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Exploring the short-term in-field performance of Recovered Nitrogen from Manure (RENURE) materials to substitute synthetic nitrogen fertilisers

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ABSTRACT

Possible amendments to the European Commission's Nitrates Directive, such as the proposal of 'RENURE' criteria for the use of mineral nitrogen (N) bio-based fertilisers (BBFs) as a substitute to the Haber-Bosch derived chemical N fertilisers, is expected to open novel avenues for BBF use in the near future. Short and long-term testing of the RENURE materials in field trials will provide comprehensive insights into their crop response and environmental impacts. In this study, three potential RENURE materials (ammonium nitrate (AN) from stripping/scrubbing, ammonium sulphate (AS) from air scrubbing of pig stables, and pig urine (PU) from separated manure system) were tested in an NVZ to evaluate their short-term N effects. Although the trial experienced some weather-related effects, the selected RENURE materials performed comparably to the chemical N fertiliser with respect to agronomic yield (fresh $yield_{RENURE\ materials}$ = 28 – 32 tonne ha^{-1}; fresh $yield_{Synthetic\ NPK}$ = 32 tonne ha^{-1}) and post-harvest residual nitrate in soil. The soil biota response analyses demonstrated that, microbial communities responded well to the application of RENURE materials, whereas nematode communities were more structured after AS application in comparison to the chemical N fertiliser. Overall, this short-term trial exhibited comparable performance of tested RENURE materials to the Haber-Bosch-derived N fertiliser, and long term trials are recommended for further result validation.

1. Introduction

Livestock manure and slurries, when poorly managed, are known to cause considerable amounts of methane, ammonia (NH_3) and nitrous oxide emissions [1]. Along with the protection of land and groundwater sources from nutrient leaching, nutrient recovery from animal manure and other biomass streams has garnered attention due to the emphasis laid on the reduction of fossil fuel dependency and mining of limited natural resources [2–4]. Agricultural nutrients from different biomass streams (manure, food and organic wastes etc.) can be recovered using several market-ready technologies to procure bio-based fertilisers (BBFs). Many studies regarding the performance of BBFs in comparison to synthetic mineral fertilisers have shown promising results, indicating the potential of BBFs to be used as synthetic fertiliser substitutes [5–8]. Although, legalities involved in fertiliser use (e.g. the Nitrates Directive (ND) (91/676/EEC)) can impede the market-uptake of BBFs, foreseen revisions in these regulatory policies are expected to enhance avenues for increased BBF usage by end-users. For instance, despite the fact that the ND has been an effective environmental legislation for the past 30 years, one of its fundamental principles is that, regardless of the processing applied to animal manure, it retains the legal status of 'manure' (and thus as an animal by-product / waste). This hinders the possibility of optimally deriving and refining mineral fertilisers from manure as 'added-value products'. Unconceived three decades back, manure biorefinery technologies have undergone substantial technological development since the drafting of this legislation. In order to address this legal constraint, the European Commission's Joint Research Centre (JRC)-led 'SAFEMANURE' study has put forth harmonised criteria that could allow nitrogen (N) fertilisers, partially or completely derived from manure through processing, to be used as synthetic fertiliser substitutes in the Nitrate Vulnerable Zones (NVZs), above the legal ceiling of 170 kg N ha^{-1} y^{-1} [9]. Known as the 'recovered nitrogen from manure' (RENURE) materials, this criteria has been proposed for those N-rich manure-derived materials that meet the standards to act as 'chemical fertilisers' (produced by the Haber-Bosch technology) as defined in the ND. The guiding principles defining the RENURE criteria proposal

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emphasises on an important goal – that the implementation of RENURE be entirely in tandem with the main objective of the ND, i.e. reduction and prevention of water pollution caused by nitrates (NO_3^--N) from agricultural sources.

