior to the onset of the experiment in 2013.

| Parameters | 0–30 cm | 30–60 cm | 60–90 cm |
|---------------------|---------|----------|----------|
| % sand | 41.0 | 34.5 | 35.2 |
| % silt | 9.2 | 9.6 | 5.5 |
| % clay | 49.8 | 55.9 | 59.3 |
| % C _{org} | 1.83 | 0.60 | 0.62 |
| % N _{org} | 0.27 | 0.15 | 0.15 |
| pH H ₂ O | 6.3 | 6.1 | 5.7 |
| pH KCl | 5.2 | 4.6 | 4.1 |

2.2. Experimental design

Nine different fertilization treatments classified into three schemes (biogas residues, chemical fertilizers and their combination) (Table 2) were evaluated and compared to an unfertilized control and to each other. The biogas residues used are a product of the biomethanation plant on site (Ferme du Faascht) and the substrates utilized are mainly liquid and solid bovine manure, crop residues (silage maize and grass) and organic wastes from the food industry. In this study, four different biogas residue fractions were tested separately, while the raw fraction was also tested in combination with chemical fertilizers (Table 2). The chemical characteristics of the BR fractions (Table 3) were measured on applied batches using standard analytical methods and the fertilizer amount was adjusted accordingly (based on their nitrogen content) prior to field application. The solid and liquid fractions were obtained with the use of a screw press separator equipped with a 500 µm sieve (FAN Separator PSS 3.2-520, GmbH, Germany), while a belt dryer (DORSET, The Netherlands) was used to dry the raw digestate to produce granules of 5–7 mm. The chemical fertilizers tested were ammonium nitrate (13.5% NH₄⁺-N, 13.5% NO₃⁻-N), ammonium sulphate (21% NH₄⁺-N, 24% S) and potassium nitrate (17.4% K, 13.7% NO₃⁻-N). The fertilization treatments were applied in a randomized block design with four replicates per treatment. A total of 56 plots were arranged in 8 parallel rows consisting of 7 plots each. Each plot has dimensions of 10 m by 2 m, separated by a mowed strip of 1 m horizontally and 0.5 m vertically to minimize the edge effect (Fig. 2). Two out of the eleven different treatments initially tested in this field trial were not evaluated in the current paper (treatments 6 & 10, as shown in Fig. 2). The fertilizers were weighed and applied by hand aiming the highest possible uniform distribution across the plots. The solid and dried fractions of biogas residues, as well as the chemical fertilizers, were evenly distributed over the plots. The raw and liquid fractions were dispersed with a watering can with a mounted shovel-shaped deflector. The nitrogen rates of the fertilizers tested were 230 kg N ha⁻¹ yr⁻¹ and 350 kg N ha⁻¹ yr⁻¹ in accordance with the maximum admissible doses in the Walloon region (Grant et al., 2011) and the average annual N inputs in Belgium (280 kg ha⁻¹ in the form of chemical fertilizer and manure from application and grazing) (Jensen et al., 2011). Of the nine different treatments, only raw digestate (RD) and ammonium nitrate (AN) were tested for both N rates. The total nitrogen dose was divided into instalments allowing the adjustment of fertilizer input to crop demand during the permissible period of application (1st February to 15th September). The main field management practices that took place during the experimental period of 2013 to 2015 and the amount of nitrogen added each time during fertilization are displayed as a timetable in Fig. 3.

2.3. Residual soil mineral nitrogen

To assess the nitrogen leaching potential of eleven fertilization treatments and compare them to an unfertilized reference (control) and to each other, soil samples of 0–30, 30–60 and 60–90 cm were collected with a hand auger during the autumn and after the last harvest of each year (Fig. 3). Five cores were collected for each plot and each depth, and were mixed and stored at 4 °C (for <24 h) until the analysis. Prior to the analysis for NO₃-N and NH₄-N, the samples were homogenized through sieving with a mesh of 8 mm. Soil aliquots of 30 g were mixed with 150 ml of KCl solution (0.1 M) and placed on a rotating shaker for 30 min before allowing them to stand for an additional 30 min. (Moniteur Belge, 2013). The extract was allowed to settle and the supernatant was used for the quantification of the nitrate and ammonium content in the soil by the colorimetric method of continuous flow analysis (CFA) on a SKALAR SAN + 1050 (ISO11732 for ammonium and ISO 13395 for nitrate). The water content of the soil samples was measured through gravimetry (105 °C, 24 h) and was taken into

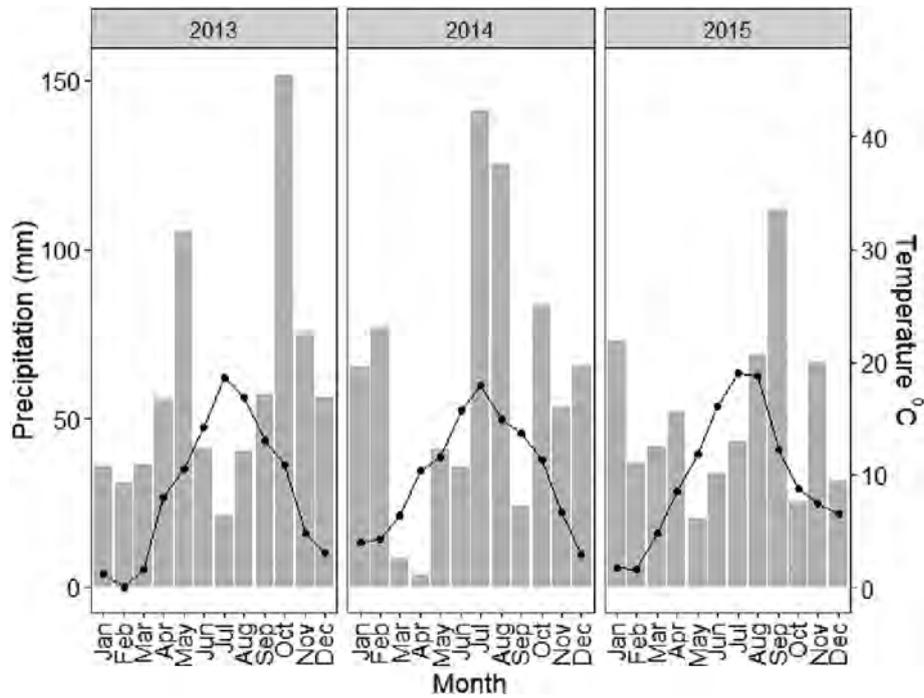


Fig. 1. Trends of cumulative monthly rainfall (as bars) and average monthly air temperature (as dots) in the area of Arlon during the study (as recorded at the meteorological station of Useldange in Luxembourg).

account to express the NO₃-N and NH₄-N values in terms of dry soil weight.

The contribution of each fertilization treatment to the accumulation of nitrate was assessed through the total residual nitrate concentration detected in the soil profile (0–90 cm) based on the difference method and according to Eq. (1):

$$\text{Fertilizer Contribution to Nitrate Accumulation (FCNA, \%)} = (N - N_0) / F \times 100 \quad (1)$$

Where: N = Total nitrate concentration in fertilized plot
 N₀ = Total nitrate concentration in unfertilized control plot
 F = Quantity of nitrogen applied

2.4. Nutrient use efficiency

To obtain the total annual yield, grass was harvested four times per year (Fig. 3) with a Haldrup F-55 grass harvester and the aboveground biomass from each plot was collected and weighed. The harvested

grass was dried at 55 °C for 48 h to obtain the dry matter (DM) content for further calculations. The impact of the various fertilization treatments and the two nitrogen rates on the nutrient use efficiency (NUE) measurements was assessed according to Dobermann (2007). As NUE is known to manifest considerable spatial and temporal variability (Fixen et al., 2015), agronomic efficiency and apparent recovery efficiency were calculated as average values.

The dry biomass weight was used to calculate the *Agronomic Efficiency (AE)* of each fertilization treatment through the difference method and according to Eq. (2):

$$AE \text{ (kg DM kg}^{-1} \text{ N)} = (Y - Y_0) / F \quad (2)$$

Where: Y = Yield of aboveground grass biomass of fertilized plot
 Y₀ = Yield of aboveground grass biomass of unfertilized control plot
 F = Quantity of nitrogen applied

Sub-samples of the dry biomass were ground and used for the calculation of the nitrogen uptake per unit of nitrogen applied using the Near Infrared Reflectance Spectroscopy (NIRS) method. The NIRS-based analysis is a standard method used by the accredited soil and forage analytical laboratory of the “Centre de Michamps” in the Walloon region. Two replicates of 5 g each are used for the analysis and the

Table 2
 Experimental treatments classified into three schemes and two nitrogen doses.

| Treatment N ^{0a} | Abbreviation | Treatment name | Total N Input (kg ha ⁻¹) | Scheme |
|---------------------------|--------------|---|--------------------------------------|---------------------------|
| 1 | CTR | Control | 0 | N/A |
| 2 | RD 230 | Raw Digestate | 230 | Biogas Residue |
| 3 | LD 230 | Liquid Digestate | 230 | Biogas Residue |
| 4 | SD 230 | Solid Digestate | 230 | Biogas Residue |
| 5 | DD 230 | Dried Digestate | 230 | Biogas Residue |
| 7 | AN 230 | (NH ₄)(NO ₃) | 230 | Chemical |
| 8 | RD + AS 350 | Raw Digestate + (NH ₄) ₂ SO ₄ | 350 (230 + 120) | Biogas Residue + Chemical |
| 9 | RD + PN 350 | Raw Digestate + KNO ₃ | 350 (230 + 120) | Biogas Residue + Chemical |
| 11 | RD 350 | Raw Digestate | 350 | Biogas Residue |
| 12 | AS 350 | (NH ₄) ₂ SO ₄ | 350 | Chemical |
| 13 | PN 350 | KNO ₃ | 350 | Chemical |
| 14 | AN 350 | (NH ₄)(NO ₃) | 350 | Chemical |

^a Corresponds to each tested treatment depicted in Fig. 2.

Table 3
 Mean values of the chemical characteristics of the BR fractions tested over three years.

| Parameters | RD | LD | SD | DD |
|---------------------------------|------|------|-------|-------|
| N _{tot} | 5.30 | 5.27 | 7.27 | 21.76 |
| NH ₄ ⁺ -N | 2.73 | 3.10 | 1.86 | 0.00 |
| C:N | 3.80 | 3.79 | 19.63 | 15.83 |
| DM% | 6.66 | 6.84 | 36.71 | 90.54 |
| OM% | 4.0 | 3.82 | 24.38 | 54.38 |
| K ₂ O | 3.21 | 3.24 | 5.63 | 38.90 |
| MgO | 0.54 | 0.58 | 6.58 | 8.74 |
| P ₂ O ₅ | 2.25 | 2.38 | 8.21 | 32.89 |
| CaO | 3.88 | 4.14 | 21.63 | 51.63 |
| pH | 8.19 | 7.85 | 8.51 | - |

RD: Raw digestate; LD: Liquid phase of screw press (500 μm) separated digestate; SD: Solid phase of screw press separated digestate; DD: Dried raw digestate on a belt dryer.

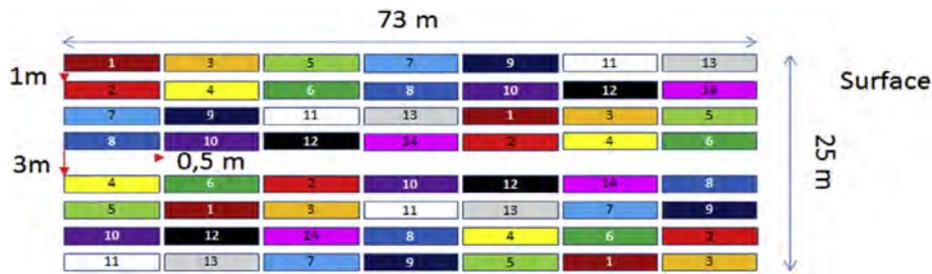


Fig. 2. Experimental site specifics and randomized block design of treatments and their replicates ($n = 4$). Treatments 6 & 10 were not evaluated in the current paper.

analytical precision for N content in plant biomass is 0.5%. The NIRS calibration is maintained and continuously updated with new analytical references obtained from the REQUASUD network and fed in a centralized database which contained over 1 million of validated sample data (<http://www.requaconsult.requasud.be/>). Based on the data obtained, the *Apparent Recovery Efficiency (ARE)* was calculated by the difference method and according to Eq. (3):

$$\text{ARE (\%)} = (U - U_0) / F \times 100 \quad (3)$$

Where: U = Nitrogen uptake in aboveground grass biomass of fertilized plot

U_0 = Nitrogen uptake in aboveground grass biomass of unfertilized control plot

F = Quantity of nitrogen applied

2.5. Statistical analysis

All data exploration and statistical analyses were performed with R software (Version 3.4.1). All statistics were considered significant at the threshold $\alpha = 0.05$ ($n = 4$). The data describing the yield and aboveground biomass nitrogen content complied with the assumptions of Gaussian distribution and hence, a repeated measures two-way analysis of variance (ANOVA) was performed with the use of the *psycho* package (Makowski, 2018) to determine the effects of the treatment, year and their interaction, on the dry mass yield and nitrogen content of the forage. When the results indicated statistical differences, a *post hoc* pair-wise comparison was conducted using Tukey's HSD adjustment.

On the other hand, data describing the residual soil mineral nitrogen did not fulfil the normality condition of the Shapiro Wilk test. Therefore, the rank transformation of data preceded a repeated measures two-way

analysis of variance to determine whether fertilization treatment, year and their interaction had a significant effect on the residual nitrate and ammonium concentrations in the soil profile. When statistical significance was indicated, a post-hoc analysis on ranks using Tukey's HSD adjustment was performed with the use of the *lsmeans* package (Lenth, 2016) to detect significantly different means.

3. Results

3.1. Residual soil nitrate and ammonium distribution in the soil profile

The major mineral nitrogen form detected in the soil profile, after the last harvest each autumn, was nitrate (NO_3^- -N). Overall, residual nitrate concentration was higher in the top soil layer of 0–30 cm for all the treatments and all years compared to the two deeper layers of 30–60 and 60–90 cm (Fig. 4). Following the application of the biogas residue fractions, the residual nitrate concentration in the top soil layer did not exceed 16 kg ha^{-1} , even at the maximum fertilization rate of 350 kg N ha^{-1} , over the three years. Most importantly, the concentration of nitrate in the two deeper soil layers did not exceed 5 kg ha^{-1} . Treatments within the chemical and combinatorial schemes led to a greater accumulation of nitrate in the soil profile, demonstrating significant differences with BRs ($P < 0.001$). In 2015, following the application of PN 350, nitrate concentration reached 87 kg ha^{-1} at 30–60 cm and 50 kg ha^{-1} at 60–90 cm. The nitrate concentration was lower following the application of combinatorial treatments, compared to chemical fertilizers as the sole N source, with RD + PN 350 reducing nitrate to 22 kg ha^{-1} at 30–60 cm depth in 2015. A strong year effect ($P < 0.001$) was observed for the chemical treatments (except for AN 230) across all depths as nitrate concentration during 2015 in some cases was tenfold compared to 2013 and 2014. Furthermore, the impact of the N rate supplied as $(\text{NH}_4)(\text{NO}_3)$ was apparent in 2015 as the



Fig. 3. Timetable of field management practices during 2013–2015. The amount of nitrogen added each time during fertilization is indicated in the yellow boxes. Treatments applied at the rate of 230 kg N ha^{-1} were divided into three instalments, while those applied at the rate of 350 kg N ha^{-1} were divided into four instalments per year. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

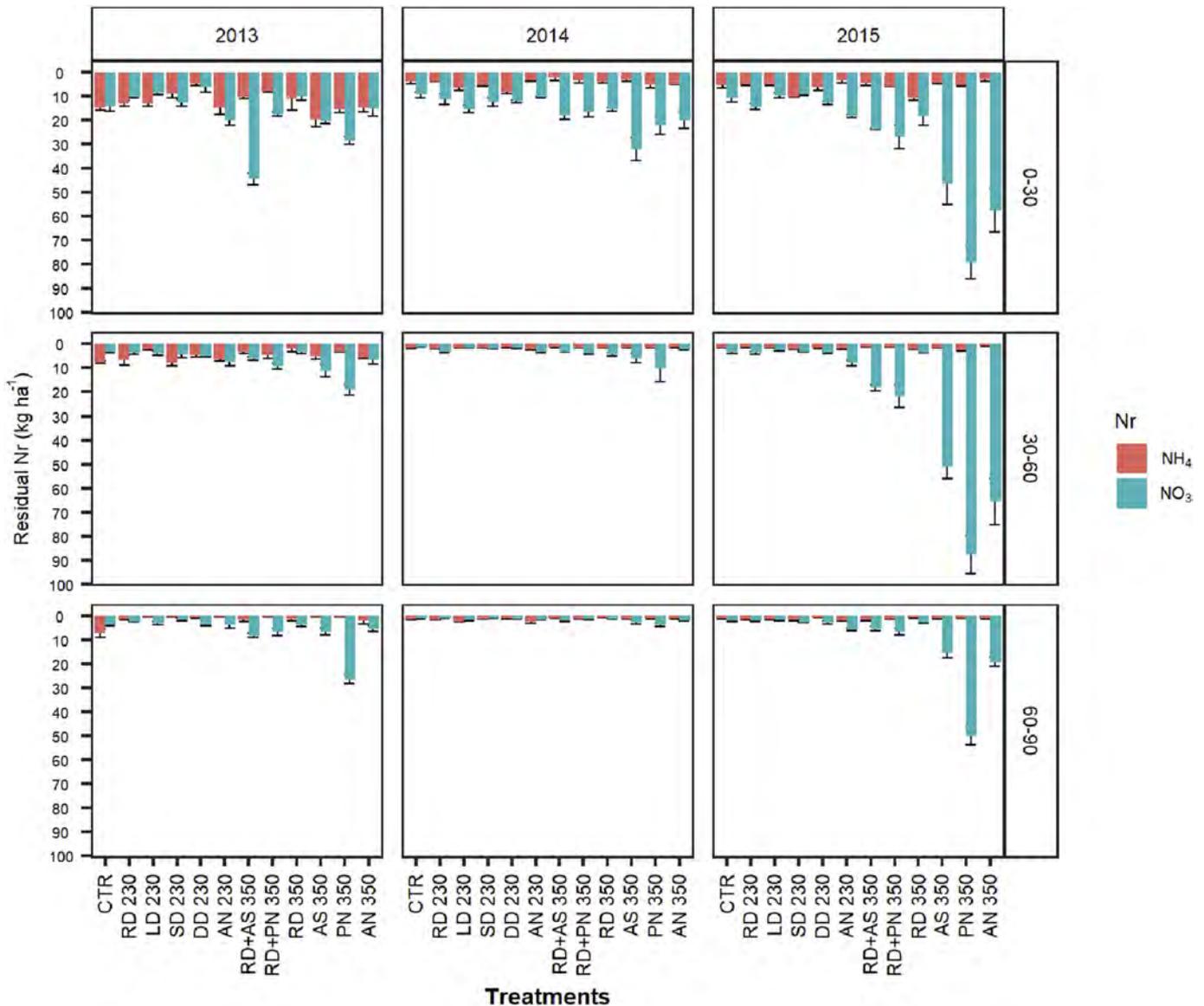


Fig. 4. Residual soil mineral nitrogen (Nr: Reactive nitrogen forms: NO_3^- and NH_4^+) as detected in the three soil depths in autumn of each year after the application of each treatment (CONTROL: No treatment; RD 230:Raw Digestate; LD 230:Liquid Phase; SD 230:Solid Phase; DD 230:Dried Digestate; AN 230:(NH_4)(NO_3); RD + AS 350: Raw Digestate + (NH_4) $_2$ SO $_4$; RD + PN 350:Raw Digestate + KNO $_3$; RD 350:Raw Digestate; AS 350:(NH_4) $_2$ SO $_4$; PN 350:KNO $_3$; AN 350:(NH_4)(NO_3)).

accumulation and progressive downward movement of nitrate was significantly higher ($P < 0.01$) under the application of 350 kg of N ha^{-1} (AN 350) compared to 230 kg of N ha^{-1} (AN 230). In contrast, the two N rates tested through raw digestate (RD 230 & RD 350) application did not have any effect on the nitrate concentration in any of the soil layers and years.

