



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 $5 \text{ cm} \times 5.5 \text{ cm}$ ANKOM filter bags with 25 mg

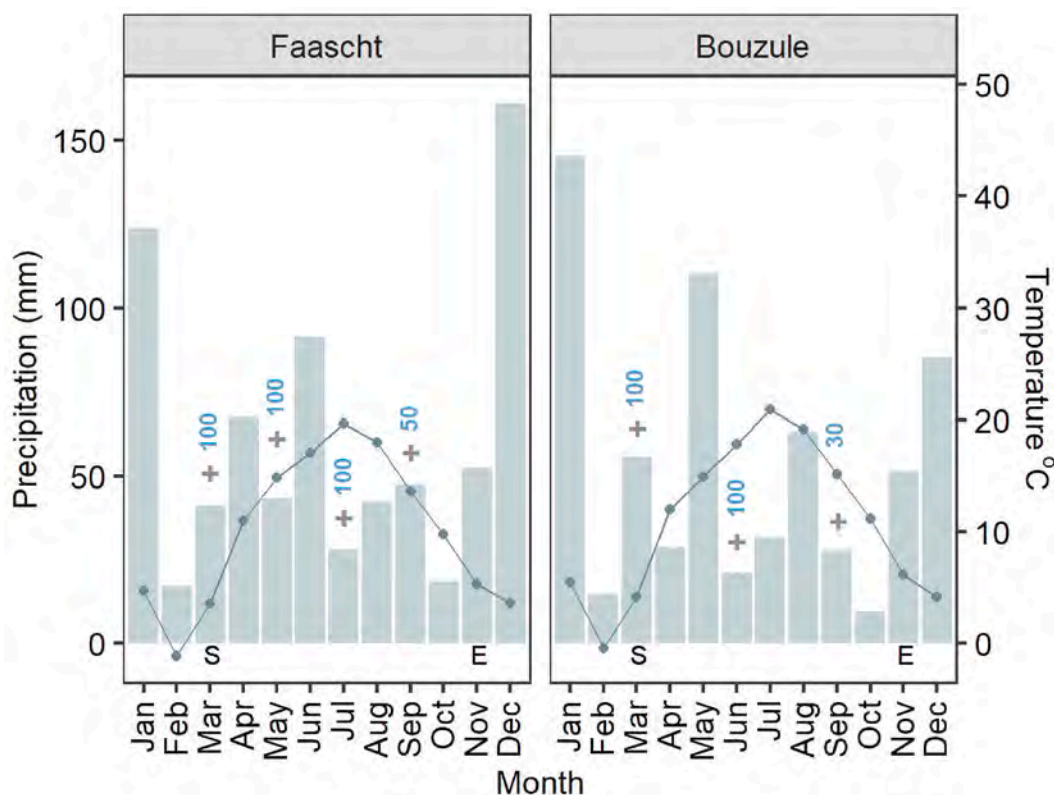


Fig. 1. Cumulative monthly rainfall (bars) and average monthly air temperature (dots and lines) at the locations of the *Ferme de Faascht* (as recorded at the nearby meteorological station in Useldange, Luxembourg) and *Ferme de La Bouzule* (as recorded at the INRAE meteorological station in Champenoux, 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,