In order for the RENURE criteria proposal to be adopted into actual legislation, further scientific evidence is required to substantiate and validate the environmental and agronomic performance of manure-derived BBFs in comparison to their synthetic N counterparts. Nutrient Use Efficiency (NUE) conveys the nutrient assimilation capacity of plants, i.e. the N uptake in plants relative to the total N that was applied. By comparing the NUE of a candidate RENURE material to that of the chemical fertiliser, determination of the nitrogen fertiliser replacement value (NFRV), defined as the amount of chemical fertiliser saved while using a bio-based alternative [10,11], can be calculated. The higher NUE of chemical fertilisers combined with their lower susceptibility to N leaching made the Haber-Bosch derived (and equivalent) chemical N fertilisers to be used as a yardstick for comparison of candidate RENURE materials [12].

Candidate RENURE materials are categorised into different priorities as top, medium and low priority materials. The top priority RENURE materials with a high likelihood of making the practical substitution of synthetic fertilisers, include scrubbing salts, i.e. N obtained from manure by partial conversion into volatile NH_3 (stripping) followed by recapturing (scrubbing) the extracted NH_3 into soluble ammonium (NH_4^+) using a low pH solution (sulphuric, nitric or phosphoric acid to produce ammonium sulphate, ammonium nitrate, and (di-) ammonium phosphate, respectively). NH_4^+ salts from off-gases (e.g. ammonium sulphate from air scrubbers) are high quality products defined as RENURE materials and have been evaluated as equivalent to chemical fertilisers. Low priority materials include untreated manure, liquid-solid separated manure without treatment, etc. Thus, this legislative amendment could give N-rich BBFs like the top priority (ammonium nitrate (AN) from stripping-scrubbing of liquid fraction (LF) of digestate and ammonium sulphate (AS) from air scrubbing of stables) and low priority (pig urine separated from separated manure system) RENURE materials, the much requisite nudge towards their increased market-uptake. The agronomic efficiency of BBFs will greatly influence its market uptake, among other factors [13]. Though there are a few controlled pot and greenhouse experiments performed on RENURE materials like the NH_4^+ salts (AN and AS) [7,14], presently, literature lacks research performed on field-scale trials for the fertilisers tested in this study; there is only one previous trial done for AN [14] and none for PU. Thus, the current study aims to investigate the agronomic (crop yield, N uptake and NFRV) and environmental (residual nitrate post-harvest to assess risks for leaching of applied N) performance of the above-mentioned RENURE materials under relevant operational field conditions.

Furthermore, the most relevant risks include soil fertility, biological pathogens, etc. among others. To this end, it is significant to investigate the response of soil bacterial, fungal and nematode communities to the application of RENURE materials, using high throughput sequencing technologies. Healthy, functioning soil ecosystems are central to the successful production of agricultural crops. A single gram of soil can contain up to 10 billion microorganisms, belonging to thousands of different species, forming a complex network of trophic interactions [15]. Many factors can influence soil microbial community structure and composition, such as soil properties, including pH, texture, moisture and compaction, as well as climate, vegetation, land management and nutrient availability [16]. Bacteria and fungi are the most abundant microorganisms in soil and provide a multitude of key ecosystem services such as organic matter decomposition, nutrient cycling, mineralisation, N fixation and maintaining soil quality health, among others [17]. Likewise, healthy populations and diversity of soil micro fauna are essential for maintaining soil food networks. Nematodes are microscopic roundworms and the most abundant and widespread animals in nature [18]. They can be used as bioindicators of environmental conditions and change [19–22] since they are representative of their habitats, and due to their high abundance, feeding behaviours and diversity [23]. Plants growing in soil with bacteria and bacterial feeding nematodes absorb more N than plants growing in soil with only bacteria [24]. Bacterivorous nematodes play a significant role in N mineralisation [25–28], nutrient cycling [29] and enhancing N availability [30]. Nematodes that easily spread and colonise their habitats are known as colonisers and enrichment opportunists. These are bacterial feeders that reproduce and increase in numbers rapidly [31]. Nematodes with the opposite life strategies, known as persisters, contain species with larger individuals and are characterised by long life spans and low reproduction rates. The reduction of persisters, such as those in the order Dorylaimida, is a sign of soil environmental disturbance [32]. Soil community diversity (α-diversity) is an indicator of soil health [33] and the loss of diversity poses a major ecological threat, as it is associated with the loss of essential soil ecosystem functions [34]. Additionally, variation in overall soil microbial community structure (ß-diversity) may influence and disrupt some of the vital ecosystem processes provided by soils [35]. Due to these factors, investigation of the soil biota response to RENURE material application was deemed significant.