Ammonium detected in the soil profile remained below 10 kg ha^{-1} for all treatments and depths, with the exception of the top soil layer in 2013 when most treatments fluctuated between 10 and 20 kg ha^{-1} . No significant differences were noted between the control plot and the treatments, or among treatments within the same year. In addition, neither year, nor treatment by year interaction was noted. Overall, ammonium concentration was highest in the top soil layer for all treatments over the three years (on average 80% of the total NH_4 was detected in 0–30 cm) and showed no progressive movement in depth (Fig. 4).

3.2. Treatment contribution to residual soil nitrate

The contribution of the treatments within the biogas residue scheme to the accumulation of residual nitrate in the soil (0–90 cm) was at the

same level as the unfertilized control, while in the first year of the experiment, their percentage contribution was lower than that observed for the control (Fig. 5). All treatments within both chemical and combinatorial schemes had a significantly ($P < 0.01$) higher contribution to the accumulation of nitrate in the soil compared to the biogas residue fractions, with the highest contribution observed in 2015 for the chemical fertilizers. About 60% of the nitrate detected in the soil in 2015 following the application of PN 350 was attributed to the treatment. Partial substitution (65%) of PN 350 by raw digestate in the same year reduced this contribution to 17%. In addition, the rate of total N applied did not affect the percentage contribution of the raw digestate (RD 230 and RD 350) in any of the three years of the trial, whereas in 2015, AN 350 showed a contribution to the accumulation of residual soil nitrate that was five times higher than AN 230.

3.3. Impact of treatment on forage yield and agronomic efficiency

Overall, the yields of the harvested grass were not affected significantly by the treatment within the same year. The yields produced under the application of biogas residue fractions were slightly

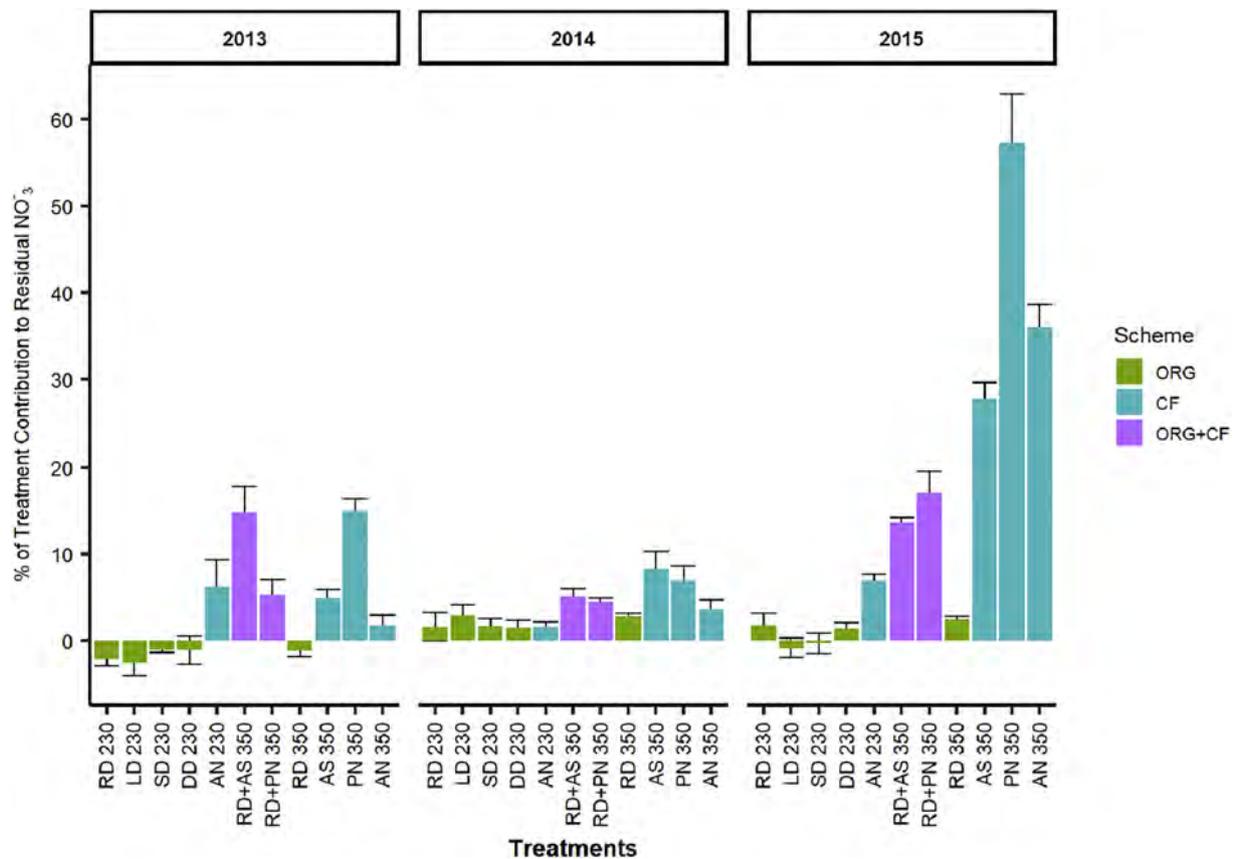


Fig. 5. Contribution of the fertilization treatments to the accumulation of residual nitrate in the soil profile (0–90 cm) (RD 230:Raw Digestate; LD 230:Liquid Phase; SD 230:Solid Phase; DD 230:Dried Digestate; AN 230:(NH₄)(NO₃); RD + AS 350: Raw Digestate + (NH₄)₂SO₄; RD + PN 350:Raw Digestate + KNO₃; RD 350:Raw Digestate; AS 350: (NH₄)₂SO₄; PN 350: KNO₃; AN 350: (NH₄)(NO₃)). The treatments are grouped in three schemes (BR: Biogas Residues; CF: Chemical Fertilizers; BR + CF: Biogas Residues + Chemical Fertilizers).

lower (in 2013 and 2015) but not statistically different to those obtained under the application of chemical fertilizers and combinatorial treatments (Fig. 6). Statistically significant differences were observed only between the control and chemical and combinatorial treatments in 2013 and 2015. Substituting chemical fertilizers with biogas residues up to 65% showed no significant reduction in the aboveground biomass produced when compared to the chemical fertilizers as the sole nitrogen source. However, between years, the grass yields showed significant differences ($P < 0.001$), with 2014 yielding the highest biomass, giving on average 12 tons of DM ha⁻¹ under the application of BRs and CFs, with RD 230 performing better than all the chemical treatments, and the control producing approximately 9.5 tons of DM ha⁻¹. In 2015, the yield obtained was the lowest for the three-year period, with the performance of CFs and BR fractions showing no statistical differences. In addition, the rate of nitrogen (230 and 350 kg ha⁻¹) did not have any impact on the grass yield within the same year, neither for raw digestate (RD 230 and RD 350) nor for ammonium nitrate (AN 230 and an 350), thus indicating a plateau effect.

The average Agronomic Efficiency calculated for all three years did not exceed 14 kg of DM kg⁻¹ of N applied for most of the treatments tested. However, the scheme of chemical fertilizers demonstrated the highest efficiency in 2013 and 2015 (Fig. 6). The rate of nitrogen applied as raw digestate did not affect the AE, except in 2014 when the efficiency of RD 230 was double the efficiency of RD 350. On the other hand, for ammonium nitrate (AN 230 and AN 350), the impact of the N rate on the crop yield increase per kg of nitrogen applied, revealed higher efficiency when the N rate was 230 kg ha⁻¹, reaching almost 20 kg kg⁻¹ in 2013 and 16 kg kg⁻¹ in 2015.

3.4. Impact of treatment on plant nitrogen uptake and apparent recovery efficiency

Nitrogen uptake by the aboveground biomass was strongly affected by the fertilization treatment ($P < 0.001$), the trial year ($P < 0.001$) and their interaction ($P < 0.05$). More specifically, in 2013, all the treatments within the chemical fertilization scheme had a great positive impact on the nitrogen uptake, with AS 350 reaching 272 kg of N ha⁻¹. Nitrogen uptake under the application of the chemical and combinatorial treatments was not statistically different. The biogas residue fractions demonstrated the lowest performance with nitrogen uptake ranging from 140 kg ha⁻¹ up to 173 kg ha⁻¹ but did not differ significantly from the combinatorial treatments. However, BRs (except SD 230 and DD 230) and chemical fertilizers showed strong differences regarding nitrogen uptake under the same nitrogen rate in 2013. The following year, there were no statistical differences either between the control and the treatments or among treatments. In 2015, nitrogen uptake was the lowest for all the treatments compared to the two previous years. BRs had no significant differences either with the combinatorial treatments or with the chemical treatments under the same nitrogen rate. The tested rates of 230 kg ha⁻¹ and 350 kg ha⁻¹ had no significant effect for either raw digestate or ammonium nitrate (Fig. 7).

The average Apparent Recovery Efficiency was highest in 2013 and lowest in 2014 for all treatments. Overall, the highest aboveground nitrogen uptake per unit of nitrogen applied was observed for the treatments within the chemical scheme, with AN 230 being the most efficient treatment. More specifically, in 2013, almost 50% of the nitrogen detected in the aboveground plant biomass was attributed to AN 230 (increased N uptake by 109 kg ha⁻¹), while RD + PN 350 increased nitrogen content by 57 kg ha⁻¹ and SD 230 by 43 kg ha⁻¹. In 2014, only

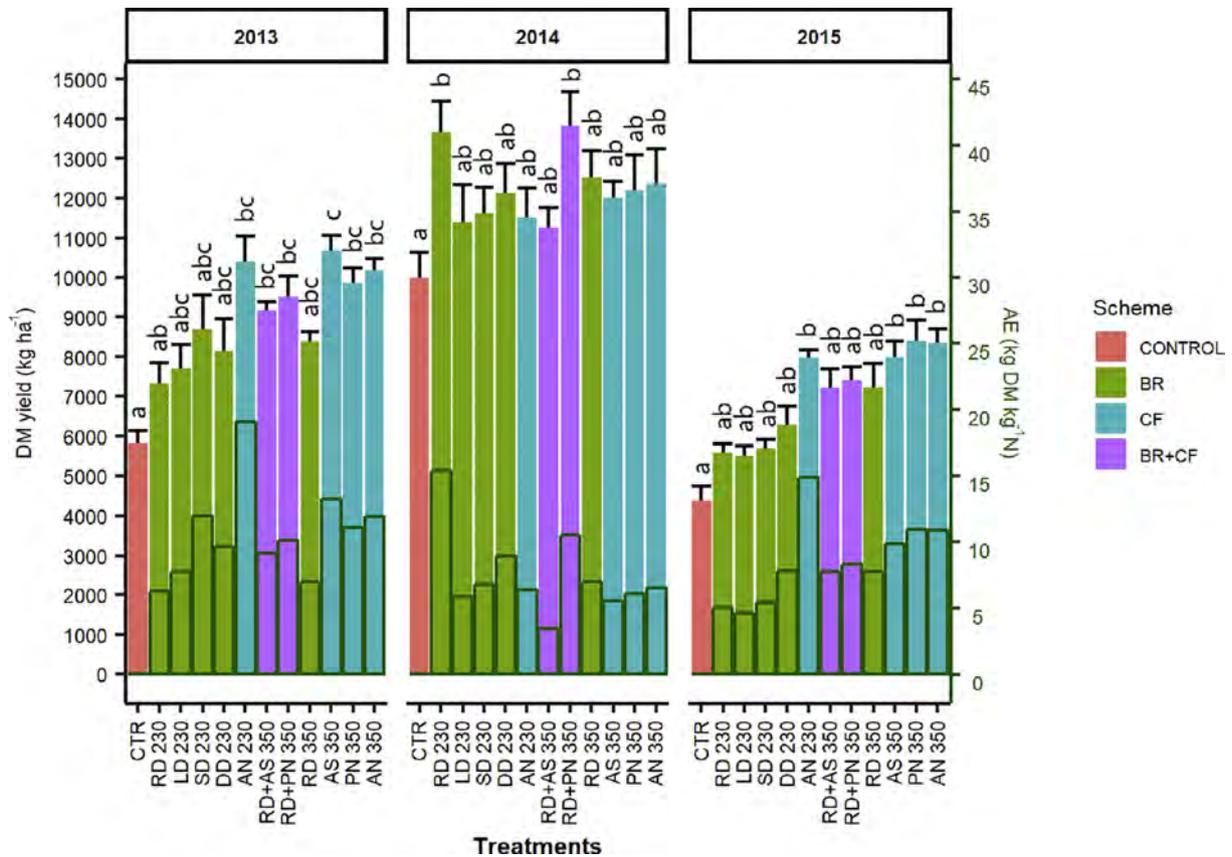


Fig. 6. Yield of harvested forage (DM: dry mass, bare bars) and average Agronomic Efficiency (AE, bars with outlines) per year and per treatment (CONTROL: No treatment; RD 230:Raw Digestate; LD 230:Liquid Phase; SD 230:Solid Phase; DD 230:Dried Digestate; AN 230:(NH₄)(NO₃); RD + AS 350:Raw Digestate + (NH₄)₂SO₄; RD + PN 350:Raw Digestate + KNO₃; RD 350:Raw Digestate; AS 350: (NH₄)₂SO₄; PN 350: KNO₃; AN 350: (NH₄)(NO₃)). Yield data with the same letter in a column are not significantly different.

32 kg of 198 kg, 7 kg of 177 kg and 9 kg of 173 kg ha⁻¹ of N uptake by the grass was attributed to fertilization with AN 230, RD + PN 350 and SD 230, respectively. In the last year, the chemical treatments increased N uptake the most (54 kg ha⁻¹ for AN 230) compared to combinatorial treatments (22 kg ha⁻¹ for RD + AS 350) and BR fractions (22 kg ha⁻¹ for DD 230). Overall, the ARE was higher for (NH₄)(NO₃) when applied at the rate of 230 kg ha⁻¹, while for the raw digestate the ARE was slightly higher at the rate of 350 kg ha⁻¹ with the exception of 2014 (Fig. 7).

3.5. Effect of precipitation on nitrate accumulation and agronomic performance

To understand how nitrogen utilization and losses may be affected by a changing climate variable such as precipitation, while keeping in mind the strong year effect observed in the previous analyses, we explored the influence of the annual rainfall on residual soil nitrate, DM yield and N uptake of the aboveground plants. The cumulative amount of residual nitrate detected within 90 cm of soil under the application of BR fractions was not affected by the annual precipitation and demonstrated the same response as the unfertilized control plot. In contrast, nitrate concentration measured in the plots treated with chemical fertilizers revealed the implication of the annual rainfall in the accumulation of nitrate. More specifically, the trend observed indicates that low annual precipitation (as recorded in 2015) can be conducive to nitrate accumulation when fertilization is performed with high rates of chemical fertilizers. However, nitrate accumulation in the soil under low precipitation was moderated when 65% of chemical fertilizers were substituted by raw digestate (Fig. 8).

Regarding the dry mass yield – for all the treatments tested, but also for the unfertilized control – the highest yield was noted in 2014 when

rainfall was highest (722 mm), while the lowest yield was harvested in 2015 when precipitation was almost half (604 mm) the regional average (1014 mm yr⁻¹). This observation implied a trend for an increasing yield as annual precipitation increased. The concentration of nitrogen detected in the aboveground plant parts was the lowest for all the treatments and the control when the recorded annual precipitation was only 604 mm in 2015. For the biogas residue fractions and the control, the increase of rainfall (707 and 722 mm in 2013 and 2014, respectively) led to an increase of plant nitrogen content. However, for the chemical and combinatorial treatments, nitrogen uptake reached a peak when annual rainfall was 707 mm but further elevation of precipitation to 722 mm led to the reduction of nitrogen uptake (Fig. 8).