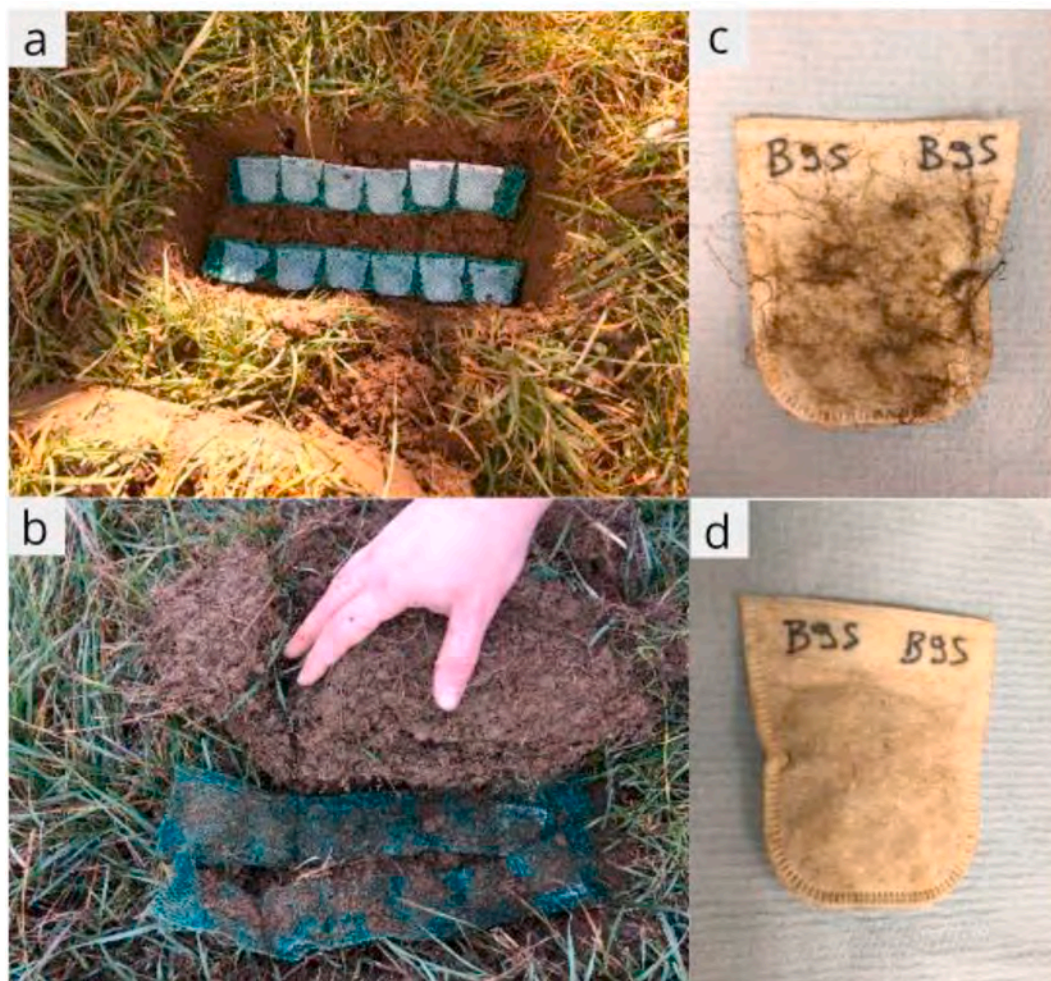


Fig. 2. a) ANKOM filter bags filled with granulated biogas residue (top row) and rooibos tea (bottom row), and placed inside nylon net pockets before the initiation of the incubation at the beginning of March 2018, b) bags retrieved at the end of November 2018, after nine months (270 days) of incubation 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 glomalins – 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