Thus, this research strives to evaluate the agronomic potential of three candidate RENURE materials by comparing their performance to the Haber Bosch-derived chemical fertiliser ammonium nitrate by examining the short-term agricultural and environmental impacts of RENURE material application. This was done by assessing:

i) the yield and NUE of crops fertilised with the RENURE materials
ii) the environmental impacts like residual NO_3^--N and response of soil bacterial, fungal and nematode communities to the RENURE material application

2. Materials and methods

2.1. Field and soil characteristics

The field covering a surface area of 2.04 ha, had a soil profile categorised as a Z.c.h. soil-type with sandy texture, a somewhat dry drainage class (signs of rust deeper than 60 cm) and a postpodzol B horizon [36]. As uniform soil conditions are not guaranteed on a field of this size, a preliminary screening was done to eliminate the divergent patches of the field and to allow the organisation of blocks with similar soil conditions. Ensuring homogeneity in soil properties throughout the field, thus eliminating the effect of soil on results, would help in determining significant differences between treatments, if any. Initially the field was split into 39 sectors (18×15 m), and was scanned using the following three techniques: (i) drone imaging of preceding crop (Ryegrass), (ii) penetrologger measurements to test soil compaction and (iii) physico-chemical characterisation of soil (0-30 cm). Based on the normalized difference vegetation index (NDVI) and normalized difference red edge index (NDRE) images, and chemical analysis of the top soil layer (Table 1), three most divergent sectors (32, 33 and 36) were excluded from the trial (Figure 1). The results of the penetrologger measurements were used to consign similar sectors to the same block.

The field used for the trial was situated on a mixed farm that combines cattle breeding and fodder production with extensive vegetable cropping (spinach, carrots, salsify, early potatoes…). Crop history on the field over the last decade(s) was determined by a three year rotation dominated by silage maize (two out of three years). Whenever possible, Italian ryegrass was sown after harvesting the maize to harvest a single cut in the following year. Sometimes, this single cut of grass was replaced by spinach. The other main crop in rotation is early potatoes (once every three years), also followed by Italian ryegrass. In two out of three years, organic fertiliser (mostly cattle slurry) at the rate of 45-50 tonnes ha^{-1} is applied, depending on the N content of the slurry.

Table 1
An overview of the soil physico-chemical characterisation (0-30 cm) per block prior to crop cultivation. Values for calcium (Ca), magnesium (Mg), sodium (Na), potassium (K), phosphorus (P) and sulphur (S) are the plant-available results obtained from extraction with ammonium lactate.

Parameters	Units	Block 1	Block 2	Block 3	Block 4	Target Value
pH (KCl)		5.6	6.0	6.0	5.0	5 – 5.5
Texture		Sand	Sand	Sand	Sand	
NH_4^+-N	kg ha^{-1} dried soil	12	14	11	10	n.a.
NO_3^--N	kg ha^{-1} dried soil	16	22	16	13	n.a
Organic C	%	0.7	0.8	0.8	0.8	1.2 – 1.9
Ca	mg/100g dried soil	82	89	87	65	63 – 240
Mg	mg/100g dried soil	12	14	13	8.5	6 – 10
Na	mg/100g dried soil	0.4	0.5	0.3	0.6	2.7 – 5.9
K	mg/100g dried soil	24	21	28	22	10 – 18
P	mg/100g dried soil	64	67	66	67	10 – 18
S	mg/100g dried soil	1.1	1.2	2.2	1.3	2.3 – 3