4. Discussion

4.1. Potential nitrate leaching

Nitrogen loss through nitrate leaching from the root zone of agricultural lands has a negative impact on the groundwater quality (Marchi et al., 2016) and can reduce soil fertility, thus affecting crop yields (Jabloun et al., 2015). In general, grasslands have a large nitrogen uptake capacity and therefore show a low nitrate leaching risk (Cameron et al., 2013). The nature and rate of the fertilizers used in this grassland experiment and environmental factors such as precipitation, as well as their interaction, seem to determine the soil profile content in mineral nitrogen, mainly in the form of nitrate. More precisely, nitrate concentration was scheme-specific and found to be higher under the application of chemical and combinatorial schemes compared to biogas residues. However, in the interpretation of our results, we should keep in mind the high spatial and temporal variability that characterizes soil systems, and the possible contribution of the manual application of fertilizers to

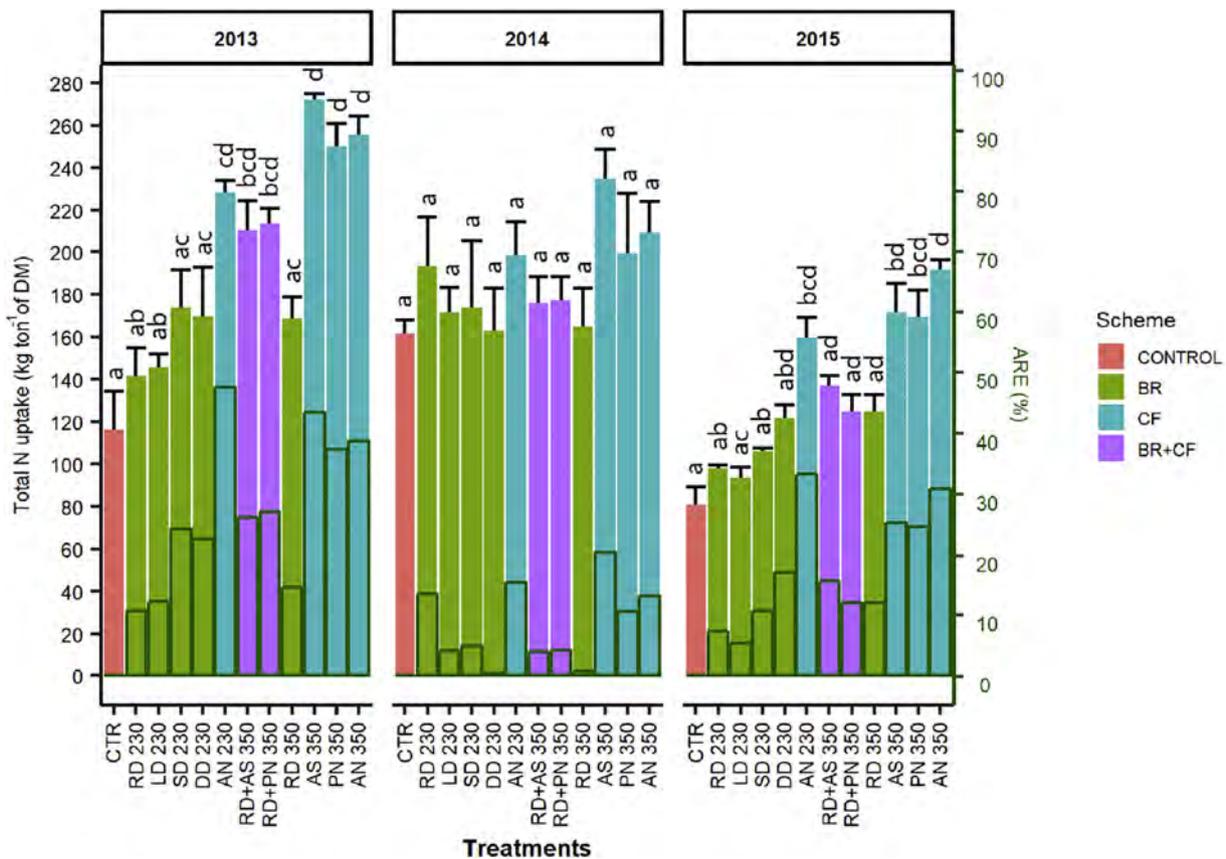


Fig. 7. Total N recovered by aboveground plant biomass (bare bars) and average Apparent Recovery Efficiency (ARE, bars with outlines) per year and per treatment (CONTROL: No treatment; RD 230:Raw Digestate; LD 230:Liquid Phase; SD 230:Solid Phase; DD 230:Dried Digestate; AN 230:(NH₄)(NO₃); RD + AS 350: Raw Digestate + (NH₄)₂SO₄; RD + PN 350: Raw Digestate + KNO₃; RD 350:Raw Digestate; AS 350: (NH₄)₂SO₄; PN 350: KNO₃; AN 350: (NH₄)(NO₃)). Total N uptake data with the same letter in a column are not significantly different.

the spatial heterogeneity of the soil. We believe that precise treatment application, frequent sampling and a higher number of replicates in such field trials could provide more reliable data and further reinforce the findings.

The accumulation of nitrate in the soil depends greatly on environmental factors such as water availability, which dictates transformations and losses of nitrogen in the soil (Wang et al., 2006), while allowing N uptake by the vegetation (Yao et al., 2018). The results of the current study revealed the accumulation of nitrate in all three soil depths, when precipitation amounted to almost half (604 mm in 2015) of the annual rainfall for the region (1014 mm yr⁻¹), under the application of chemical and combinatorial fertilizers (Fig. 8). Similarly, other studies investigating the impact of precipitation on nitrate leaching concluded that there was a negative correlation between high precipitation and nitrate concentration in the extracted soil solution (Huang et al., 2018; Jabloun et al., 2015), which may be attributed to the dilution effect (Beaudoin et al., 2005), and the enhanced denitrification promoted under the anaerobic conditions established in saturated soils (Philipot et al., 2007). However, nitrate accumulation in the soil profile and its loss through leaching and denitrification, could vary in soils of differing texture, under disparate rainfall distribution during seasons or extreme rainfall events and temperatures.

Furthermore, the quantity of the fertilizers applied has a great potential to increase nitrate leaching (Yao et al., 2018; Delin and Stenberg, 2014) especially if it exceeds the uptake capacity of the plants (Guillard et al., 1995). However, in this study, this was the case only for chemical fertilizers but not for BRs as the sole nitrogen source. Increasing the nitrogen rate from 230 kg ha⁻¹ to 350 kg ha⁻¹ per year for ammonium nitrate, in 2015, led to the vertical distribution of nitrate and tripled its accumulation in the top soil layer (0–30 cm), increased it by

tenfold at 30–60 cm, and quadrupled it at 60–90 cm (Fig. 4). Therefore, potential nitrate leaching is significantly elevated with the excess application of chemical fertilizers (Lenka et al., 2013), particularly in combination with decreasing water availability (Huang et al., 2018). In contrast, increasing the rate of applied nitrogen in the form of raw digestate from 230 kg ha⁻¹ to 350 kg ha⁻¹ per year showed no accumulation or progressive movement of nitrate in the soil profile when annual precipitation was low (2015). Walsh et al. (2012) also demonstrated that the application of mineral fertilizer on grassland led to greater NO₃⁻ leaching than cow slurry-based liquid digestate, when levels of soil nutrients exceeded plant requirements. These findings are also in agreement with the results obtained by Svoboda et al. (2013), who showed that the application of chemical fertilizer as calcium ammonium nitrate (CAN), at high nitrogen rates (360 kg ha⁻¹), resulted in nitrate concentrations above the EU drinking water threshold, while corresponding BRs, obtained through co-fermented pig slurry/maize, remained below that threshold and at significantly lower levels compared to CAN. These results strongly confirm our hypothesis that – in the short-term – BRs applied as the sole nitrogen source and at the maximum rate of 350 kg N ha⁻¹ yr⁻¹ do not increase the potential nitrate leaching risk in contrast to chemical fertilizers. However, to be able to make estimates about the long-term consequences, it is necessary to consider the repeated application of BRs and increased mineralization of accumulating organic N. The lack of data from long-term grassland experiments does not allow the full evaluation of the nitrate leaching potential of BRs in the long-term. Nevertheless, this risk could possibly be mitigated or eliminated with the proper adjustment of the supplementary chemical fertilizers. Substituting chemical fertilizers such as KNO₃ and (NH₄)₂SO₄ by 65% with raw digestate has the potential to reduce the accumulation of nitrate in the

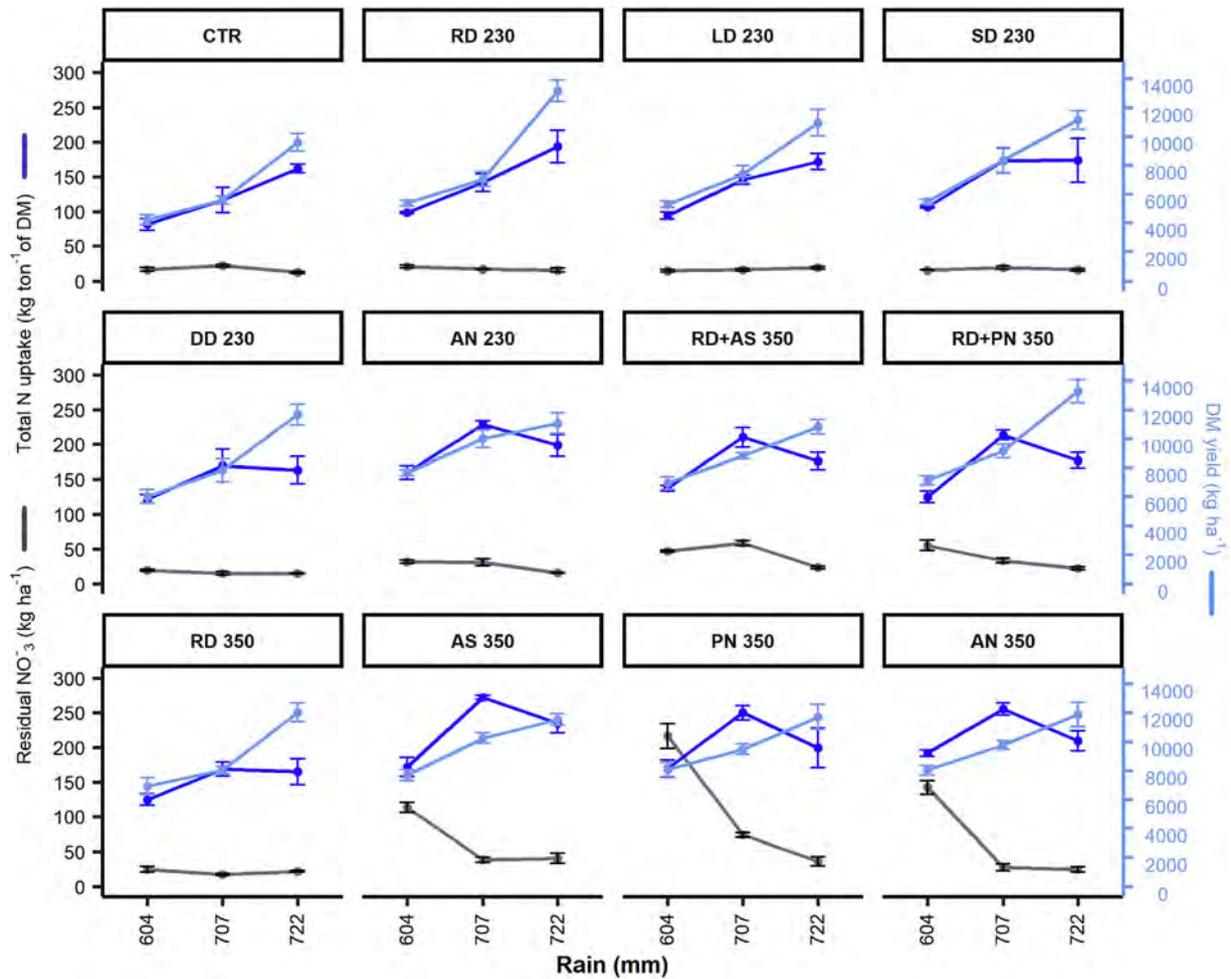


Fig. 8. Residual nitrate, dry mass yield and nitrogen uptake (mean \pm se, $n = 4$) under changing annual precipitation (2013: 707 mm, 2014: 722 mm, 2015: 604 mm) per treatment tested (CONTROL: No treatment; RD 230:Raw Digestate; LD 230:Liquid Phase; SD 230:Solid Phase; DD 230:Dried Digestate; AN 230:(NH₄)(NO₃); RD + AS 350:Raw Digestate + (NH₄)₂SO₄; RD + PN 350:Raw Digestate + KNO₃; RD 350:Raw Digestate; AS 350: (NH₄)₂SO₄; PN 350: KNO₃; AN 350: (NH₄)(NO₃)).

soil by 60% during dry periods. This finding indicates that most of the nitrate load in the soil is explained by the proportion of chemical fertilizers in the combinatorial treatments tested. Furthermore, this could be partially attributed to the high content of BRs in ammonium, which inhibits the nitrification rate by restricting the activity of *Nitrobacter* in the soil (Monaghan and Barraclough, 1992). To reach a conclusion on the potential of partially replacing chemical fertilizers by BRs, long-term field experiments under various climatic conditions and different substitution rates are required. Moreover, our results do not confirm the hypothesis put forward by other researchers (Kirchmann et al., 2007) claiming a high nitrate loss risk due to the asynchrony of crop demand and nitrogen release from the organic matter when fertilizing with organic fertilizers such as digestate or manure.

Lastly, of particular interest is the elevated concentration of residual nitrate in the soil, recorded under the application of ammonium sulphate (AS 350). Despite the fact that ammonium sulphate (21% NH₄⁺ and 24% SO₄²⁻) does not introduce any nitrate in the soil upon application, it is among the treatments that demonstrated the highest nitrate accumulation in the soil profile, particularly in the last year of the trial. As ammonium is the main form of nitrogen contained in ammonium sulphate, a behaviour similar to ammonium-rich BRs would be anticipated in the soil. This observation could possibly be explained by the sulphate-reducing ammonium oxidation (SRAO) process, first assumed by Fdz-Polanco et al., 2001. The SRAO process has not been considered under *in situ* conditions and could provide an insight to biological reactions taking place in agroecosystems between NH₄⁺ and SO₄²⁻.

4.2. Agronomic performance

Regarding the agronomic performance of the fertilization schemes tested in this experiment, it was demonstrated that BR fractions have the potential to maintain yields in the grasslands of the Walloon Region and potentially provide nitrogen in the long-term due to the slow mineralization of organic N. Overall, for all three years of this field trial, the aboveground biomass yield obtained under the application of the various fractions of BRs was in close proximity with the yield produced under the use of combinatorial treatments, as well as the chemical fertilizers as the sole source of nitrogen (Fig. 6). Similar to our findings, other studies have shown that the use of BRs as the sole source of nitrogen or the partial substitution of chemical fertilizers by a variety of organic fertilizers can maintain nutrient supply and crop yield at similar levels to mineral fertilizers (Sigurnjak et al., 2017; Sapp et al., 2015; Andruschkewitsch et al., 2013; Alburquerque et al., 2012; Walsh et al., 2012; Zhang et al., 2009). Furthermore, with respect to the rate of nitrogen applied (230 and 350 kg ha⁻¹), for both raw digestate and ammonium nitrate, our results support the findings of other studies indicating that higher fertilizer rates do not guarantee higher yields (Sestak et al., 2014), but may also have an adverse effect on nitrogen use efficiency (Yang et al., 2017).

The nitrogen content of the aboveground dry biomass was significantly affected by the fertilization scheme, mainly in the first year of the trial, with grass N content being the highest under the application of chemical fertilizers, indicating their higher plant availability (Fig. 7).

This could be partly explained by the fact that nitrogen contained in BRs is mainly in the form of organic nitrogen and NH_4^+ -N (50 to 80% of the total N). Therefore, the slow release of organically bound N in the first year of application may result in lower utilization by plants (Gutser et al., 2005) while NH_4^+ -N adsorption on the soil matrix may reduce its availability and entail plant root growth towards this nitrogen source. However, the composition of biogas residues may depend on factors such as the type of feedstock and degradability, retention time and conditions within the digester, as well as on further post-digestion treatments. Finally, under warm and aerated soil conditions, NH_4^+ -N is susceptible to rapid transformation and assimilation by the soil microbial biomass, and therefore is available to plants only for a short time. The latter can be supported if we take into account the low nitrogen uptake observed under the application of the BR fractions and their lower contribution to nitrate accumulation in the soil profile compared to the unfertilized control in the first year of the experiment (Fig. 5). Hence, we posit that BRs, in contrast to chemical fertilizers, act mainly via the soil N pool (Gutser et al., 2005) by enhancing the activity and biomass of soil microorganisms (Nkoa, 2014), leading to the increased immobilization (Larsen et al., 2007) of available N. In line, Abubaker et al. (2015) concluded that application of BRs on soil could limit nitrogen availability to plants in the short-term. Over the following two years, the biogas residue fractions tested had no statistical differences with the chemical and combinatorial treatments, something that could be ascribed to the residual N effect, observed also in other studies evaluating the nitrogen fertilizer replacement value of biogas residues (Cavalli et al., 2016) and manure (Schröder, 2005). This outcome suggests that the low degradation rate of organic N contained in BRs has the potential to gradually supply the soil with nutrients required for plant growth, particularly plants with a constant nitrogen demand, such as perennial forage grasses (De Boer, 2017). It should be noted that in our experiment, fertilization was done by hand and the fertilizers were applied to the soil surface rather than incorporated into the soil to reduce nitrogen losses through ammonia volatilization. Gericke (2009) estimated that emissions following BR application were between 7 and 24% of the applied NH_4^+ -N. We believe that the employment of more suitable application methods of BRs (injection, trailing-shoe), that minimize their exposure to air and maximise their contact with the topsoil, could possibly lead to higher nitrogen uptake by the plants. Finally, we should keep in mind that the belowground biomass of grasslands encompasses the main pool of organic matter, with root turnover playing a critical role in nutrient cycling (Fiala, 2010). Therefore, to obtain a comprehensive view of the utilization and physical allocation of the plant available nitrogen, experiments that consider the form of N provided and simultaneously examine the aboveground and belowground biomass content would be illuminating.

Nitrogen use efficiency indicators, such as the agronomic efficiency and apparent recovery efficiency, revealed a plateau effect under higher fertilization rates, while less than half of the nitrogen applied was retained in the biomass. Particularly for BR fractions, nitrogen use efficiency may be underestimated in short-term trials as plant nutrient requirements may not be met shortly after their application due to residual effects. These observations suggest that local farmers could increase their economic benefits by reducing the fertilization rate both for BRs and chemical fertilizers without having a negative impact on the agronomic performance. In many farming systems, the increase of chemical fertilizers has failed to increase nitrogen use efficiency proportionally, instead leading to environmental problems (Shen et al., 2013). Beside the fertilization rates, another factor that can affect the nutrient use efficiency of fertilizers and consequently the yield production is the deficiency or imbalance of essential plant nutrients, as well as their synergistic and antagonistic interactions (Rietra et al., 2017). Knowledge of these interactions would be of particular interest for biogas residues since they are rich in plant macro- and micro-nutrients. Therefore, experiments that consider application time, nitrogen amount, nutrient composition of BRs and the nature of nutrient

interactions could help to optimize BR use and fine-tune their application according to different conditions and plant requirements.

At last, the results of this study indicated a relationship between the annual precipitation and yield performance, with all the fertilization schemes tested demonstrating a uniform response. The aboveground biomass yield was significantly higher and treatment-independent when the annual rainfall was high, while it was reduced up to 40% in some cases when the precipitation was low (Fig. 8). According to this field experiment, plant biomass nitrogen was also affected by the annual rainfall. More specifically, plots treated with BRs revealed a positive response to elevating annual precipitation, while plots treated with chemical and combinatorial treatments indicated a dilution effect when annual rainfall exceeded 707 mm (Fig. 8). It is well known that alterations in the rainfall patterns and the annual mean directly affect the crop yields (Jabloun et al., 2015), the utilization of nutrients and thus, the productivity of agricultural ecosystems (Han et al., 2015). Taking into account our findings, along with the broader nutrient base of BRs, their utilization could be of particular interest in tropical soils with low organic matter content and other deficiencies. Also, in countries where agriculture is limited by inadequate and erratic rainfall, such as in sub-Saharan countries, the use of BRs could possibly unburden soils from nitrate accumulation due to the use of chemical fertilizers, enhance the water holding capacity and increase carbon sequestration (Smith et al., 2014). Although our observations help in better understanding the behaviour of BRs and compare it to that one of chemical fertilizers, these results should not be extrapolated to all soil types, climatic conditions and management systems without local investigation.