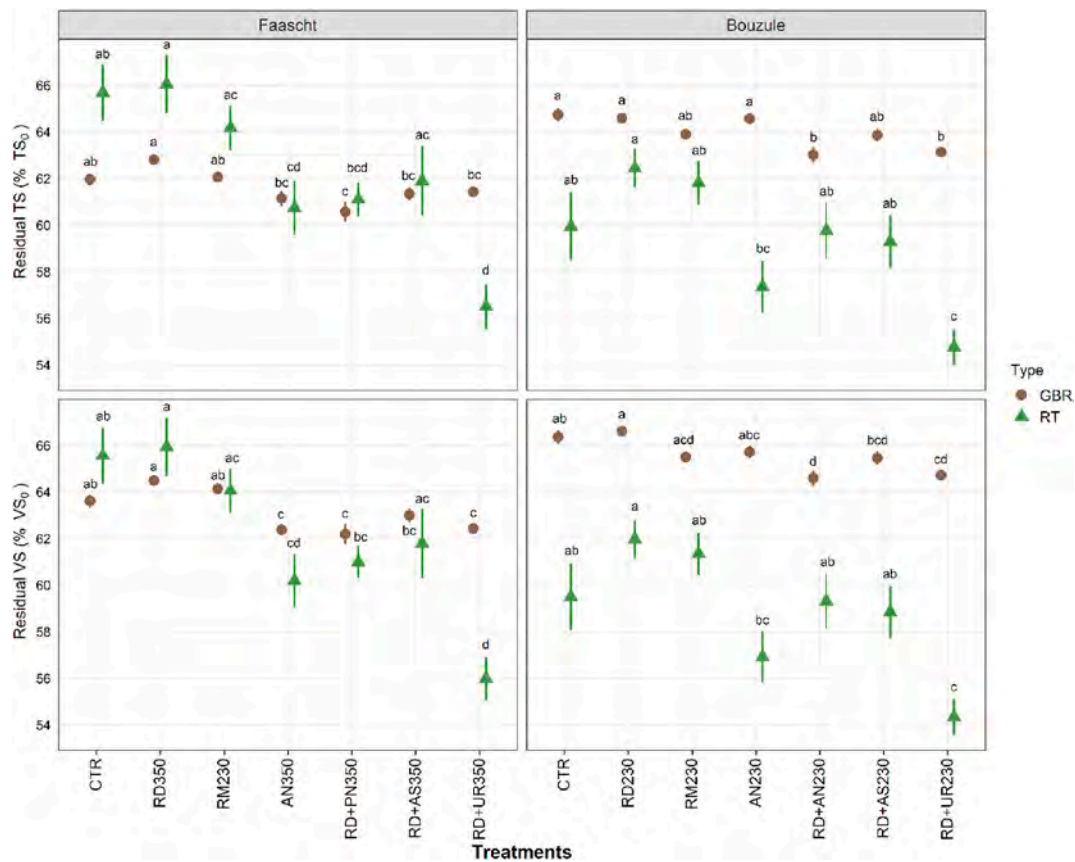


Fig. 3. Residual total solids (TS) and volatile solids (VS) in decomposed granulated biogas residue (GBR) and rooibos tea (RT) expressed on a dry matter basis as a percentages of the initial total solids (TS_0) and initial volatile solids (VS_0), respectively, in the two experimental grasslands (mean \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