n.a.: not available

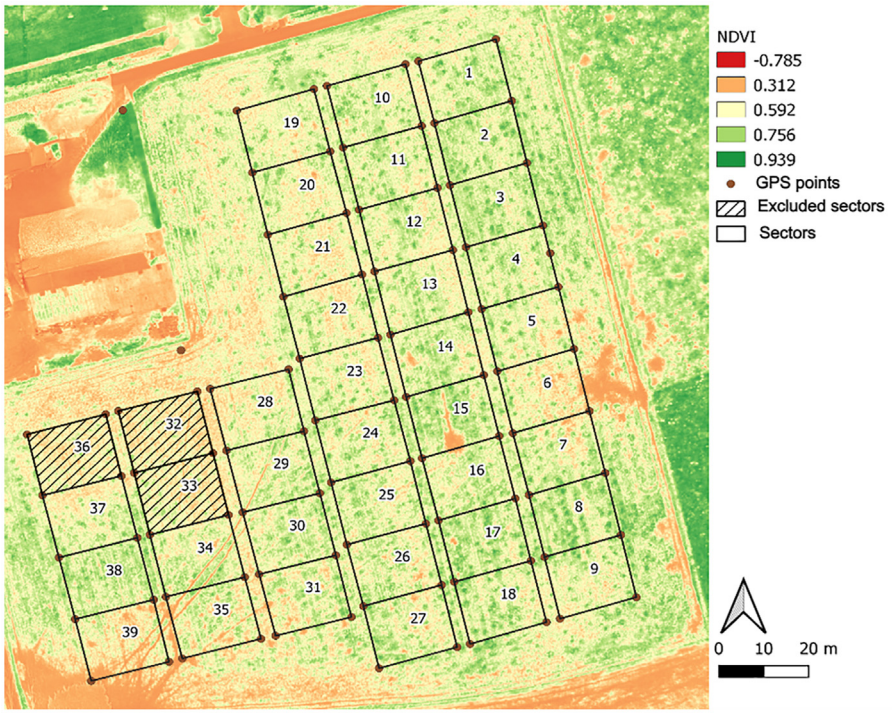

Fig. 1. Representation of the field in 39 sectors used for pre-screening to determine field variabilities. The sectors 1-9, 10-18, 19-27 and 28-39 are assigned to blocks 1, 2, 3 and 4 respectively. The three shaded sectors (32, 33 and 36) were excluded on the basis of pre-screening.

2.2. Origin and composition of RENURE materials

The RENURE materials tested in the field trial were AN, AS and PU. The AN was obtained from the stripping-scrubbing unit of a pig farm with an anaerobic digestion (AD) plant, located in Gistel, Belgium. The farm has a capacity of 11000 fattening pigs with a manure treatment capacity of 60000 tonnes y^{-1}. The input for AD treatment includes different types of animal manure (65% pig manure, 17% solid fraction (SF) of pig manure and 9% horse manure) and food waste (9%). The resulting digestate is separated into SF and LF by centrifugation, after which the LF is subjected to NH_3 stripping and scrubbing, recovering N in the form of AN. AS was collected from a pig stable that utilises an acid scrubber to capture the NH_3-rich indoor air. The process is done by capturing the volatile NH_3 in its gaseous form by an acid scrubber, which is a reactor filled with inorganic packing material, with large porosity and specific area [14]. After collection, both products were stored outside, in a closed but non-isolated, white, semi-light transmitting plastic container for five weeks between collection and trial installation. Samples of BBFs were taken for analyses four weeks before trial installation. Before sampling, the containers were mixed by shaking them with a forklift. PU was collected from a source-based manure separation system (Vermeulen Dobbelaere Welfare System ((VeDoWS) animal housing system)) located at a Flemish pig farm. In the manure separation system, the urine is collected in a shallow cellar constructed beneath a slatted floor where solid faeces is separated by scraping off on a daily basis, and the liquid urine trickles down to a separate collection channel (personal communication, VeDoWS). A first sample was taken from the storage pit at the stable and was analysed three weeks before trial installation and the amount of PU applied in the trial was calculated based upon this first analysis. The PU was transported by an official transporter using a tank-car. During transport (just before the first sampling) and when the tank of the trial fertiliser machine was filled at trial installation, the PU was mixed. At trial installation (after fertilisation), a second sample was taken and analysed. AN and AS were analysed only once since both are mineral in nature. In the case of pig manure ((PM), positive organic reference used in trial), considerable difference in the N content was observed at both sampling periods (Table 2).