4.3. Health and safety risks associated with biogas residue application

Beside the environmental and agronomic benefits that may result from the application of biogas residues on agricultural lands, the adverse effects of their use – such as chemical and biological contamination potential – are of paramount importance for the farmers and end users. The effective recycling of organic wastes back into the soil presupposes BRs not only rich in nutrients but also safe for living organisms. BR quality with respect to health and safety depends mainly on the organic wastes used as feedstock and the conditions within the reactor. In biogas plants such as this one, where animal manure is one of the main input materials, the monitoring of BR quality is important. Discussions around the benefits and risks of BRs are very controversial with the main concerns being the spread of pathogens, the accumulation of heavy metals and the presence of organic pollutants and antibiotics. To control the quality of biogas residues intended for land application, in Europe, regulations set up limit values for these contaminants, and provide lists with materials that are suitable as feedstock for the anaerobic digestion process (EU, 2002).

This study was focused on the short-term environmental and agronomic impact of biogas residues when applied on a permanent grassland. As this has been an ongoing field experiment since 2013, we are currently investigating the impact of the various biogas residue fractions, produced at the biogas plant on site (Ferme du Faascht, Attert, Belgium), on the diversity and abundance of autochthonous soil bacteria implicated in nitrogen cycling processes, as well as the potential of soil contamination by pathogens, by exploiting 'omic' approaches. From the existing literature, it is well known that the process of anaerobic digestion, even under the mesophilic temperature of 39 °C, can eliminate or reduce pathogens such as *Escherichia coli*, *Salmonella* and *Listeria* contained in animal effluents (Goberna et al., 2011). Recent studies have reported plant disease suppression (Fuchs et al., 2008), as well as pesticidal (Jothi et al., 2003; Kupper et al., 2006) and insecticidal activity (German National Academy of Sciences Leopoldina, 2012) of biogas residues. However, more resistant microorganisms such as spore-forming *Clostridium perfringens* and prions, may survive even after thermophilic digestion (Elmerdahl Olsen and Errebo Larsen, 1987; Franke-Whittle and Insam, 2013). Despite the potential risk,

under no circumstances does anaerobic digestion increase the spreading risk of bacterial pathogens, especially when compared to undigested manures (Insam et al., 2015). With a retention time of 40 days and a mesophilic operational temperature of 38 °C for the manure based biomethanation process established in our biogas plant, we hypothesize an adequate sanitation of the produced digestate – particularly for the dried biogas residue fraction produced after post-treatment on a belt dryer. Phytotoxicity due to the heavy metals, organic pollutants and antibiotics contained in BRs is another major concern. Their concentration is also believed to decrease after anaerobic digestion (Möller and Müller, 2012; Varel et al., 2012), although not all of the contaminants can be sufficiently controlled. Maunuksela et al. (2012), with the use of plant bioassays, tested the phytotoxicity of various biogas products in concentrations used in the field conditions, proving their suitability as fertilizers. Various treatment processes of biogas residues are available to ensure that the end products meet the requirements of environmental regulations (Adam et al., 2018). However, there is a need for long-term field experiments regarding heavy metal and organic pollutant build-up in soil and plant tissues. Overall, the composition and quality of BRs intended for fertilization should be closely monitored. Confidence in the safety of biogas residues is expected to promote their use as biofertilizers.

5. Conclusions

During this three-year study, biogas residue fractions did not demonstrate any nitrate leaching risk and remained nitrogen rate- and precipitation-independent as opposed to chemical fertilizers. The partial substitution of chemical fertilizers by raw digestate at the level of 65% was proven to reduce the accumulation of residual nitrate in the soil. The application of BRs as the sole nitrogen source, as well as in combination with chemical fertilizers, demonstrated their potential to maintain yields and nitrogen uptake and concurrently mitigate nitrate leaching risk in this grassland system.

However, more comprehensive studies are required to understand the long-term consequences of repeated biogas residue application on environmental pollution and agronomic performance, and to implement their use in a wider context. Field trials that will shed a light on the destiny of the organic nitrogen accumulated in the soil under fertilization with BRs and the processes that determine its availability in the long-term are in need. Together with quality and safety assessment, different fertilization rates tested in various soils and climatic conditions could help to optimize the use of BRs in agriculture. Finally, global modelling studies regarding climate change – such as the elevation of atmospheric CO₂ and unpredictable weather – should be put in perspective to better predict the repercussions of different fertilization practices on the N cycle and plant production in the future.

Keeping in mind the advantages and disadvantages of the biogas residues and chemical fertilizers evaluated in this field experiment, we can state with optimism that substitution of chemical fertilizers by biogas residues could be an effective approach to an eco-friendly nutrient management in similar agricultural systems.

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Research article



Biogas residues in the battle for terrestrial carbon sequestration: A comparative decomposition study in the grassland soils of the Greater Region

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ABSTRACT

The recycling of biogas residues resulting from the anaerobic digestion of organic waste on agricultural land is among the means to reduce chemical fertilizer use and combat climate change. This in sacco decomposition study investigates (1) the potential of the granulated biogas residue fraction to provide nutrients and enhance soil carbon sequestration when utilized as exogenous organic matter in grassland soils, and (2) the impact of different nitrogen fertilizers on the organic matter decomposition and nutrient release processes. The experiment was conducted in two permanent grasslands of the Greater Region over one management period using rooibos tea as a comparator material. The decomposition and chemical changes of the two materials after incubation in the soil were assessed by measuring the mass loss, total carbon and nitrogen status, and fibre composition in cellulose, hemicellulose and lignin. Overall, after the incubation period, granulated biogas residue maintained up to 68% of its total mass, organic matter and total carbon; increased its content in recalcitrant organic matter by up to 45% and released 45% of its total nitrogen. Granulated biogas residue demonstrated resilience and a higher response uniformity when exposed to different nitrogen fertilizers, as opposed to the comparator material of rooibos tea. However, the magnitude of fertilizer-type effect varied, with ammonium nitrate and the combinatorial treatment of raw biogas residue mixed with urea leading to the highest organic matter loss from the bags. Our findings suggest that granulated biogas residue is a biofertilizer with the potential to supply nutrients to soil biota over time, and promote carbon sequestration in grassland soils, and thereby advance agricultural sustainability while contributing to climate change mitigation.

1. Introduction

Achieving sustainability in the agricultural sector could contribute to the mitigation of climate change and environmental pollution while addressing food security challenges faced due to the constantly growing world population. Anthropogenic activities such as fossil fuel overuse, excessive fertilization and land use have altered the cycling and storage of carbon (C) and nitrogen (N), depleting the terrestrial nutrient reservoirs over time. Since nitrogen availability is the most limiting factor in

the agricultural production system and its cycle is closely linked with that of carbon, restoring these pools is of vital importance. Hence, the management of organic waste and nutrient recycling in agriculture could constitute an important strategy for achieving a non-CO₂ circular economy both inside the European Union (EU) and on a global level. Anaerobic digestion (biomethanation, AD) is an environmentally sound multipurpose process (Lukehurst et al., 2010) that allows the treatment of a wide variety of organic waste providing an alternative to fossil fuels and a substitute to Haber-Bosch-derived fertilizers in the form of biogas

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and digestate (biogas residues, BRs), respectively. Ultimately, the implementation of AD on a global scale has the potential to reduce Greenhouse Gas (GHG) emissions by 10–13% (European Biogas Association (EBA), 2019). In addition to preventing the generation of GHGs by providing alternatives to fossil fuel-based products, and offsetting GHG emissions inherent to the landfilling, composting or storage of organic wastes (Smith et al., 2007), the utilization of biogas residues in agriculture also has the potential to provide nutrients and promote terrestrial carbon sequestration, thus enhancing the capacity of agricultural soils to produce food and feed.

Since the dawn of civilisation, the input of exogenous organic matter into agricultural soils, through the incorporation of agricultural residues, the application of manure and other organic waste, has been a means of replenishing the organic matter stocks (Hartley and Singh, 2018) while improving soil quality and productivity, biodiversity, water retention capacity and nutrient recycling, among others (Abdullahi et al., 2018; Cai et al., 2019; Diacono and Montemurro, 2010; Lal et al., 2015). However, a plethora of agricultural practices and global environmental changes have manipulated the factors controlling the decomposition processes in the soil (Swift et al., 1998), leading to a fundamental alteration of the C and N cycling in terrestrial ecosystems (Cotrufo et al., 2010). Today, in the scope of climate change mitigation, besides improving soil fertility and agronomic productivity, the addition of organic matter to soil is considered as a measure to reduce CO₂ in the atmosphere through carbon sequestration and its long-term storage as resistant soil organic matter (Kirkby et al., 2016). The main mitigation potential lies in soil carbon sequestration in depleted agroecosystems, as well as in systems that already have high stocks, by preserving or increasing them (ADEME, 2015). According to the Intergovernmental Panel on Climate Change (IPCC), agriculture has one of the most significant near-term (by 2030) greenhouse gas mitigation potentials, with 90% of it arising from increased soil C sequestration (Smith et al., 2014; Smith et al., 2007). In the same context, the “4 per 1000” international programme, signed by 150 countries in the framework of the Paris Climate Agreement, aspires to increase global soil organic matter stocks by 0.4% per year (Minasny et al., 2017). In the same vein, a long-term framework has been proposed by the EU to expand and maintain permanent grasslands in order to reduce CO₂ emissions and promote carbon sequestration (European Commission, 2010). Given the fact that grasslands occupy 40.5% of the terrestrial area (Bai et al., 2010; Suttie et al., 2005) and are known to have a greater nutrient uptake capacity (Cameron et al., 2013) and soil organic carbon sequestration potential than cropland (Lal et al., 2015; Conant et al., 2001), there is an imperative need to understand how different management practices may influence the long-term C responses of grassland soils (FAO, 2010).

In an attempt to gather firm scientific evidence on the benefits of biogas residues, many studies have shown their fertilizing properties and beneficial effects on agricultural soils (Chiew et al., 2015; Koszel and Lorencowicz, 2015; Nkoa, 2014; Odlare et al., 2008; Sapp et al., 2015), often accompanied by a negligible polluting potential (Svoboda et al., 2013; Tsachidou et al., 2019a,b; Tshikalange et al., 2020; Walsh et al., 2012). However, to maximize the environmental benefits of biogas residues and ensure their optimal and wide use in agricultural systems, we need to be able to estimate their stability and nutrient release rates in agricultural soils (Maynaud et al., 2017; Diacono and Montemurro, 2010). Both the decomposition and nutrient value of the biogas residues depend on the type and initial composition of the substrate utilized in the biogas plant (Bareha et al., 2018), and the changes that it undergoes during the course of anaerobic digestion, and can be hard to predict. It has been demonstrated that the organic fraction of biogas residues, post-anaerobic digestion, is much more recalcitrant than the input feedstock, leading to the stabilization of organic matter and a lower organic matter degradation rate after field application (Cayuela et al., 2010; Möller, 2015; Veeken et al., 2017). Therefore, besides their fertilizing properties, biogas residues being rich in stable organic matter – particularly their solid fraction – could also promote

carbon sequestration and long-term storage in the soil. Nevertheless, due to the wide range of feedstock types used in the biogas plants, the composition and quality of biogas residues may vary among the plants creating concerns regarding their safety and polluting potential. Different environmental risks associated with their use as fertilizers have led to the establishment of legal constraints, with the European Nitrates Directive 91/676/EEC being one of the earliest instruments put in place to control agricultural pollution (EEC Council Directive, 1991).

Regarding nutrient cycling in terrestrial ecosystems, there is ongoing controversy concerning the extent to which nitrogen fertilization can increase soil organic matter and carbon sequestration, and therefore contribute to the mitigation of climate change (Ehtesham and Bengtson, 2017). While the usual assumption is that N input promotes organic matter accumulation and C sequestration in the soil, there are also studies demonstrating the opposite, where mainly losses of soil organic carbon were observed (Khan et al., 2007; Ladha et al., 2011), particularly under excessive nitrogen application (Poffenbarger et al., 2017; Singh, 2018). These contradictory findings may be attributed to the different experimental settings, data assessment and the complex web of interactions taking place in the soil system. Related questions arise regarding the effect of the N fertilizer source on organic matter decomposition and carbon sequestration – but also on nitrogen release and accumulation – due to the growing dependence of modern agriculture on synthetic N fertilizers and the need to transit to more environmentally friendly substitutes. The type, amount and application method of the N fertilizer is of great importance, particularly when it comes to soil biota such as bacteria and fungi, which are agents of both the decomposition and synthesis of organic matter, and are responsible for the transformation and cycling of essential nutrients.

To this end, if we intend to manage C and N stocks in agricultural soils and remove GHGs from the atmosphere, we need to identify and understand the factors and mechanisms that regulate organic matter decomposition. For this study, we have employed the *in sacco* method to evaluate the organic matter stability and composition changes of granulated biogas residue (GBR) incubated in grassland soil profile under the application of different nitrogen fertilizers over one management period, using rooibos tea (RT) as a comparator material. Conducting this experiment, we aimed to study the property of GBR to provide nutrients and promote carbon sequestration in grassland soils, thus extending the list of biogas residue benefits. We also aimed to determine whether the decomposition process and fate of the quality variables of GBR are affected by the source of the nitrogen fertilizer applied to the grassland. Finally, since equivalent analytical data on the same type of material were not available in the literature, rooibos tea was chosen as a comparator material previously evaluated in litter decomposition studies (Keuskamp et al., 2013) to better showcase the impact of different N sources on the decomposition process (Bridgham and Ye, 2013).

To the best of the authors' knowledge, the scientific literature is missing *in situ* and *in sacco* studies investigating the decomposition and nutrient fate of biogas residues and their potential to sequester carbon when utilized as biofertilizers or soil improvers on agricultural soils. In this first attempt, our purpose was to provide the scientific evidence needed to substantiate the stability and dual property of GBR in the scope of climate change mitigation, address the controversy regarding organic matter build-up and carbon sequestration under nitrogen fertilization, and make this information available to farmers, policy- and decision-makers around the world.

2. Materials and methods

2.1. Site description

The experiment was conducted in two permanent grasslands of the Greater Region in 2018. One of the grasslands is located in the commune of Attert, a Walloon municipality of Belgium and is adjacent to the

biogas plant “Ferme de Faascht” (49.745665° N; 5.812498° E). The climate in this region is classified as oceanic (Cfb), with a mean annual rainfall that amounts to 938 mm and an average annual temperature of 8.5 °C. The second grassland is located in Champenoux, France and is adjacent to the biogas plant “Ferme de La Bouzule” (48.4421° N; 6.1934° E). The climate here is also classified as Cfb, with a mean annual rainfall of 753 mm and an average annual temperature of 9.5 °C. The mean monthly temperature and cumulative monthly precipitation recorded in 2018 are presented for both sites in Fig. 1. The profile and chemical characteristics of both grassland soils are summarized in Table 1. In both grasslands, there is an experimental set up (randomized block design) that tests various nitrogen fertilization treatments for their polluting potential and agricultural performance. These trials have been in place since 2013 at Faascht and since 2014 at Bouzule. For this experiment, we selected seven treatments, with three replicates per treatment in each grassland, representing four different N fertilizer types – biogas residues, manure, chemical fertilizers, combinatorial treatments of biogas residues and chemical fertilizers – and an unfertilized control. The chemical fertilizers used were ammonium nitrate (13.5% $\text{NH}_4^+\text{-N}$, 13.5% $\text{NO}_3^-\text{-N}$), ammonium sulphate (21% $\text{NH}_4^+\text{-N}$, 24% S), potassium nitrate (17.4% K, 13.7% $\text{NO}_3^-\text{-N}$) and urea (46% $(\text{NH}_2)_2\text{CO}$). The chemical characteristics of the raw biogas residue fraction (RD) and raw manure (RM) tested were measured on applied batches using standard analytical methods and the fertilizer amount was adjusted based on their nitrogen content (RD: $\text{N } 6.30 \text{ mg g}^{-1}$; RM: $\text{N } 2.7 \text{ mg g}^{-1}$) prior to field application. The nitrogen rates of the fertilizers tested were $230 \text{ kg N ha}^{-1} \text{ yr}^{-1}$ and $350 \text{ kg N ha}^{-1} \text{ yr}^{-1}$ in accordance with the maximum admissible doses in the Greater Region (Grant et al., 2011) and the average annual N inputs in the EU (Jensen et al., 2011). The specifics of the selected fertilization treatments are presented in Table 2. The fertilization was performed in

Table 1

Soil characteristics of the two experimental grasslands measured in the 0–30 cm soil layer prior to the incubation period.

| Parameters | Site | |
|-----------------------------|---------|---------|
| | Faascht | Bouzule |
| % sand | 41 | 36 |
| % silt | 9 | 37 |
| % clay | 50 | 28 |
| % C_{org} | 1.8 | 3.5 |
| % N_{total} | 0.3 | 0.4 |
| % CaCO_3 | ND | 45 |
| pH H_2O | 6.3 | 7.6 |
| pH KCl | 5.2 | 7.1 |

ND: below detection limit.

instalments during the permissible period from March to October (Fig. 1).