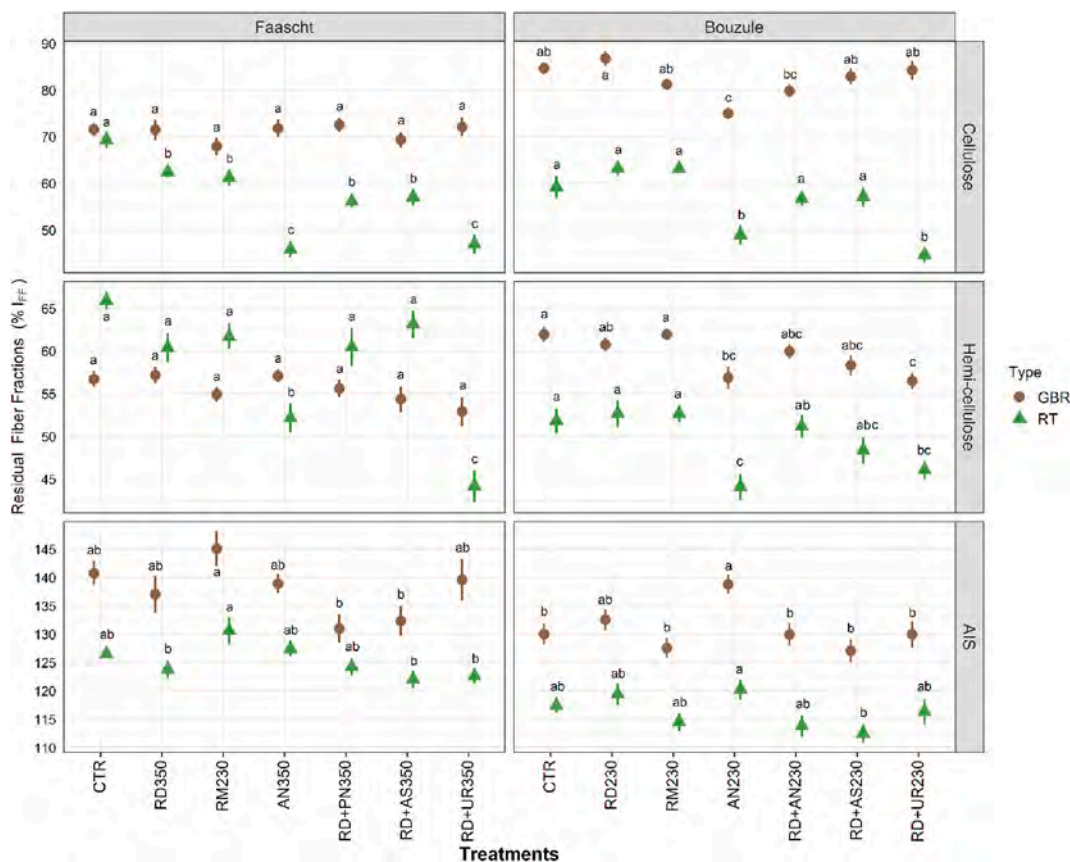


Fig. 4. Residual cellulose, hemi-cellulose and acid-insoluble substance (AIS) in decomposed granulated biogas residue (GBR) and rooibos tea (RT) expressed as a percentage of each initial fibre fraction (I_{FF}) (mean \pm standard error, $n = 9$). Different lowercase letters indicate a significant difference 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

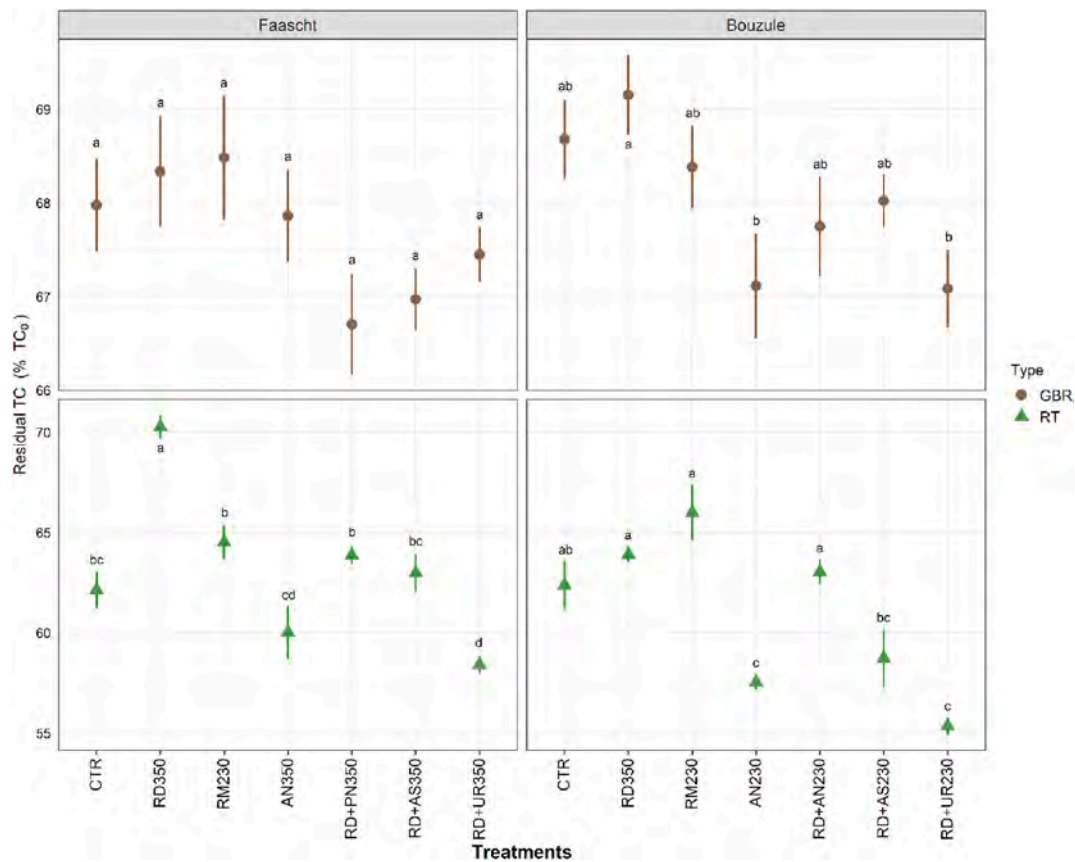


Fig. 5. Residual total carbon (TC) in decomposed granulated biogas residue (GBR) and rooibos tea (RT) expressed as a percentage of the initial total carbon content of the unexposed material (TC_0) (mean \pm standard error, $n = 3$). Different lowercase letters indicate a significant difference between 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