For determination of Kjeldahl N, organic N was converted into NH_4^+-N using a destruction with H_2SO_4 and then measured using a steam-distillation and titration. This was followed by measurement of NH_4^+-N

Table 2
An overview of the physico-chemical characterisation of PM and the tested RENURE materials

Products	Sampling date	Sampling time	Sampling location	NO_3^--N (g kg^{-1})	NH_4^+-N (g kg^{-1})	Total N (g kg^{-1})	Total P (g kg^{-1})	Total K (g kg^{-1})	Total S (g kg^{-1})
PM	4/03/2019	Before trial installation	Stable	< LoD	6.9	12	3.7	7.6	1.9
PM	23/04/2019	After fertilisation		< LoD	4.9	8.0	2.2	6.6	1.9
PU	4/03/2019	Before trial installation	Stable	< LoD	3.0	4.3	0.40	2.7	0.60
PU	24/04/2019	After fertilisation		< LoD	4.2	5.0	0.30	4.2	0.60
AN	15/04/2019	Before trial installation	On site storage	43	43	86	n.d	n.d.	0.50
AS	18/03/2019	Before trial installation	On site storage	< LoD	34	34	n.d.	n.d.	38

PM: Pig manure; PU: Pig urine; AN: Ammonium nitrate; AS: Ammonium sulphate; LoD: Limit of Detection= 0.00066 g kg^{-1}
n.d.: not determined

Table 3
Overview of tested treatments: amount of BBFs and synthetic fertilisers, and their nutrient content applied (in kg ha^{-1}) from each treatment (dose of BBFs based upon their sampling after fertilisation).

Treatments	Product applied (kg ha^{-1})				Total nutrients applied (kg ha^{-1})			
	Synthetic N	Synthetic P	Synthetic K	BBF	N	P_2O_5*	K_2O*	SO_3**
Unfertilised control	-	-	-	-	-	-	-	-
Mineral PK	-	234	837	-	-	108	250	351
Mineral NPK	503	234	837	-	151	108	250	351
PM	-	-	446	12700	102	108	250	248
AN	-	234	837	1760	152	108	250	352
AS	-	234	417	4488	150	108	250	427
PU	-	159	461	35500	178	108	250	248

* Nutrients from both, BBFs and synthetic fertilisers
** Since K_2SO_4 contains both, K and S, all treatments exceeded crop demand of S, since priority was given to maintaining the K fertilisation. In the case of AS, K fertilisation was done using KCl, to avoid excessive S addition from K_2SO_4

content using steam-distillation. MgO was added until an alkaline reaction occurs and the steam-distillation emits the NH_4^+ bound as ammonium borate, which is then titrated with hydrochloric acid (HCl). P and K were only measured on PM and PU since AN and AS are pure N products. The analysis was done by incinerating the samples at 550°C, the ashes were then digested in nitric acid (HNO_3) and measurement was done by an Inductively coupled plasma-Atomic emission spectroscopy (ICP-AES, Optima 8300, Perkin Elmer, USA). Total S was measured on all samples by digesting the samples in solution of aqua regia (6 ml HCl and 2 ml HNO_3) and measurement with ICP-AES (Table 2).

2.3. Trial set-up and fertilisation strategies

This short-term trial was conducted between the end of April to the end of September, 2019. Each plot of the trial measures 6×8 m. The trial set-up was done by including four replicates of each RENURE material. The two negative controls (N-unfertilised), i.e. unfertilised control, and treatment with mineral phosphorus (P), potassium (K) and sulphur (S) were tested in eight replicates for statistical accuracy. All other treatments, including the two positive references (N-fertilised), i.e. synthetic NPK (Haber-Bosch-derived chemical ammonium nitrate (30% N) and PM, were tested in four replicates. The N fertilisation advice for the field was set at 150 kg N ha^{-1} based upon the chosen crop, i.e. maize (Zea mays), field characteristics, historical management practices on the field and soil available mineral nitrogen at the time of sowing. Because of slight discrepancies between the samples taken from storage before trial installation (used to calculate the doses to be applied) and the samples taken from the fertiliser machine at trial installation, differences in N-doses between treatments can be observed (Table 3). On all plots except the unfertilised control, mineral P, K and S fertilisers were applied to supply slightly more than the requisite nutrient content by the crop to ensure that the plant growth could only be limited by N deficiency. The mineral P fertiliser used was triple superphosphate. Mineral K and S were applied as potassium sulphate (K_2SO_4) on all plots except the unfertilised control and where treatment with AS was applied. Because AS contains large amounts of S, K fertilisation was done with potassium chloride (KCl) and no additional mineral S was applied.