2.2. Experimental design

In this study, we implemented the *in sacco* technique – commonly used for predicting the digestibility of roughages by cattle (Fonseca et al., 1998) and in litter decomposition studies (Keuskamp et al., 2013) – in two grasslands to assess on one hand, the stability and nutrient fate of GBR in the soil, and on the other hand, the decomposability of exogenous organic matter in response to different N fertilizers over one management period, using rooibos tea (Lipton rooibos tea; 93% rooibos; EAN: 87 22700 18843 8) as a comparator material. Prior to the implementation, both substrates were weighed with a precision of three decimals and filled in inert 5 cm × 5.5 cm ANKOM filter bags with 25 μm

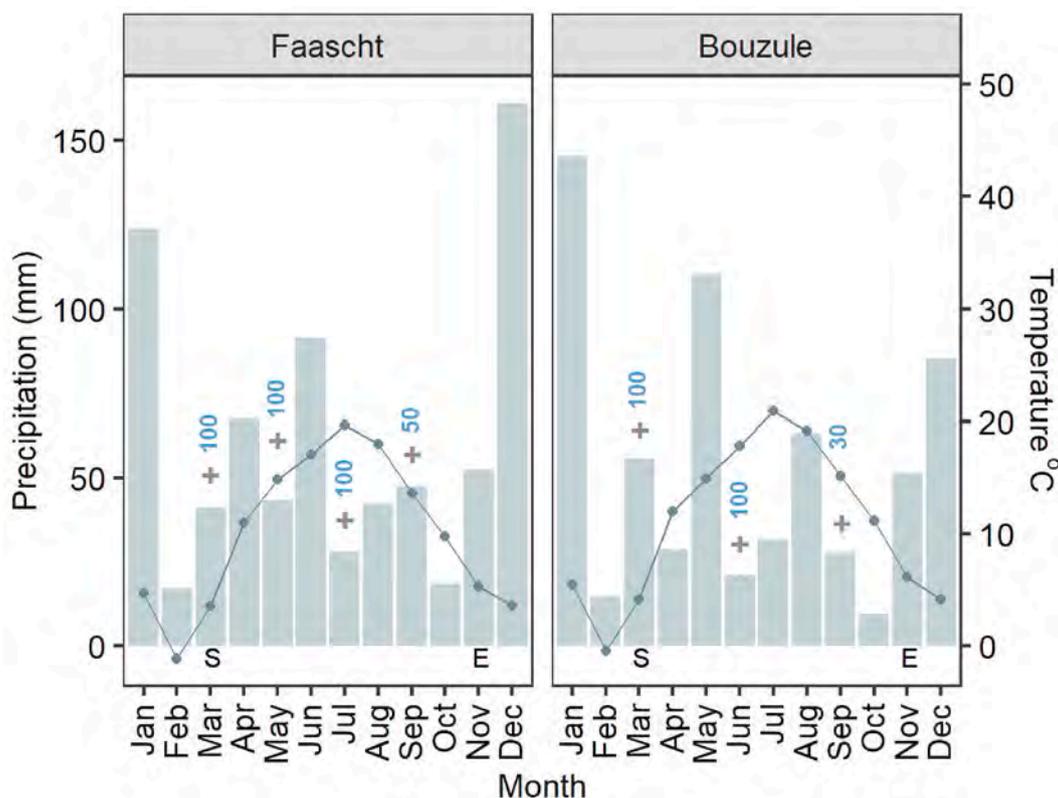


Fig. 1. Cumulative monthly rainfall (bars) and average monthly air temperature (dots and lines) at the locations of the *Ferme de Faascht* (as recorded at the nearby meteorological station in Useldange, Luxembourg) and *Ferme de La Bouzule* (as recorded at the INRAE meteorological station in Champenoux, France) during 2018. The letters above the month axis indicate the start (S) and end (E) of the incubation period at both sites. Crosses “+” indicate the months in which fertilizers were applied during the management period. Treatments applied at the rate of 230 kg N ha^{-1} were divided into three instalments, while those applied at the rate of 350 kg N ha^{-1} were divided into four instalments. The application rates (kg ha^{-1}) are indicated above “+”.

Table 2

Treatments selected at the two experimental sites and the total nitrogen units applied during the management period per year since the installation of the experiments.

| Fertilization Treatments (Identification Code) | Total N Input (kg ha ⁻¹) | |
|--|--------------------------------------|----------|
| | Faascht | Bouzule |
| Control (CTR) | 0 | 0 |
| Raw Biogas Residues (RD350) | 350 | 230 |
| Raw Manure (RM230) | 230 | 230 |
| Ammonium Nitrate (AN350) | 350 | 230 |
| Raw Biogas Residues + Potassium Nitrate (RD + PN350) | 230 + 120 | – |
| Raw Biogas Residues + Ammonium Nitrate (RD + AN230) | – | 170 + 60 |
| Raw Biogas Residues + Ammonium Sulphate (RD + AS350) | 230 + 120 | 170 + 60 |
| Raw Biogas Residues + Urea (RD + UR350) | 230 + 120 | 170 + 60 |

porosity (Ankom Technology – F57 Filter Bags) that still allowed the free movement of the materials in the bags (ca. 3 g of GBR and 2 g of RT). The 25 µm meshing of the bags facilitates microbial colonization (Yin et al., 2019) and gas exchange between the soil and the bag content while limiting material loss and contamination (Huntington and Givens, 1995). In addition, it restricts the entry of plant roots, mesofauna and macrofauna into the bags (Karberg et al., 2008; Robertson and Paul, 2000). All bags were assigned a unique code and were marked with a permanent ANKOM marker for identification. The bags were placed in

nylon net pockets to immobilize them and allow their easy retrieval from the soil after the period of incubation. To allow the material exposure to the active topsoil, the bags were placed at 10 cm depth. For this purpose, a soil block of approximately 40 cm × 20 cm × 10 cm depth was carefully removed from the centre of each experimental plot with a spade and a set of six bags for each material was placed in parallel inside the soil cavity and then covered back up with the block of soil (6 bags × 2 material types per plot × 3 replicates) as shown in Fig. 2a. All bags remained in the soil over one management period of nine months (270 days), from the beginning of March to the end of November 2018. After their removal from the soil (Fig. 2b), all bags were allowed to air dry at room temperature and the adhered soil particles and plant roots were removed from the surface of the bags before the analyses (Fig. 2c and d).

2.3. Granulated biogas residue

The granulated biogas residue material under investigation is a product of the biomethanation plant located at the “Ferme de Faascht”, where the substrates utilized are mainly liquid and solid bovine manure, crop residues (silage maize and grass) and organic waste from the food industry. The hydraulic retention time in this biogas plant is slightly over 100 days, allowing for an efficient anaerobic digestion of the substrates. To obtain GBR, the raw biogas residue fraction was dried on a belt dryer (DORSET, The Netherlands) to produce dry granules of 5–7 mm in size. The initial quality and chemical composition of GBR and RT,

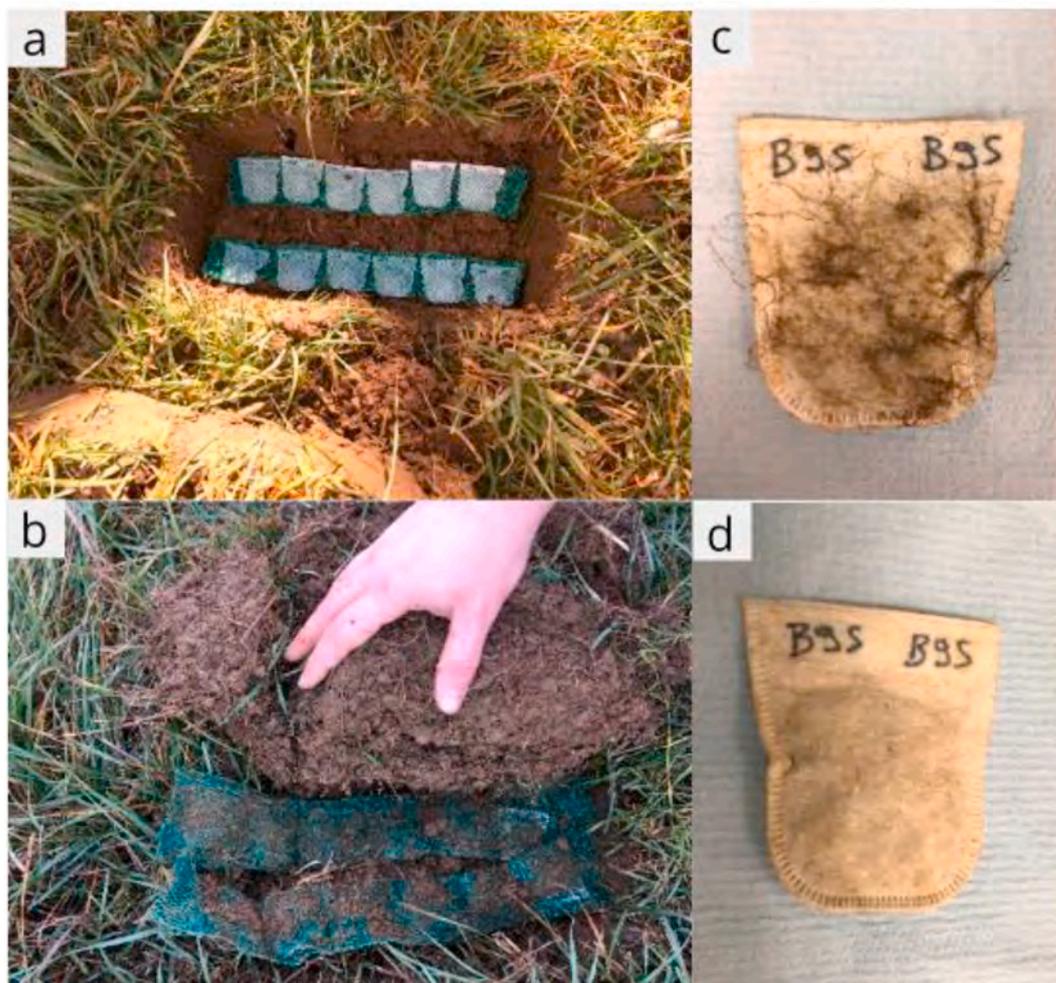


Fig. 2. a) ANKOM filter bags filled with granulated biogas residue (top row) and rooibos tea (bottom row), and placed inside nylon net pockets before the initiation of the incubation at the beginning of March 2018, b) bags retrieved at the end of November 2018, after nine months (270 days) of incubation in the grassland soil, c) filter bag covered with soil and plant roots after excavation and d) after removal of soil particles and roots.

prior to their exposure to soil, were analysed using standard methods and are detailed in Table 3. All aspects of initial material chemistry except lignin content were significantly different between the two material types. The fraction of GBR was selected due to a set of properties including high content in organic matter, inherent stability, and physical characteristics that allow the safe storage, handling, transportation and application, and consequently, its high marketing potential, as opposed to other biogas residue fractions and organic waste traditionally applied to agricultural land that are difficult to manage (Hansen and Henriksen, 1989). Regarding the comparator material selected, the fermentation process followed to obtain the commercially available product of RT allows a better side-by-side evaluation of these stabilized materials.

2.4. Total solids, volatile solids and mass loss determination

The gravimetric method was employed to assess the content in total solids (TS), volatile solids (VS) and the mass loss of GBR and RT from the bags during decomposition in the soil. The filled bags were weighed one by one to 0.001 g precision before and after field incubation (subtracting the weight of each empty bag) to calculate the mass difference ($n = 18$). A representative number of samples (one retrieved bag per plot per material) were oven-dried at 70 °C to a constant mass (Keuskamp et al., 2013) to allow the calculation of the total solids (dry matter, DM) without altering the organic matter, particularly the fibres (O'Kelly, 2014), and were then combusted in a muffle furnace at 550 °C for 3 h (loss-on-ignition, LOI) to allow the calculation of volatile solids (VS) (as an approximation of organic matter) and inorganic matter (ash) (Hoogsteen et al., 2015) contained in the samples after incubation. The remaining total solids, volatile solids and ash were calculated for each bag and the data were expressed as a percentage of the total solids, volatile solids and ash occurring in each material before exposure.

The residual relative total solids (R_{TS}) and volatile solids (R_{VS}) following field exposure were calculated according to Eq. (1) and Eq. (2), respectively:

$$\%R_{TS} = (TS_t / TS_0) \times 100 \quad (1)$$

where TS_0 is the oven-dried total solids mass of non-incubated samples, and TS_t is the oven-dried total solids mass of the incubated samples.

$$\%R_{VS} = (VS_t / VS_0) \times 100 \quad (2)$$

where VS_0 is the gasified volatile solids mass of non-incubated samples, and VS_t is the gasified volatile solids mass of the incubated samples.

2.5. Fibre composition

The cellulose, hemi-cellulose and lignin contents of the initial and decomposed granulated biogas residue and rooibos tea were determined

Table 3

Initial composition of the major components in granulated biogas residue and rooibos tea (mean \pm standard error, $n = 3$).

| Components | Granulated Biogas Residue | Rooibos Tea |
|----------------------|-------------------------------|-------------------------------|
| TS (% FW) | 92 \pm 0.00 ^a | 96 \pm 0.00 ^b |
| VS (% TS) | 66 \pm 0.00 ^a | 98 \pm 0.00 ^b |
| Ash (% TS) | 34 \pm 0.00 ^a | 2 \pm 0.00 ^b |
| Total C (% TS) | 32.8 \pm 0.6 ^a | 46.6 \pm 0.1 ^b |
| Total N (% TS) | 3.2 \pm 0.1 ^a | 0.8 \pm 0.1 ^b |
| C/N | 10.3 \pm 0.1 ^a | 55.8 \pm 4.1 ^b |
| Cellulose (% TS) | 9.44 \pm 0.97 ^a | 27.23 \pm 0.73 ^b |
| Hemicellulose (% TS) | 24.74 \pm 0.57 ^a | 11.73 \pm 0.13 ^b |
| Lignin (% TS) | 16.36 \pm 0.32 ^a | 17.17 \pm 0.40 ^a |
| Lignin/N | 5.13 \pm 0.24 ^a | 20.56 \pm 1.28 ^b |
| Lignin-N (% Lignin) | 2.57 \pm 0.10 ^a | 0.98 \pm 0.02 ^b |

FW: Fresh Weight; TS: Total Solids; VS: Volatile Solids.

Different lowercase letters indicate significant differences between the two materials ($p < 0.05$).

according to the analytical methods proposed by ANKOM Technology using the ANKOM A2000 Fiber Analyzer digestion apparatus. Three bags per plot for each material were randomly selected, the samples were ground in a mill on a 2 mm screen (FOSS, CT 293 CyclotecT), and sequential extractions were performed on 0.5 g of dried sample in ANKOM filter bags. Neutral detergent fibre (NDF), which is the residue containing predominantly hemicellulose, cellulose, and lignin, was obtained after digestion in a neutral detergent solution (ANKOM Technology, solution FND20) with heat-stable alpha-amylase (FAA, ANKOM Technology) and sodium sulphite (FSS, ANKOM Technology). Subsequently, the NDF fraction was digested in a sulphuric acid (1 N) and cetyl trimethylammonium bromide (CTAB, 20 g/l) solution, resulting in the acid detergent fibre fraction (ADF), which consists predominantly of cellulose and lignin. Finally, the ADF fraction was digested in a concentrated sulphuric acid solution (72% by weight) (ANKOM Technology - FSA72) providing the acid detergent lignin (ADL) fraction which consists mainly of lignin and other recalcitrant compounds. The analysis was performed in triplicates for each bag selected ($n = 9$). The absolute amounts of cellulose, hemi-cellulose and lignin before and after decomposition in the soil were calculated on a DM basis and were expressed as percentages of each initial fibre fraction contained in the GBR and RT before field incubation according to Eqs. (3) and (4):

$$\%FF = (W_3 - (W_1 \times C_1)) / (W_2 \times DM) \times 100 \quad (3)$$

where %FF is each relative extracted fibre fraction (NDF, ADF or ADL) used to calculate the content in hemi-cellulose, cellulose and lignin, W_1 is the weight of the empty filter bag to be filled with material, W_2 is the weight of the air-dried sample to be extracted, W_3 is the weight of the oven-dried filled bags after the extraction process, C_1 is the empty filter bag correction (final oven-dried weight/original blank bag weight) and DM is the dry matter correction. Hemi-cellulose was calculated by subtracting ADF from NDF. Cellulose was calculated by subtracting ADL from ADF while lignin corresponded to the remaining ADL fraction.

$$\%R_{FF} = (D_{FF} / I_{FF}) \times 100 \quad (4)$$

where % R_{FF} is each relative residual fibre fraction as a percentage of the fraction initially contained in the non-incubated material, D_{FF} is the remaining mass of each fibre fraction after decomposition, and I_{FF} is the initial mass of each fibre fraction contained in the material prior to decomposition.

It should be emphasized that the sulphuric acid method employed does not discriminate between lignin and other recalcitrant compounds (Berg and Ekbohm, 1991). Hence, the ADL measured post-incubation does not refer to pure lignin but rather to a mix of lignin and other lignin-like compounds, such as humification products, fungal chitin and possibly glomalalin – a glycoprotein produced abundantly on hyphae and spores of arbuscular mycorrhizal fungi (AMF) in soil and in roots – that might have been incorporated into the material during the decomposition process (Berg, 1988). These compounds are collectively known as acid insoluble substance (AIS) (Berg, 1988) and are believed to hamper the accurate measurement of residual lignin after decomposition since they are also recorded analytically as ADL. Therefore, for the sake of simplicity, hereafter we will refer to the ADL fraction contained in the materials prior to incubation as “lignin” while referring to the ADL fraction containing lignin and other lignin-like compounds post-incubation as “AIS”.

2.6. Total carbon and nitrogen

The total carbon and total nitrogen content of the initial and decomposed samples was determined by combustion at 650 °C using an elemental LECO TruSpec CHN-analyzer. For the incubated samples, one bag per plot for each material was randomly selected and the content was ground as described above for the fibre analysis. The total C and N analysis was performed in triplicates (ca. 100 mg of ground samples per

replicate). A multipoint standard calibration of the instrument was performed using set-up standards recommended by the manufacturer (LECO, USA) prior to the analysis of the granulated biogas residue and rooibos tea samples. The remaining absolute amounts of total carbon and nitrogen after decomposition were calculated on a DM basis and expressed as a percentage of the initial total carbon and nitrogen content of the unexposed material according to Eq. (5):

$$\%R_E = (D_E / I_E) \times 100 \quad (5)$$

where $\%R_E$ is the relative residual element (total carbon or total nitrogen) as a percentage of the element initially contained in the non-incubated material, D_E is the remaining mass of the element after decomposition in the soil, and I_E is the initial mass of the element contained in the unexposed material (Table 3).

2.7. Lignin and AIS-bound nitrogen

Following the sequential fibre extraction, nitrogen bound to lignin and AIS was also determined in an attempt to evidence the incorporation of exogenous and newly synthesized nitrogen-bearing lignin-like substances and humification products during the process of decomposition. Although the building blocks of pure lignin do not contain nitrogen, some other compounds in the acid-insoluble fraction may do (Berg and Theander, 1984). The analysis was performed in triplicates ($n = 3$) and the absolute amount of AIS-bound nitrogen (AIS-N) measured after decomposition was calculated on a DM basis and expressed as a percentage of the nitrogen contained in the lignin fraction of the unexposed material (lignin-bound nitrogen, LN) according to Eq. (6):

$$\%R_{AIS-N} = (D_{AIS-N} / I_{LN}) \times 100\% \quad (6)$$

where $\%R_{AIS-N}$ is the relative residual AIS-bound nitrogen as a percentage of the lignin-bound nitrogen contained in the unexposed material, D_{AIS-N} is the residual mass of the AIS-bound nitrogen after decomposition, and I_{LN} is the mass of nitrogen contained in the lignin fraction of the unexposed material.

It should be stressed that the calculation of net N release may lead to an underestimation of the total N loss (gross N release) during the decomposition process, as the incorporation of exogenous N (gross N immobilization) can counterbalance the total N released (Pei et al., 2019). Therefore, to obtain a more realistic estimate of the remaining total N in granulated biogas residue, and to reveal any potential N release from rooibos tea that may be masked by N immobilization, we calculated the incorporation of exogenous N in the form of AIS-nitrogen (as a partial measure of gross N immobilization) according to Eq. (7):

$$\%M_{TN} = ((D_{AIS-N} - I_{LN}) / I_{TN}) \times 100\% \quad (7)$$

where $\%M_{TN}$ is the relative released total N masked by immobilized AIS-nitrogen during decomposition, D_{AIS-N} is the residual mass of the AIS-bound N after decomposition, I_{LN} is the mass of N contained in the lignin fraction of the unexposed material, and I_{TN} is the initial total nitrogen contained in the unexposed material.