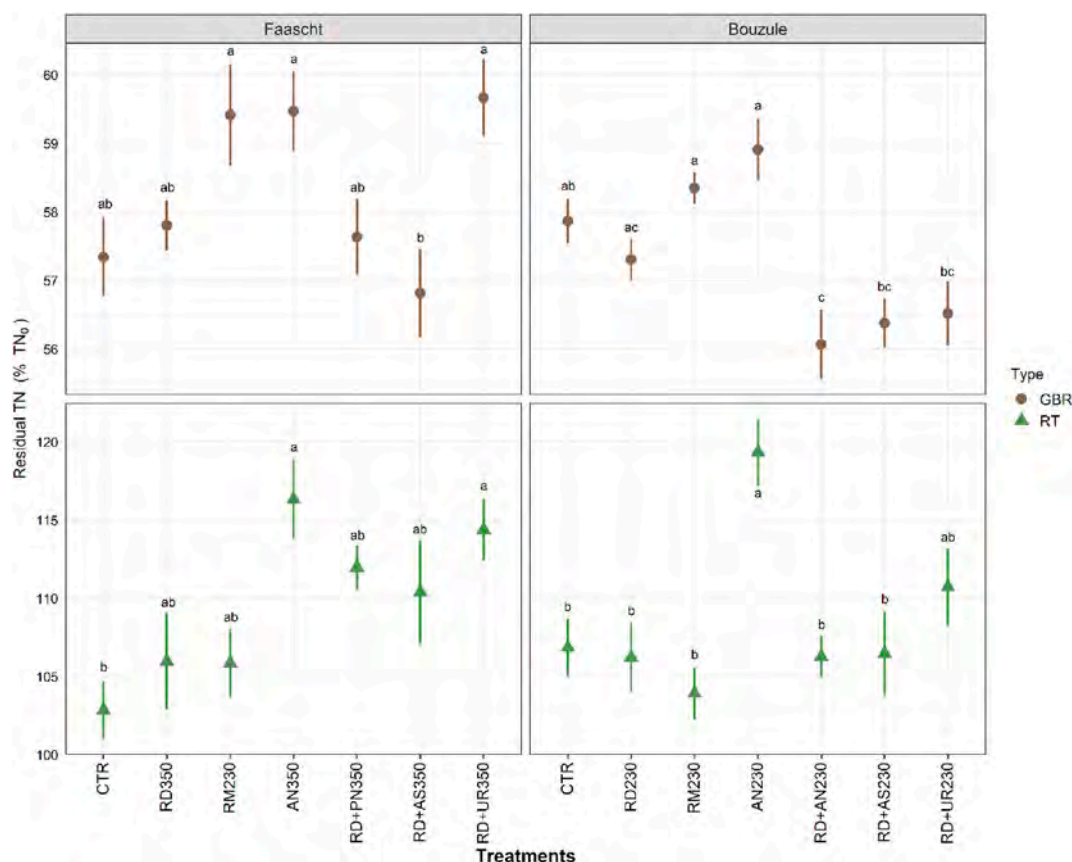


Fig. 6. Residual total nitrogen (TN) in decomposed granulated biogas residue (GBR) and rooibos tea (RT) expressed as a percentage of the initial total nitrogen content of the unexposed material (TN₀) (mean ± standard error, n = 3). Different lowercase letters indicate a significant difference between 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