Immediately after fertiliser application, the fertilisers were incorporated into the top soil layer using a spit mill. Using a heavy roll, the top soil layer was sealed again to prevent volatilisation of NH_3, and soil desiccation. A few days after fertiliser application, the field was ploughed and the maize was sown on 2nd May. Application technique of fertilisers differed based on their type. Based upon physical and chemical properties, the RENURE materials evaluated can be divided into two major categories. Pig urine has a low N content compared to chemical N fertilisers. The NH_4^+ salts (AN and AS) have higher N content, lower viscosity, and the products are free from suspended organic material. In practice, PU and PM are mostly applied using manure injectors equipped with a vacuum pump system and coulters fitted for arable land or grassland depending on the circumstances. The rate of N application was found to be lower in case of PM because N dose for application was calculated on the basis of first sampling, but considerable difference in the N content was observed in the second sampling of the product done after fertilisation (Table 2). The NH_4^+ salts were applied using hose pump systems. In practice, these systems are often combined with planting/sowing machines. Synthetic mineral fertilisers were applied broadcast by hand, and superficially incorporated into the soil before deep tillage.

2.4. Weather monitoring

Weather conditions prevailing throughout the trial duration were monitored using a weather station placed at a nearby farm, at a distance less than 500 m. Precipitation was measured by the tipping bucket method, and hourly temperature readings were logged (Figure 2). During the preliminary screening in February 2019, weather conditions were found to be exceptionally dry. Albeit normal average values, the daytime temperatures were very high, with the night experiencing an abnormally lower drop. The commencement of the trial towards April-end, 2019, was done in very dry and relatively colder weather conditions. May 2019 was colder and dryer than normal and the lower precipitation led to dryer soil conditions. After an exceptionally hot and dry

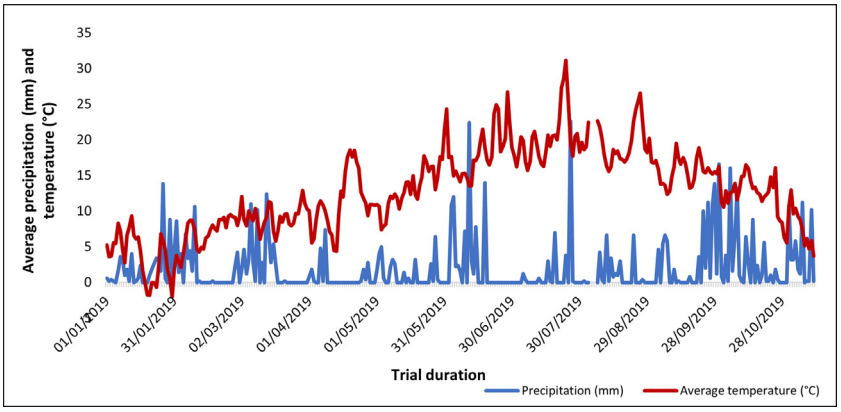

Fig. 2. Daily precipitation and average temperature conditions during the trial period.

July, the end of the month witnessed a record-breaking high in Flanders' temperature, with the weather-station registering a value of 42.94 ºC. August and September 2019 also remained very dry, with precipitation occurring only after the harvest period towards the end of September. Regular precipitation was received in this region from October until November.

2.5. Analyses of soil and plant post-harvest

2.5.1. Physicochemical characterisation

146 days after sowing, on 25th September, 2019, the maize was harvested. Yield determination was done on the net plot of size 4×6 m, using a small plot combine harvester. Aboveground biomass of each net plot was automatically weighed and chopped. Crop samples were dried at 40 ˚C for 24 hours and ground. A representative sub-sample of the chopped silage maize was taken and used for total N was analysis, which was performed using a total N analyser (Primacs, Skalar, the Netherlands).