2.8. Statistical analyses

Independent samples *t*-test was used to determine the significance of the differences in the initial chemical characteristics between granulated biogas residue and rooibos tea. One-way analysis of variance (ANOVA) and Tukey's HSD post-hoc test were used to examine the effect of N fertilization treatments on the response variables of total and organic mass loss, fibre decomposition, total carbon and N, and AIS-N status. Pearson correlation analyses were performed to determine the relationship between the different response variables for each material separately, regardless of the fertilization treatment and site, with the response variables matched on a treatment level by averaging technical

and field replicates. The standard $\alpha = 0.05$ threshold was used as a cut-off for statistical significance. All statistical analyses and data visualization were performed with R software (Version 3.6.1).

For each site, the total solids and volatile solids are reported as means \pm standard error (se) from 18 field replicates (six bags per plot \times three plots per fertilization treatment); residual fibre fractions are reported as means \pm se of 9 field replicates (three bags per plot \times three plots per fertilization treatment), and total carbon, total nitrogen and AIS-bound nitrogen are presented as means \pm se of 3 field replicates (one bag per plot \times three plots per fertilization treatment), with three technical replicates for each field replicate.

3. Results

3.1. Residual total solids and volatile solids after soil exposure

The residual TS and VS of the GBR and RT after nine months of incubation in the soil are presented in Fig. 3. In general, the relative mass loss was similar for GBR and RT in the Faascht grassland while in the Bouzule grassland the loss was higher for RT. The residual TS of GBR fluctuated between 61 and 65%, while a wider range of residual TS was observed for RT, from 55 to 66%. The same pattern was observed for residual VS, with GBR retaining 62–67% of the initial VS, while a wider range was recorded for RT, from 54 to 66%. The mass lost from RT was almost exclusively in the form of VS as a result of its initial high content in VS and low ash content (98% VS and 2% ash). On the contrary, the mass lost from GBR was comprised of both VS and ash reflecting its initial composition (66% VS and 34% ash). When assessing the impact of fertilization, overall, the maximum residual mass post-soil-incubation was recorded in the control treatment plots and in plots under the application of raw digestate and raw manure for both materials at both sites. Conversely, the highest mass loss was observed under the application of chemical and combinatorial treatments and concerned mainly RT in both grasslands. For GBR, the strongest differences in mass loss were noted between plots treated with raw digestate (RD350) and ammonium nitrate (AN350), as well as with the combinatorial treatments ($p < 0.05$) at Faascht, and between raw digestate (RD230) and the combinatorial treatments (RD + AN230 and RD + UR230) ($p < 0.05$) at Bouzule. In the same vein, ammonium nitrate (AN350 and AN230) ($p < 0.05$) and the combinatorial treatments of raw digestate mixed with urea (RD + UR350 and RD + UR230) ($p < 0.001$) in both grasslands led to the lowest residual TS and VS in rooibos tea. Lastly, considering the significantly higher initial content of RT in organic matter (VS), the relative values calculated indicate higher absolute loss of VS for RT compared to GBR.

3.2. Remaining fibre fractions

The residual fibre fractions contained in GBR and RT following decomposition, as well as their response to the nitrogen fertilizer type used, are presented in Fig. 4. On the whole, over the incubation period in the soil, GBR maintained its fibres to a greater extent than RT and demonstrated mild fertilization-induced changes in both grasslands compared to RT. The fractions of cellulose and hemi-cellulose were partially decomposed in both GBR and RT. However, GBR had substantially higher residual fractions than RT, with the nitrogen fertilizer type exerting a less significant effect on GBR cellulose and hemi-cellulose decomposition than on RT. Regarding cellulose, 68–72% and 75–86% of the initial (9.44 ± 0.97 of DM) cellulose was still present in GBR after soil incubation at the Faascht and Bouzule sites, respectively. There were no differences among treatments at Faascht in terms of cellulose decomposition, however, at the Bouzule grassland, ammonium nitrate (AN230) led to the lowest residual cellulose in GBR and to significant differences with most of the treatments. In RT, the residual cellulose fluctuated from 46 to 70% of the initial content ($27.23 \pm 0.73\%$ of DM) at Faascht and from 45 to 64% at Bouzule, with the

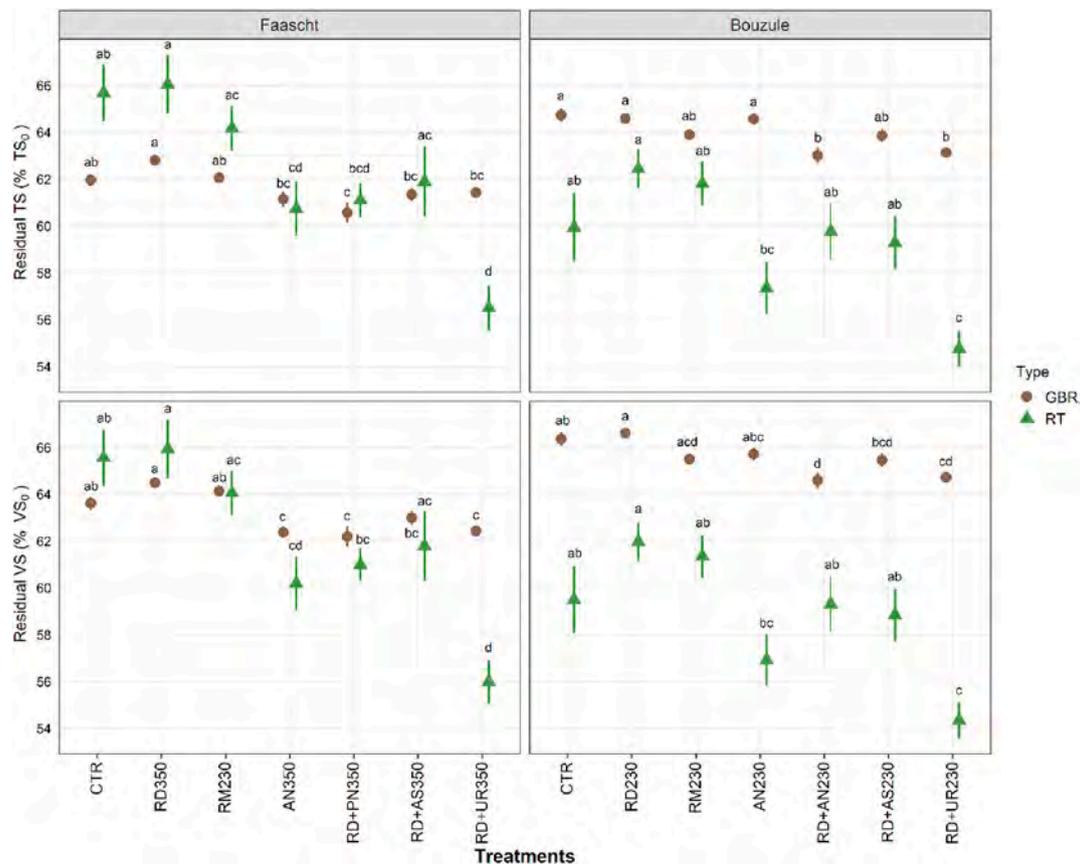


Fig. 3. Residual total solids (TS) and volatile solids (VS) in decomposed granulated biogas residue (GBR) and rooibos tea (RT) expressed on a dry matter basis as a percentages of the initial total solids (TS_0) and initial volatile solids (VS_0), respectively, in the two experimental grasslands (mean \pm standard error, $n = 18$). Different lowercase letters indicate a significant difference between the treatments ($p < 0.05$). CTR: Control; RD350 & RD230: Raw Biogas Residues; RM230: Raw Manure; AN350 & AN230: Ammonium Nitrate; RD + PN350: Raw Biogas Residues + Potassium Nitrate; RD + AN230: Raw Biogas Residues + Ammonium Nitrate; RD + AS350 & RD + AS230: Raw Biogas Residues + Ammonium Sulphate; RD + UR350 & RD + UR230: Raw Biogas Residues + Urea. The figure following the acronym of the fertilization treatment refers to the units of nitrogen applied per ha.

treatments of ammonium nitrate (AN350 and AN230) and raw digestate with urea (RD + UR350 and RD + UR230) resulting in the highest cellulose loss at both sites, especially in comparison with the unfertilized control ($p < 0.001$), raw digestate ($p < 0.001$) and raw manure ($p < 0.001$). The residual hemi-cellulose in GBR ranged from 53 to 57% of the initial content ($24.74 \pm 0.57\%$ of DM) at Faascht and from 56 to 62% at Bouzule, while in RT it ranged from 44 to 66% of the initial fraction ($11.73 \pm 0.13\%$ of DM) at Faascht and from 43 to 53% at Bouzule. The highest hemi-cellulose loss from GBR was observed at Bouzule under the treatments of ammonium nitrate (AN230) and raw digestate with urea (RD + UR230). Similarly, the lowest residual RT hemi-cellulose, at both sites, was measured under the application of ammonium nitrate (AN230 and AN350) and raw digestate combined with urea (RD + UR230 and RD + UR350) and differed statistically from most of the treatments. Keeping in mind the initial content of GBR and RT in cellulose and hemi-cellulose, these observations translate to a higher absolute loss of cellulose from RT but lower hemi-cellulose loss compared to GBR. Finally, the relative residual cellulose and hemi-cellulose showed significant positive correlation with the mass loss in both GBR ($r = 0.76$, $p < 0.05$ for cellulose and $r = 0.75$, $p < 0.05$ for hemi-cellulose) and RT ($r = 0.8$, $p < 0.01$ for cellulose and $r = 0.86$, $p < 0.01$ for hemi-cellulose), and were positively correlated with each other ($r = 0.71$, $p < 0.05$ for GBR, and $r = 0.69$, $p < 0.05$ for RT) (Table 4).

Unlike hemi-cellulose and cellulose, both the relative and absolute values of the AIS fraction (lignin and lignin-like compounds) in both the GBR and RT increased during the decomposition period. The relative AIS accumulation in GBR was higher compared to RT for all treatments and

in both grasslands. The increase in GBR, compared to the lignin contained in GBR before exposure ($16.36\% \pm 0.32$ of DM), ranged between 31 and 45% at Faascht and between 27 and 38% at Bouzule. In a similar manner, an increase of 22–31% at Faascht, and 13–20% at Bouzule was observed for RT, compared to the content of RT in lignin before exposure ($17.17\% \pm 0.40$ of DM). This increase appeared to be mostly fertilizer-independent, with no major differences among treatments. Nevertheless, given the similar initial content of both materials in lignin, these values indicate higher absolute gain in recalcitrant compounds (AIS) for GBR compared to RT.

3.3. Remaining total carbon and total nitrogen

The residual total carbon and total nitrogen contained in GBR and RT following decomposition, as well as their response to the nitrogen fertilizer type used, are visualized in Figs. 5 and 6, respectively. In general, the residual carbon in GBR ranged from approximately 67 to 69% of the initial carbon content ($32.81\% \pm 0.63$ of DM) in both grasslands, while in RT it fluctuated from 58 to 70% of the initial content ($46.59\% \pm 0.13$ of DM) in Faascht and from 55 to 66% in Bouzule. Regarding the effect of N fertilizers on the carbon content of GBR, there were no statistical differences among the treatments tested at Faascht, however, at the Bouzule grassland, the fertilizer treatments of ammonium nitrate (AN230) and raw digestate with urea (RD + UR230) led to the lowest remaining C showing significant differences with raw digestate RD230 ($p < 0.05$), which resulted in the highest remaining C among all treatments. The carbon content of the decomposed RT varied to a greater

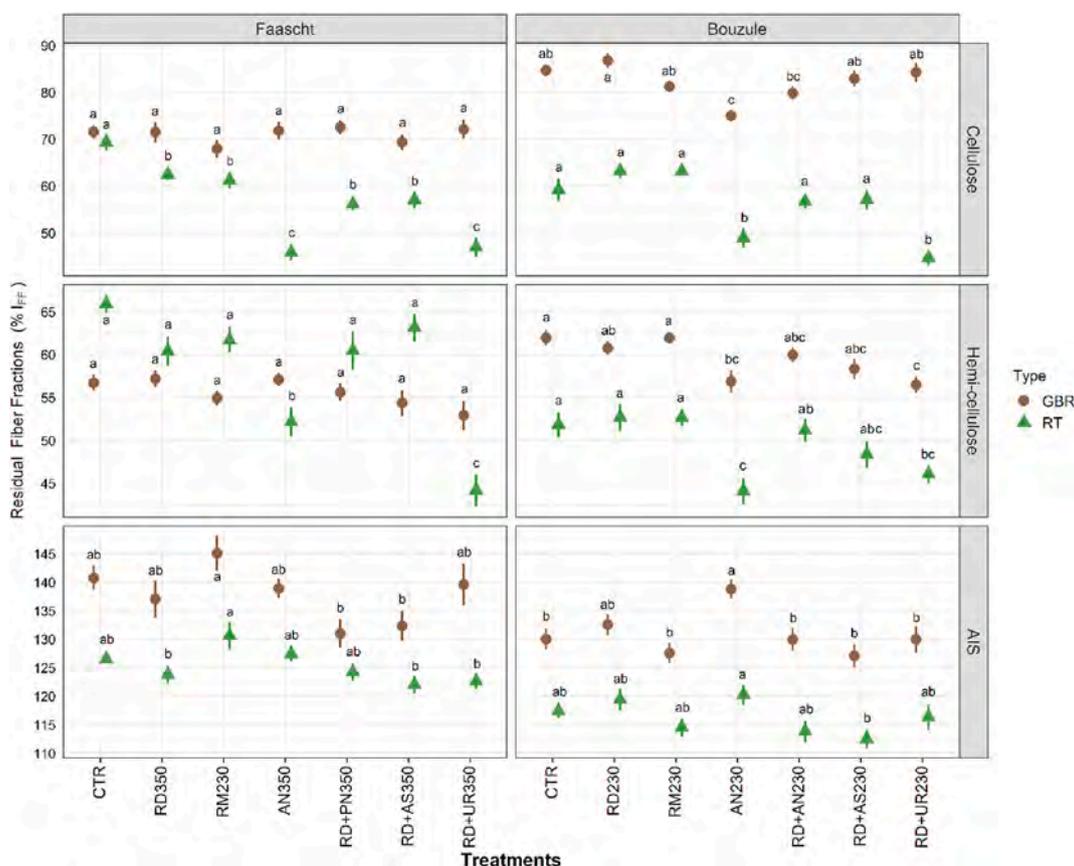


Fig. 4. Residual cellulose, hemi-cellulose and acid-insoluble substance (AIS) in decomposed granulated biogas residue (GBR) and rooibos tea (RT) expressed as a percentage of each initial fibre fraction (I_{FF}) (mean ± standard error, n = 9). Different lowercase letters indicate a significant difference between the treatments (p < 0.05). CTR: Control; RD350 & RD230: Raw Biogas Residues; RM230: Raw Manure; AN350 & AN230: Ammonium Nitrate; RD + PN350: Raw Biogas Residues + Potassium Nitrate; RD + AN230: Raw Biogas Residues + Ammonium Nitrate; RD + AS350 & RD + AS230: Raw Biogas Residues + Ammonium Sulphate; RD + UR350 & RD + UR230: Raw Biogas Residues + Urea. The figure following the acronym of the fertilization treatment refers to the units of nitrogen applied per ha.

Table 4

Pearson’s correlation coefficients (r, n = 14) among the percentage of residual total solids, volatile solids, cellulose, hemi-cellulose, acid-insoluble substance (AIS) and total carbon for granulated biogas residue (GBR) and rooibos tea (RT), separately.

| Material | Percentage of residual component | Total solids | Volatile solids | Cellulose | Hemi-cellulose | AIS |
|----------|----------------------------------|----------------|-----------------|---------------|----------------|------|
| GBR | Volatile solids | 0.99*** | 0.76* | | | |
| | Cellulose | 0.75* | 0.75* | 0.71* | | |
| | Hemi-cellulose | | | | | |
| | AIS | -0.4 | -0.39 | -0.75* | -0.5 | |
| | Total Carbon | 0.52 | 0.53 | 0.38 | 0.57 | 0.04 |
| RT | Volatile solids | 0.99*** | | | | |
| | Cellulose | 0.82** | 0.8** | | | |
| | Hemi-cellulose | 0.85** | 0.86** | 0.69* | | |
| | AIS | 0.47 | 0.51 | 0.07 | 0.57 | |
| | Total Carbon | 0.87*** | 0.86** | 0.72* | 0.66 | 0.22 |

extent and was significantly affected by the N fertilizer applied in both grasslands. More precisely, the ammonium nitrate treatment and the combinatorial treatment of raw digestate mixed with urea, at both sites, led to significantly lower residual C in the decomposed RT compared to raw digestate (RD350) (p < 0.001) and raw manure (RM230) (p < 0.01

and p < 0.001, respectively). Due to the higher initial content of RT in carbon compared to GBR, the higher relative loss measured post-decomposition also indicates a higher absolute loss. Lastly, residual C in RT was positively correlated with residual volatile solids (r = 0.86, p < 0.01) and residual cellulose (r = 0.72, p < 0.05), while residual C in GBR did not demonstrate any strong correlation (Table 4).