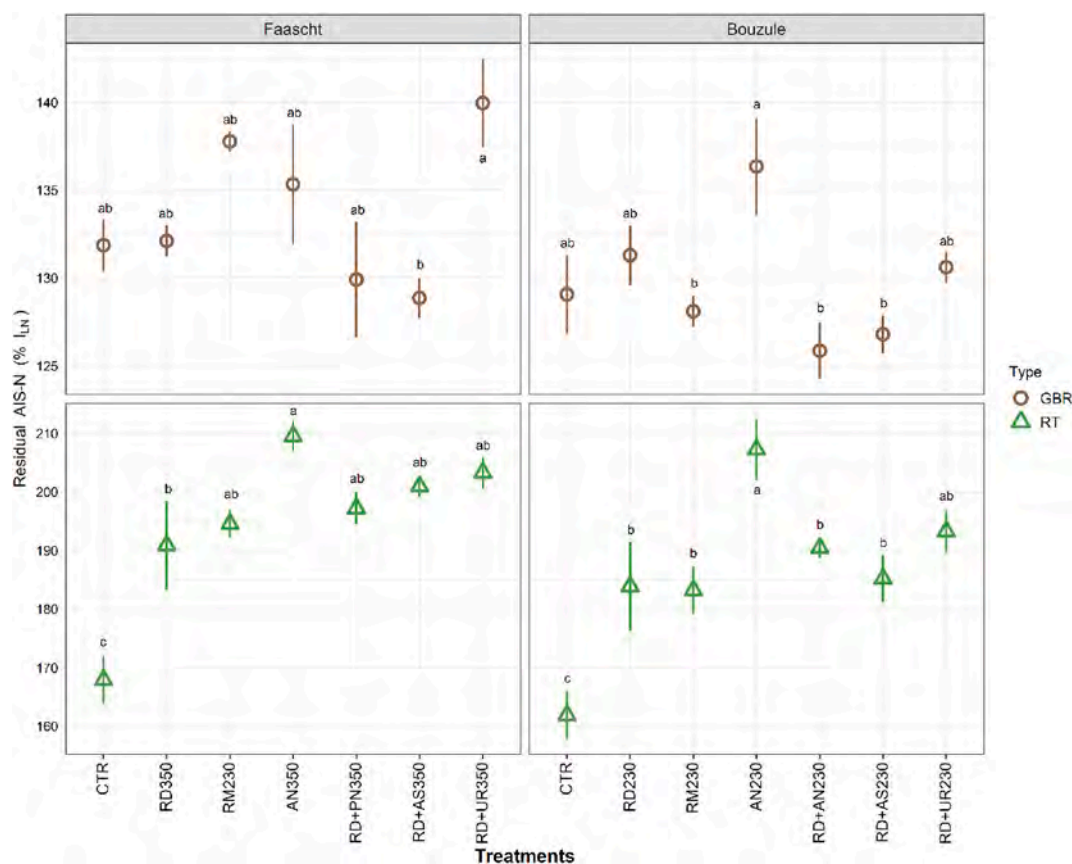


Fig. 7. Residual AIS-bound nitrogen (AIS-N) of the decomposed granulated biogas residue (GBR) and rooibos tea (RT) expressed as a percentage of the lignin-bound nitrogen contained in the unexposed material (I_{LN}) (mean \pm standard error, $n = 3$). Different lowercase letters indicate a significant 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

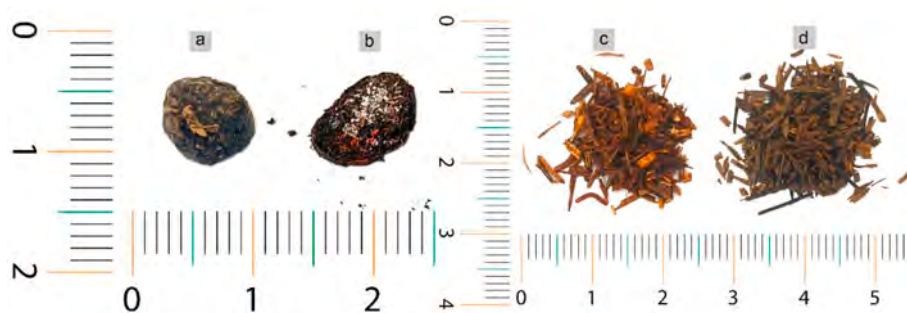


Fig. 8. a) A single biogas residue granule before incubation, b) after nine months (270 days) of incubation in the soil, c) rooibos tea before incubation and d) after nine months of incubation in the soil. The pictures of the decomposed materials were taken within 24 h of the retrieval from the soil.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