Soil samples were takenduring field pre-screening (February, 2019), two months prior to fertilisation (April, 2019) and after the maize harvest (September, 2019) in soil layers 0-90 cm (at an interval of 30 cm) for mineral N assessment and 0-30 cm for all other parameters. 15 points were sampled in each plot in a cross-shaped pattern using a 20 mm drill for the top soil layer (0-30 cm) and a 13 mm drill for the deeper layers. Within 24 hours of sampling, NH_4^+-N and NO_3^--N content were determined on fresh samples by mixing the soil samples with 200 mL 1M KCl, and the extracts were analysed using a Segmented Flow Analyzer (San^{++} continuous flow analyzer, Skalar, the Netherlands). Moisture content was determined by drying a sub-sample of the soil at 105 ˚C for 24 h. Remaining soil was air-dried and sieved using 1 mm sieves to remove the smaller roots. Analyses for pH-KCl, EC, and total elemental content were performed as described in [7].

2.5.2. Soil biota response analysis

In addition to evaluating the agronomic performance, the response of soil bacterial, fungal and nematode communities to the RENURE material application was investigated. This was achieved by high throughput sequencing technology, and specifically amplicon sequencing. Variable regions of deoxyribonucleic acid (DNA), specific to different taxonomic groups under analysis, within conserved phylogenetic regions of an organism's genome, were targeted using universal primers, amplified and sequenced. Amplicon sequencing is particularly useful in characterising diversity within environmental samples due to the accessibility of established assays, cost-efficiency, time-efficiency and availability of software packages for bioinformatic analysis [37–40].

2.5.2.1. Soil sampling, preparation for DNA extraction and DNA sequencing. Soil sampling was conducted immediately post-harvest. Nine sample cores were taken in a W pattern to a depth of 10 cm per plot, combined all as a single composite sample and stored at -20 ˚C until ready for analyses. A total of 24 composite samples were obtained (6 treatments×4 replicate plots). Defrosted samples were sieved through a 2 mm mesh for thorough homogenisation. For bacterial and fungal analyses, 0.25 g sub-samples were used for DNA extraction and processed immediately. For nematode analysis, 25 g sub-samples of soil were shaken in 25 ml of deionised water for 10 minutes at 95 rpm and centrifuged for 2 minutes at 3500 rpm. The supernatant was discarded, and the remaining material was dried overnight at 28 ˚C in Petri dishes before thoroughly homogenising again using a mortar and pestle. Finally, 0.25 g sub-samples were used for further processing. Total DNA was extracted from the 0.25 g soil sub-samples using the Qiagen DNeasy® PowerSoil® Pro kit, as per the manufacturer's instructions. Total DNA quality and quantity were assessed by a NanoDrop™ instrument and agarose gel electrophoresis using 1% agarose gels, before outsourcing to a sequencing company (Novogene Ltd. U.K.). Bacterial 16S V4-V5 region rRNA, fungal ITS1 region rRNA and nematode 18S V4 region rRNA were sequenced using 515F and 907R [41,42], ITS5-1737F and ITS2-2043R [43], and MN18F and 22R [44] primer pairs, respectively, on Illumina paired-end platform. Primer sequences are supplied in supplementary material (Table S1).

2.5.2.2. Sequence data analysis. Sequencing data was processed and clustered into operational taxonomic units (OTUs) based on a 97% similarity threshold by the sequencing company. In QIIME2 (version 2020.11), taxonomy was assigned to bacterial and nematode OTUs using the SILVA (release 138 SSURef_NR99) database [45], and to fungal OTUs using UNITE (version 8.2) database [46]. Fungal data was filtered to remove OTUs which did not belong to the fungal kingdom. For subsequent alpha and beta diversity analyses of bacterial, fungal and nematode communities, OTU numbers were normalised using the sequence number corresponding to the sample with the least sequences as the standard.