After nine months of decomposition, the nitrogen content of GBR and RT was found to be contrasting. While part of the total nitrogen was released from GBR during the incubation period, the relative residual nitrogen in RT indicated accumulation or conservation under all N fertilizers tested and at both sites. About 40–44% of the initial total nitrogen contained in GBR (3.19 ± 0.09 of DM) was released during incubation, in both grasslands, while RT accumulated nitrogen, increasing its initial content (0.84 ± 0.06 of DM) by 2.5–16% at Faascht and by 3.0–19% at Bouzule. Regarding the fertilizer impact on N release from GBR and N accumulation in RT, ammonium nitrate was the treatment that consistently led to the highest relative residual nitrogen in both processes. For GBR, in both grasslands, ammonium nitrate (AN350 and AN230) and raw manure (RM230) led to significantly higher relative residual N, mainly compared to the combinatorial treatments tested. For RT, ammonium nitrate led to statistically higher N accumulation (p < 0.01) compared to the control treatment at Faascht and compared to most of the treatments at Bouzule. After calculating the immobilized N as AIS-N, the gross N release from GBR was estimated to be higher than the net nitrogen release by approximately 3–5% while the gross N immobilization in RT was estimated to have masked 12–22% of the inherent nitrogen release. The relative residual total N was positively correlated with the relative accumulated AIS in GBR (r = 0.68, p

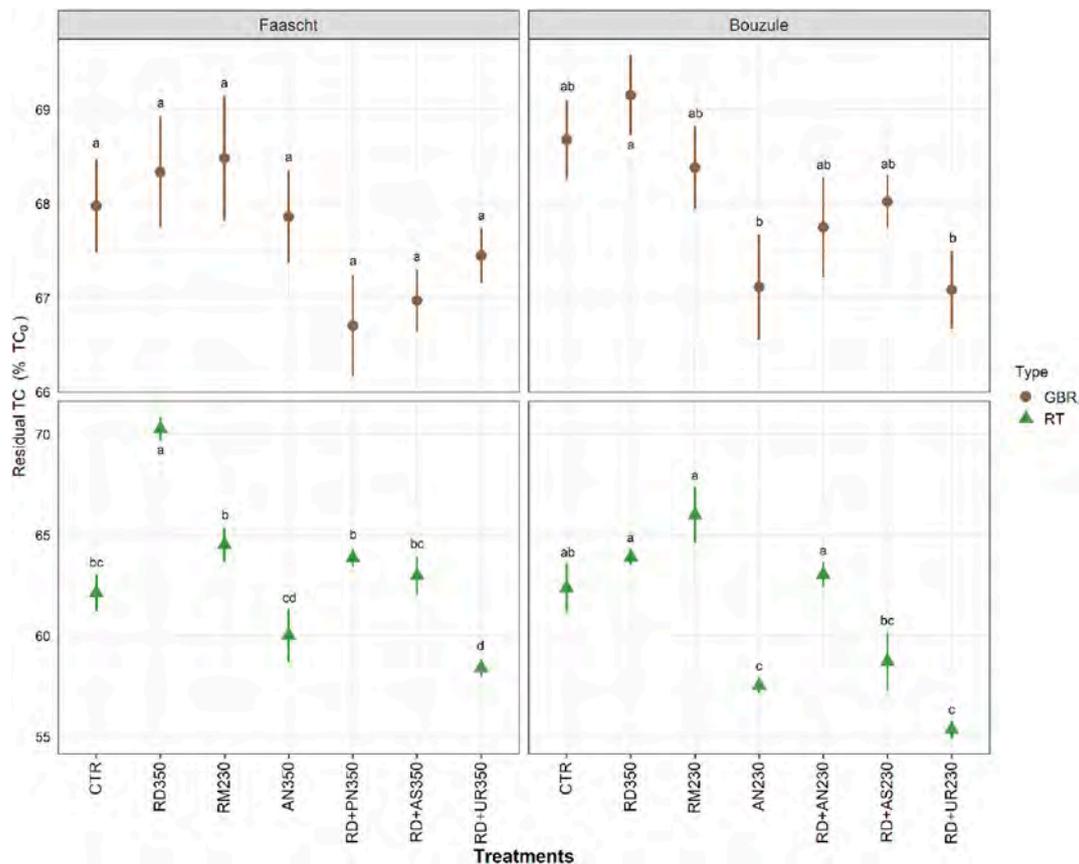


Fig. 5. Residual total carbon (TC) in decomposed granulated biogas residue (GBR) and rooibos tea (RT) expressed as a percentage of the initial total carbon content of the unexposed material (TC_0) (mean \pm standard error, $n = 3$). Different lowercase letters indicate a significant difference between the treatments ($p < 0.05$). CTR: Control; RD350 & RD230: Raw Biogas Residues; RM230: Raw Manure; AN350 & AN230: Ammonium Nitrate; RD + PN350: Raw Biogas Residues + Potassium Nitrate; RD + AN230: Raw Biogas Residues + Ammonium Nitrate; RD + AS350 & RD + AS230: Raw Biogas Residues + Ammonium Sulphate; RD + UR350 & RD + UR230: Raw Biogas Residues + Urea. The figure following the acronym of the fertilization treatment refers to the units of nitrogen applied per ha.

< 0.05) while no correlation was observed between the two in RT (Table 5).

Finally, following the C and N release at a rate of 8:1 from GBR during the decomposition process, the initial C to N ratio of the material increased from 10.3 to 11.9 at Faascht and to 12.4 at Bouzule. In the case of RT, the release of C and accumulation of N shifted the C to N ratio from 56 down to 32 at both sites.

3.4. Lignin and AIS-bound nitrogen status

Overall, the initial content of GBR and RT in lignin-bound nitrogen increased through the gain of nitrogen bearing lignin-like compounds during the process of decomposition at both sites. The relative increase was higher for RT than GBR at both sites and under all treatments tested. More precisely, the initial content of GBR in lignin-bound nitrogen (2.57 ± 0.10 of lignin) increased by 29–40% at Faascht and 26–37% at Bouzule. The lignin-bound nitrogen of RT increased by 68–110% in Faascht and 61–107% in Bouzule compared to its initial content (0.98 ± 0.02 of lignin) Fig. 7. The increase in GBR appeared to be mostly fertilizer-independent with the control treatment leading to a similar gain to most of the fertilizers tested. On the contrary, the increase of lignin-bound nitrogen in RT was greatly affected by the fertilizers applied. At both sites, all fertilization treatments led to significantly higher gain compared to the control ($p = 0.05$ to $p < 0.001$) with AN230 exerting the highest impact.

As previously mentioned, based on the incorporation of exogenous N in the form of AIS-nitrogen into the material during incubation in the soil, it was evident that the immobilized N has the potential to mask part

of the inherent N released, therefore indicating a simultaneous release and immobilization of N during the process of decomposition. The residual AIS-bound nitrogen in GBR was positively correlated with both the remaining AIS fraction ($r = 0.85$, $p < 0.001$) and total nitrogen ($r = 0.85$, $p < 0.001$) while in RT it was correlated only with the remaining total nitrogen ($r = 0.78$, $p < 0.01$) (Table 5).

4. Discussion

4.1. Decomposition of granulated biogas residue and nutrient release

Organic matter decomposition is one of the key ecological processes in ecosystems and is crucial for the supply of nutrients to a large suite of soil organisms and plants, as well as for the rate-of-rise of GHGs in the atmosphere. Traditionally, decomposition is linked to the quality of the organic material. In litter decomposition studies, quality indexes such as nitrogen and lignin content, C:N and lignin:N ratios have been associated with degradability (Cotrufo et al., 2010) and used to predict decay dynamics (Knorr et al., 2005). However, translating these relationships into a quantitative assessment of decomposition has not been an easy task (Heal et al., 1997). In contrast to the reference material of rooibos tea used in this study, and most of the litter materials in nature, the material of granulated biogas residue had a high content of inorganic matter that was released in parallel to organic soluble substances and labile compounds during the process of decomposition. As demonstrated by Adam et al. (2018), the solid fraction of biogas residues, besides its high content in organic carbon and nitrogen, is particularly rich in macronutrients such as phosphorus and potassium, at the same time

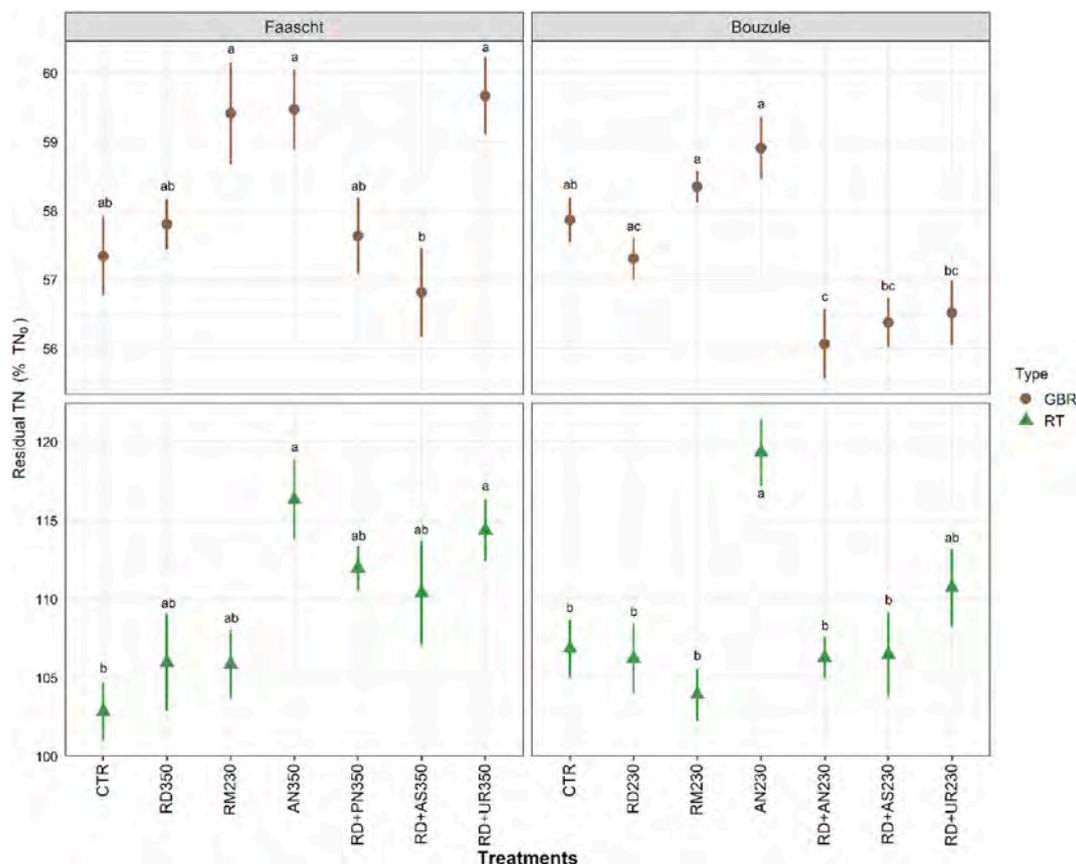


Fig. 6. Residual total nitrogen (TN) in decomposed granulated biogas residue (GBR) and rooibos tea (RT) expressed as a percentage of the initial total nitrogen content of the unexposed material (TN_0) (mean \pm standard error, $n = 3$). Different lowercase letters indicate a significant difference between the treatments ($p < 0.05$). CTR: Control; RD350 & RD230: Raw Biogas Residues; RM230: Raw Manure; AN350 & AN230: Ammonium Nitrate; RD + PN350: Raw Biogas Residues + Potassium Nitrate; RD + AN230: Raw Biogas Residues + Ammonium Nitrate; RD + AS350 & RD + AS230: Raw Biogas Residues + Ammonium Sulphate; RD + UR350 & RD + UR230: Raw Biogas Residues + Urea. The figure following the acronym of the fertilization treatment refers to the units of nitrogen applied per ha.

Table 5

Pearson’s correlation coefficients (r , $n = 14$) among the percentages of residual acid-insoluble substance (AIS), total nitrogen and AIS-bound nitrogen for granulated biogas residue (GBR) and rooibos tea (RT), separately.

| Material | Percentage of residual component | AIS | Total Nitrogen |
|----------|----------------------------------|----------------|----------------|
| GBR | Total Nitrogen | 0.68* | |
| | AIS - Nitrogen | 0.85*** | 0.85*** |
| RT | Total Nitrogen | 0.19 | |
| | AIS - Nitrogen | 0.28 | 0.78** |

retaining a large fraction of the inorganic matter (ash) contained in untreated biogas residues. In this *in sacco* field trial, about one third of the GBR total solids lost corresponded to inorganic matter, while almost 100% of the RT total solids lost corresponded to organic matter. This could potentially be an interesting characteristic of GBR to delay organic matter loss while still providing readily available nutrients to the soil microflora and vegetation. Support to the semantic role of inorganic matter in GBR could also be the lack of correlation between the relative residual total carbon and remaining mass, particularly when contrasted against RT and the significantly positive correlation observed among the same variables. In addition, other studies have demonstrated that an adequate supply of inorganic nutrients can significantly increase the humification efficiency and C sequestration in the soil (Kirkby et al., 2016). As observed here, GBR preserved over 62% of its initial total solids and over 64% of its initial organic matter, and in most cases exceeded by far the organic matter remaining in the comparator material (Fig. 3).

Regarding carbon and nitrogen, more than 66% of the initial total carbon and over 56% of the initial total nitrogen were still present in the GBR post-incubation, with the C:N ratio of the material increasing from 10 to about 12 at the two experimental sites, indicating the mineralization of N and its outflow from the bags. In the case of RT however, the residual carbon decreased to as low as 55% of its initial carbon content under the fertilization treatment of raw digestate combined with urea (Fig. 5). On the contrary, nitrogen in the RT increased both in terms of percentage and absolute value, in some cases by 10–20% of its initial nitrogen content (Fig. 6), shifting the C:N ratio of the material from 56 down to 33 for both sites and indicating the immobilization of N during organic material decomposition (Fioretto et al., 2005). This contrasting behaviour is possibly attributed to the initial nitrogen content of the two materials tested. As observed repeatedly by Berg and other researchers, the initial content of a material in nitrogen may dictate whether there will be an accumulation or release of nitrogen (Berg and Staaf, 1981; Parton et al., 2007). A somewhat uncertain threshold of 1.4% initial nitrogen concentration has been proposed based on a multitude of litter decomposition studies in which no nitrogen accumulation was observed above this concentration (Berg and Staaf, 1981). This suggestion appears to be valid for both materials tested in this experiment. Even though the amount of total C and N released is expressed as a net release, in fact, it is an underestimation of the total release (gross release), because the total loss can be counterbalanced by the immobilization of exogenous C and N (and possibly of inherent C and N) in newly formed stable complexes and by the recalcitrant structural carbon and nitrogen contained in the microbes that might have colonized the content of the bags. Such observations have been made by many researchers, mainly

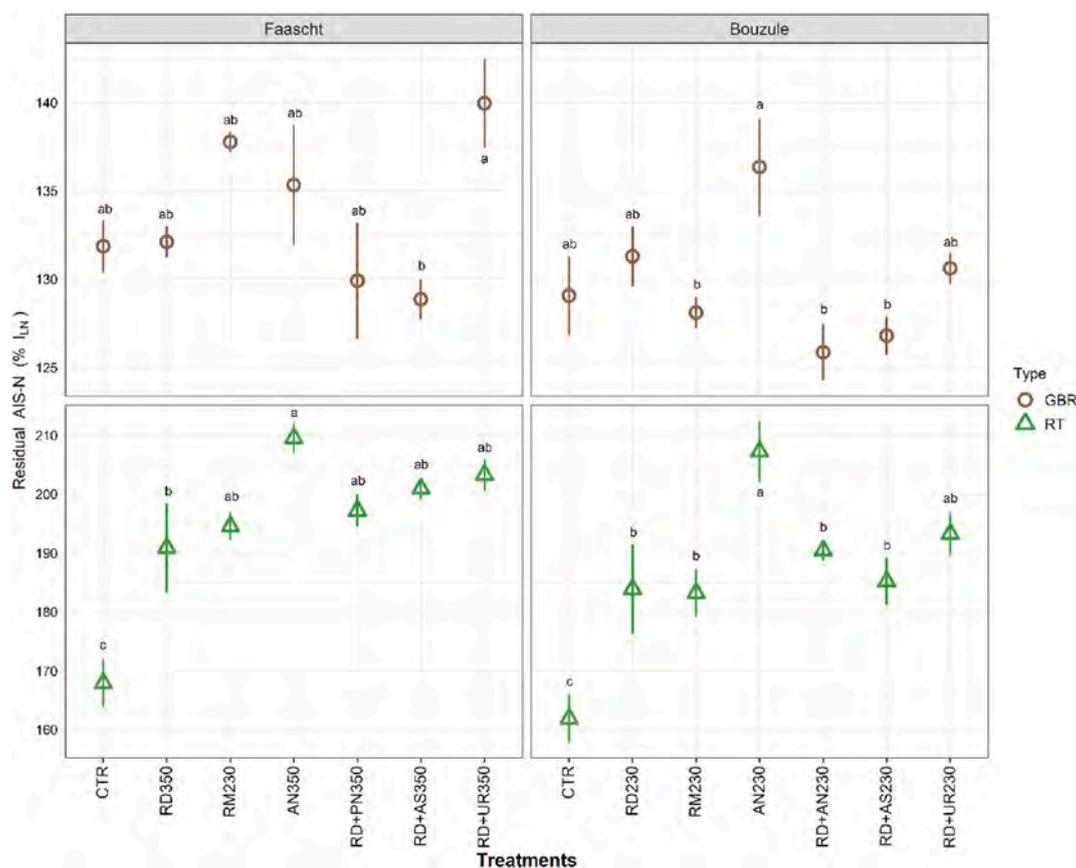


Fig. 7. Residual AIS-bound nitrogen (AIS-N) of the decomposed granulated biogas residue (GBR) and rooibos tea (RT) expressed as a percentage of the lignin-bound nitrogen contained in the unexposed material (L_N) (mean \pm standard error, $n = 3$). Different lowercase letters indicate a significant difference between treatments ($p < 0.05$). CTR: Control; RD350 & RD230: Raw Biogas Residues; RM230: Raw Manure; AN350 & AN230: Ammonium Nitrate; RD + PN350: Raw Biogas Residues + Potassium Nitrate; RD + AN230: Raw Biogas Residues + Ammonium Nitrate; RD + AS350 & RD + AS230: Raw Biogas Residues + Ammonium Sulphate; RD + UR350 & RD + UR230: Raw Biogas Residues + Urea. The figure following the acronym of the fertilization treatment refers to the units of nitrogen applied per ha.

regarding the release of N from decomposing litter (Pei et al., 2019; Zeller et al., 2000) and suggest the existence of the simultaneous release and uptake of nitrogen during the process of decomposition (Berg, 1988). This was also the case in this experiment, mainly for RT, as the immobilized nitrogen acquired through acid-insoluble substance (AIS) masked 12–22% of the inherent N released during decomposition. Based on these observations, nitrogen release from GBR since the early stages of decomposition could overcome the nitrogen immobilization problem faced soon after the incorporation of green waste into agricultural soils, which leads to an inadequate supply of nitrogen to plants. Consequently, GBR could be considered a source of nutrients mineralized over time for a large suite of soil organisms and plants, with a negligible leaching and emission potential (Tsachidou et al., 2019a, 2019b).

Material quality and consequently decomposition also depend on fibre composition and the abundance of polymers such as cellulose, hemicellulose and lignin. Lignin is known to physically protect cellulose and hemicellulose from enzymatic attack regulating the decomposition of these polymeric carbohydrates (Cooke and Whipps, 1993). High contents of lignin have a rate-reducing influence on litter decomposition, particularly when associated with high N content, as this may lead to newly formulated stable complexes (Berg and Ekbohm, 1991; Cai et al., 2021). For both materials studied, but to a greater extent for GBR, this association seems to be reflected in the increase of their acid-insoluble substance during soil incubation. The initial lignin content of GBR increased at both sites by 27–45% during soil exposure while in RT, the increase observed was in the range of 12–30%. As opposed to the increase of lignin in percentage but also in absolute value, cellulose

and hemicellulose values decreased throughout the incubation, attesting to their higher degree of degradability. These polymers can constitute not only a source of energy for soil microorganisms but also a source of carbon dioxide and methane released during their decomposition under aerobic and anaerobic conditions, respectively. This experiment witnessed the robust response of cellulose and hemicellulose in GBR to decomposition under the application of different N fertilizers, with 67–86% of the initial cellulose and 53–62% of the initial hemicellulose remaining in the GBR post-incubation. Rooibos tea though was affected to a greater extent by the fertilization treatment, with the residual cellulose and hemicellulose dropping to as low as 45% and 43% of the initial content, respectively. At this point, with respect to the protective effect of lignin on cellulose and hemicellulose degradation, it should be underlined that both materials initially had the same lignin concentration. In the case of GBR though, the intermeshed polymers displayed the expected protective mechanism by preserving, to a great extent, the least abundant polymer of cellulose (only 9.5% compared to 24.7% hemicellulose and 16.4% lignin). This protective property could possibly be attributed to the compact form of the GBR, and resembles the desired controlled-release behaviour of the environmentally friendly fertilizers developed with the use of natural materials such as chitosan, cellulose and lignin, to maximize fertilizer-use efficiency (Chen et al., 2018). These findings further advocate for the stable nature of GBR and underline their suitability as slow release biofertilizers.

Finally, owing to their organically bound nutrients, compact form and inherent stability, we posit that GBR could be used as a base dressing on bare soils, as well as a top dressing on developing crops as they do not

pose a high risk for gaseous emissions. Moreover, since they do not contain an instantly available source of nutrients, they could best be utilized as a complementary fertilizer in a combinatorial scheme, mainly targeting the long-term supply of nutrients to agricultural lands with a constant nutrient demand, such as grasslands (De Boer, 2017). Additionally, given the low nitrate leaching potential of GBR (Tsachidou et al., 2019a,b; 2019a) due to the organically bound nitrogen, GBR could best be utilized in Nitrate Vulnerable Zones. The main concern regarding nutrient loss from GBR is associated with ammonia volatilization during the thermal drying of biogas residues to obtain GBR, mainly if the air washer system is underperforming.

4.2. Carbon sequestration potential of granulated biogas residue

Soil organic matter is the largest terrestrial nutrient and carbon reservoir, and is critical for ecosystem sustainability (Frey, 2019; Schlesinger and Bernhardt, 2013). The size of the carbon pool depends on the balance between the formation of soil organic matter from decomposition by soil organisms and its mineralization to inorganic carbon (Cotrufo et al., 2015). One of the challenges concerning C sequestration is its residence time in the soil. To increase its mean residence time and slow the rate of its release back into the atmosphere through decomposition and mineralization, certain aspects should be considered, such as the decomposability, chemical composition, physical structure for better protection and the soil depth of the deposition. The results obtained from this investigation evince the suitability of GBR to efficiently address all the points above. GBR characteristics such as its shape, compactness, inherent stability and composition are considered to have contributed towards the observed slow decomposition of carbon polymers such as cellulose and hemi-cellulose, and the high percentage of remaining carbon in the matrix. We postulate that the spherical shape and compactness of GBR create a physical barrier leading to the protection of organic matter and resistance to mineralization, similar to soil aggregates enclosing organic matter (ADEME, 2015). The potential protective effect could be supported if we notice the intact granules retrieved post-incubation (Fig. 8b) and the significantly higher decomposition recorded for the finely shredded comparator material of rooibos tea. This can also be based on findings that relate litter decay to leaf toughness, width and specific leaf area in litter decomposition studies (Gallardo and Merino, 1993; Gillon et al., 1994). Finally, these characteristics resemble the properties of the recalcitrant biochar substance which is also promoted as a climate change mitigation tool when utilized as a soil amendment (Case et al., 2014; Majumder et al., 2019; Ramlow and Cotrufo, 2018).

Another important aspect of carbon sequestration is recalcitrance. Refractory structural compounds, such as lignin, are known to be the primary building blocks of stable organic matter, mainly due to their insolubility and low degradability. Lignin protects the easily accessible carbon sources such as cellulose and hemicellulose, and prolongs the presence of carbon in the soil. Hence, digestion of feedstock rich in

lignin, such as crop residues and energy crops, could produce GBR with high recalcitrant carbon longevity. However, the transformation of existing compounds into novel recalcitrant compounds is the main mechanism that creates stable organic matter rather than the conservation of resistant compounds (Prescott, 2010). The results obtained from the current experiment regarding lignin decomposition appear to be in line with the aforementioned mechanism. After nine months of incubation in the soil, the lignin fraction had increased in the form of AIS in both GBR and RT by up to 45% and 30%, respectively. Analysis of the lignin- and AIS-bound nitrogen also revealed the increase of lignin-associated N by 25–40% in GBR and by 60–110% in rooibos tea. This was interpreted to mean that some of the inherent nitrogen and nitrogen from an external source was incorporated into lignin-like compounds, which were analytically recorded as AIS (Berg, 1988). Besides the formation of lignin-like substances during decomposition, we posit that recalcitrant C and N were carried into the bags via the resilient compounds of chitin and glomalin originating from fungi (Frey et al., 2000), such as arbuscular mycorrhizal fungi (AMF), which might have invaded the bags to absorb nutrients, particularly phosphorus, from the GBR. Indeed, on a side note, ongoing work on fungi identification in the decomposed samples revealed the copious colonization of GBR by fungi, but not of rooibos tea (Fig. 8b and d). This information may indicate that the AIS and N associated with it emanate from different recalcitrant N-bearing compounds at a different ratio in the two materials. Therefore, even though it is assumed that stable organic matter is formed primarily from recalcitrant compounds such as lignin, this could underline the importance of stabilized soil organic matter formation from labile components, mainly in the form of microbial products and biomass (Cotrufo et al., 2015).

4.3. Nitrogen fertilization impact on decomposition and C sequestration

The intensive use of N fertilizers in modern agriculture is motivated by the economic benefits of high yields and has generally been perceived to sequester soil organic carbon (Khan et al., 2007). However, over the last decade there has been an ongoing controversy regarding the extent to which nitrogen fertilization can increase carbon sequestration in agricultural ecosystems (Ehtesham and Bengtson, 2017). The long-term fertilization of grassland soils with chemical fertilizers is a common management practice that aims to produce plant biomass (Cenini et al., 2015) and may influence nutrient cycling, C sequestration and GHG emissions (Smith et al., 2013). Keeping in mind that grasslands have the potential to store more C than arable land (Conant et al., 2001), there is an urgent need to understand how chronic nitrogen fertilization may affect organic matter decomposition and the ability of grassland soils to sequester C. Long-term field studies support the view that the optimum application of N fertilizers can build up organic matter in the soil, as opposed to high N application rates that lead to mineralization and the loss of soil organic matter (Green et al., 2006; Liang et al., 2019; Singh, 2018). Besides the rate of N fertilizer, the source of N is also considered



Fig. 8. a) A single biogas residue granule before incubation, b) after nine months (270 days) of incubation in the soil, c) rooibos tea before incubation and d) after nine months of incubation in the soil. The pictures of the decomposed materials were taken within 24 h of the retrieval from the soil. The size of the granules and rooibos tea fannings are displayed in mm.

an important parameter and should be investigated regarding its impact on organic matter decomposition and C sequestration in grassland soils (Fornara et al., 2013).

In the present decomposition experiment, GBR and RT responded differently to the type of N fertilizers tested in the two grasslands. Overall, the total mass and organic matter of GBR was affected to a lesser extent by N fertilizer type compared to RT. After nine months of incubation in the soil of the Faascht grassland (pH = 6.3), GBR and RT had similar relative remaining mass and organic matter, with RT demonstrating higher relative residual values than GBR under no fertilization and organic fertilization, as opposed to chemical and combinatorial treatments. On the contrary, in the slightly alkaline and calcareous soil (pH = 7.6) of Bouzule grassland, the relative remaining mass and organic matter were significantly higher for the GBR regardless of the fertilization treatment compared to RT, which underwent the highest decomposition under the chemical and combinatorial treatments, in agreement with the observation made in the Faascht soil. In accordance with other studies, the long-term application of chemical fertilizers may have led to a significant increase of the soil microbial biomass (Geisseler and Scow, 2014), which in turn could imply an increase in microbial decomposer activity (Singh, 2018). Despite the great differences observed under the prism of fertilizer type, based on the varying decomposition of both materials under no fertilization in the two different grasslands, it is likely that other factors such as soil specifics may have influenced the decomposition process. The initially different soil pH and content of carbonate at the two grasslands may be partly responsible for the differences observed between the two sites for the same material. As a pH above 7 is known to increase microbial biomass and activity, and a pH below 5 has the opposite effect (Singh, 2018), further acidification of the less alkaline Faascht grassland soil due to high N fertilization rates could also pose an indirect influence on decomposition. However, this appears to have affected only the material of rooibos tea, reflecting the lower and higher decomposition expected in the soil of the Faascht and Bouzule sites, respectively. The opposite was observed for GBR with higher mass and organic matter remaining in the alkaline soil, regardless of the fertilizer type. This could be connected to increasing fungal abundance in soils with a lower pH (Aciego Pietri and Brookes, 2009) and their stronger preference for phosphorus-rich sources such as GBR. Hence, these observations suggest the existence of complex interactions between fertilizer type, rate and soil bio-physico-chemical properties. Nevertheless, the influence of fertilizer type on total mass and organic matter loss is evident in both grassland soils and for both materials, with RT clearly displaying the severe impact of chemical fertilizers such as ammonium nitrate and urea.

Nitrogen fertilization, particularly the long-term addition of N may effectuate changes on the decomposition of polymer carbohydrates such as cellulose, hemi-cellulose and lignin by altering the activity of cellulose- and lignin-degrading enzymes (Cenini et al., 2015), and facilitating the formation of more recalcitrant lignin-like compounds (Prescott, 2010). Overall, after nine months of decomposition, we observed the retardation of lignin decomposition and the accumulation of recalcitrant lignin-like compounds, in both materials and grasslands but without any major differences between the treatments that could be attributed to the addition of N or the type of N fertilizer (Sjöberg et al., 2004). The relative and absolute value of AIS was higher for GBR than RT, given the fact that their initial lignin concentration was not significantly different. This could be explained partially by GBR being a source rich in nutrients that attracts soil microorganisms, mainly P-seeking fungi, which form various acid-insoluble lignin-like substances. AIS accumulation appears to be more composition and site-specific, rather than fertilizer type and rate-dependant. On the contrary, the cellulose and hemi-cellulose polymers were partially degraded after nine months of incubation in the soil. In general, GBR cellulose and hemi-cellulose were more resistant to the N fertilizer type, compared to RT. Fertilizer type, and more specifically ammonium nitrate and the combinatorial treatment of raw

biogas residue mixed with urea strongly promoted both cellulose and hemi-cellulose decomposition in RT, and in both grasslands, particularly when compared to the unfertilized and organically fertilized RT. A similar observation was made for GBR with the same treatments promoting hemi-cellulose decomposition and ammonium nitrate enhancing cellulose decomposition in the Bouzule grassland. This outcome supports evidence from other grassland studies advocating for the stimulation of cellulose degrading enzymes by the addition of N (Ajwa et al., 1999; Fog, 1988; Keeler et al., 2009) and underlines the importance of the N fertilizer type. The lack of major differences between the N fertilization treatments regarding the decomposition of GBR cellulose and hemi-cellulose may be partially assigned to the importance of the shape and structure of the granules that restrict accessibility to decay factors and slow down the rate of decomposition. Interestingly, in contrast to the accelerating effect of chemical fertilizers on carbohydrate polymer decomposition, the organic fertilizers tested (raw biogas residue fraction and raw manure) resembled the influence exerted by the unfertilized control plots, leading to the highest residual cellulose and hemi-cellulose observed in both materials and both grasslands.

Finally, a pronounced accelerating effect of ammonium nitrate and the fertilization mix containing urea, and a decelerating effect of the organic fertilizers on the overall C release from the decaying organic matter was observed for both GBR and RT at both sites. In line with our findings, other fertilization experiments with urea and ammonium-based fertilizers denoted a positive priming effect leading to the enhanced decomposition of plant materials and soil organic carbon mineralization (Hamer et al., 2009; Green et al., 2006; Soponsathien, 1998). Concurrently, incubation studies of soils amended with organic matter revealed a negative priming effect explained by the preferential substrate utilization of added organic matter over soil organic matter (Guenet et al., 2010). We ascribe the different impact of organic and chemical fertilizers on decomposition and carbon mineralization mainly to the shift in soil microbial communities and functions caused by the long-term application of these fertilizers (Guo et al., 2019; Leff et al., 2015). Inter alia, we consider the inconsistent carbon and nutrient input to be another important factor shaping the differences observed between organic and chemical fertilizers (Blagodatskaya and Kuzyakov, 2008). Although the main perception is that the abundant carbon source provided by organic fertilizers enhances microbial activity and accelerates the mineralization of carbon (Li et al., 2018; Ribeiro et al., 2010), we posit that it leads to the build-up of newly formed stable organic carbon, offsetting the mineralization of pre-existing organic matter, whereas the application of chemical fertilizers promotes the decomposition of pre-existing organic matter as it is the sole source of organic carbon and nutrients, thus further depleting the soil carbon pools (Blagodatskaya and Kuzyakov, 2008; Fontaine et al., 2011; Kirkby et al., 2014). Overall, our results showcase that nitrogen fertilization is among the management practices that can have a strong influence on the fate of organic carbon in grassland soils. There is a need to determine how N fertilization could be most effective for maintaining or sequestering C and which type of N fertilizer has the greatest capacity to promote it. Hence, since N fertilization is practiced widely across the globe, it is wise to assess the impact of fertilization in agriculture by comparing different types of N fertilizers to each other rather than comparing them to an unfertilized control (Khan et al., 2007).

4.4. Method limitations and future work

Even though the *in sacco* technique is a reliable method for studying the decomposition of a multitude of materials in many biological systems, such as terrestrial ecosystems, it comes with several trade-offs. The mesh size of the bag is a crucial parameter that determines the accessibility and exclusion of organisms based on their dimensions. In this first attempt to understand GBR stability and nutrient fate, we concentrated on microbial decomposition by selectively excluding mesofauna and macrofauna from entering the bags, which could eliminate or erase

differences attributed to fertilization treatment and other factors. However, we should not disregard the important role of soil fauna to regulate decomposition by controlling the abundance and diversity of the microbial community colonizing non-confined organic matter (Cotrufo et al., 2010). The mesh size of 25 µm used in the current study can also prevent plant roots from intruding the bags while minimizing contamination by soil particles and material loss during the period of decomposition. To gain further insight into the decomposition of GBR, we could exploit this trade-off by using bags of different mesh sizes to evaluate the contribution of different soil organisms to decomposition and nutrient release (Coleman et al., 1999; Swift et al., 1998).

Another trade-off of the method is the compartmentalization and physical separation of the material from the system as well as the limited movement of the material in the microenvironment of the bag, as opposed to the non-confined organic matter occurring naturally in the system. This contributes to the creation of a microclimate, possibly increasing the moisture concentration and limiting the aeration inside the bag. Therefore, the decomposition of organic matter is not entirely a function of its initial composition, environmental conditions and fertilization treatments, but also of technical aspects of the method, creating a microclimatic artefact. Interestingly, studies comparing the *in sacco* method to the direct observation of non-confined material decomposition revealed a higher mass loss when using the former (De Santo et al., 1993; Kurz-Besson et al., 2005). Nevertheless, it is assumed that the results of *in sacco* studies will reflect trends that are characteristic of non-confined organic material, hence allowing for comparisons of different materials, sites, and experimental manipulations (Wieder and Lang, 1982).

Given the wide range of biotic and abiotic modulators involved in soil organic matter decomposition, with an unpredictable impact on soil carbon storage and release, further research is required to understand this complex process. As changes in soil organic matter and stable carbon can only be determined in long-term observations, to assess and quantify the contribution of GBR and other solid biogas residue fractions to the build-up of organic carbon, we need to follow and characterize the gradual changes of material quality over time. In addition, the varying composition of biogas residues due to the wide range of substrates utilized at different proportions in various biogas plants, may be another point for investigation, to identify the blends with the highest capacity to promote carbon sequestration in agricultural soils. Furthermore, since separating the effect of environmental factors and management practices from substrate composition may be challenging, establishing long-term studies under variable conditions and in differently managed agroecosystems could help to unravel the complex interactions taking place in the soil during the decomposition of organic fertilizers.

As a next step in this investigation, we aim to isolate and identify the bacteria and fungi that have colonized both GBR and RT during these nine months in the soil, focusing our interest particularly on the arbuscular mycorrhizal fungi. Acknowledged to have a great potential for promoting C sequestration in the soil while significantly contributing to plant growth and nutrition through the hyphal transport of immobile mineral ions such as phosphorus, AMF have received much attention. We posit that the fertilization of grassland soils with biogas residues, and particularly with GBR and other P-rich solid fractions, is a management practice that has the potential to maximize the production and sequestration of resilient C derived from AMF (Frey, 2019; Wilson et al., 2009) and its exudates (Rillig, 2004), thus contributing to stable soil organic carbon pools and greenhouse gas mitigation.

5. Conclusions

This study provides the first insight into the decomposition process of granulated biogas residue in grassland soils, which was strictly mediated by soil microorganisms in order to conserve differences that could otherwise be erased by larger soil organisms. In this initial step, we demonstrated the stability and dual property of the GBR to (1) provide

nutrients to soil organisms, and (2) promote terrestrial carbon sequestration by increasing the recalcitrant organic matter in the form of lignin-like compounds during the early stage of decomposition. Additionally, we shed some light on the impact of different types of N fertilizers on the overall decomposition process, and assessed their potential to affect carbon release and sequestration. The material of GBR was refractory to decomposition and was affected to a lesser extent by the different nitrogen sources during incubation in the soil as opposed to the comparator material of rooibos tea. Ammonium nitrate and the fertilization mix containing urea promoted the decomposition and carbon release from both materials, while the top application of raw biogas residue and raw manure had the opposite effect.

We conclude that GBR can be used as a slow release source of nutrients for the soil biota, and as a means to enhance soil health, promote carbon sequestration in the soil and slow down the emission of carbon dioxide into the atmosphere, which in turn can alleviate climate change. This information may add to the list of benefits of biogas residues and promote their wide use as fertilizers and soil improvers. Further studies to help predict the rate of solid biogas residue decomposition and release of nutrients in the soil under different environmental and management conditions could be a key step towards relaxing the stringent restrictions for biogas residue-based fertilisers.

We trust that this prelude will be a stimulus to further investigations into the dual property of solid biogas residue fractions as a long-term source of nutrients to plants and soil organisms, and a wherewithal to sequester carbon, thus contributing towards agricultural sustainability, circularity, and climate change mitigation.

Author contribution

Bella Tsachidou: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization; Christophe Hissler: Supervision, Writing – review & editing; Anaïs Noo: Investigation; Sébastien Lemaigre: Resources; Benjamin Daigneux: Investigation; Jérôme Gennen: Investigation; Stéphane Pacaud: Investigation; Isabelle F. George: Supervision; Philippe Delfosse: Conceptualization, Supervision, Writing – review & editing, Funding acquisition

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Declaration of competing interest

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

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