2.6. Calculations and data analysis

The calculation of apparent nitrogen recovery (ANR) and NFRV [47–49], NUE and nitrogen replacement use efficiency (NRUE) was done as shown in equations 1-4. The negative control mentioned in equation 1 refers to the treatment with mineral PK.

$$\text{ANR} = \frac{(N\ uptake\ TREATMENT\ (kg\ ha^{-1})) - (N\ uptake\ CONTROL\ (kg\ ha^{-1}))}{Total\ N\ applied\ TREATMENT\ (kg\ ha^{-1})} \quad (1)$$

$$\text{NFRV} = \frac{ANR\ BBF}{ANR\ synthetic\ N\ fertiliser} \quad (2)$$

$$\text{NUE} = \frac{N\ uptake\ (kg\ ha^{-1})}{Total\ N\ applied\ (kg\ ha^{-1})} \quad (3)$$

Table 4
Mean ± stdev of maize yield, N uptake, apparent nitrogen recovery (ANR), nitrogen fertiliser replacement value (NFRV), nitrogen use efficiency (NUE) and nitrogen replacement use efficiency (NRUE) for tested treatments (n=8 : unfertilised control and PK control; n=4 : other treatments), where, the different lowercase letters indicate significant differences between treatments.

Treatment	Fresh yield (tonne ha^{-1})	Dry yield (tonne ha^{-1})	N uptake (kg ha^{-1})	ANR	NFRV	NUE	NRUE
Control	32 ± 6.8	13 ± 2.4	150.6 ± 28^{ab}	-	-	-	-
PK Control	30 ± 5.9	12 ± 2.2	140.6 ± 26^{a}	-	-	-	-
NPK	36 ± 7.5	13 ± 2.4	195.5 ± 21^{c}	0.36 ± 0.14	-	1.3 ± 0.14^{ab}	-
PM	27 ± 4.8	10 ± 2.2	151.1 ± 28^{abc}	0.10 ± 0.28	0.29 ± 0.76	1.5 ± 0.28^{b}	1.1 ± 0.21^{b}
AN	32 ± 9.9	11 ± 3.0	171.9 ± 41^{abc}	0.21 ± 0.27	0.57 ± 0.74	1.1 ± 0.27^{ab}	0.87 ± 0.21^{ab}
AS	37 ± 6.7	12 ± 2.2	189.1 ± 37^{bc}	0.32 ± 0.25	0.89 ± 0.69	1.3 ± 0.25^{ab}	0.97 ± 0.19^{ab}
PU	28 ± 3.0	9.4 ± 1.9	150.1 ± 18^{ab}	0.05 ± 0.10	0.15 ± 0.29	0.85 ± 0.10^{a}	0.65 ± 0.08^{a}

PK Control: Synthetic phosphorus and potassium; NPK: Synthetic nitrogen, phosphorus and potassium; PM: Pig manure; AN: ammonium nitrate; AS: Ammonium sulphate; PU: Pig urine

$$\text{NRUE} = \frac{NUE\ BBF}{NUE\ synthetic\ N\ fertiliser} \quad (4)$$

Data for yield, N uptake, ANR, NFRV, NUE, NRUE and residual NO_3^--N were processed using the statistical program IBM SPSS Statistics, version 27.0. The parametric one-way ANOVA test was performed to evaluate significant differences for each of the aforementioned parameter, followed by the Tukey's post-hoc test to identify the differences between individual treatments, for all parameters except N uptake. Since Tukey's test failed to show the significant differences between groups as indicated by the ANOVA, the Waller-Duncan post-hoc test was used in case of N uptake. Normality within and variance between treatments were determined by the Shapiro-Wilk test and Levene's test respectively.

Levels of bacterial, fungal and nematode diversity were assessed by Observed OTU and Shannon alpha diversity indices and statistically compared by Kruskal-Wallis H test. Beta diversity was measured by both, weighted and unweighted Unifrac distances, followed by permutational multivariate analysis of variance based on 999 Monte-Carlo permutations. Differences between the nematode communities were detected via a nonparametric test Anosim (Analysis of Similarity) and MetaStat. Statistical analysis of bacterial and fungal abundances were performed in IBM SPSS Statistics, version 25. One-way ANOVA with Tukey's Honestly Significant Difference (HSD) pairwise comparisons were used to detect significant differences between treatment groups in the relative abundances of bacteria and fungi at specific taxon levels. When data violated the homogeneity of variance or normality assumptions, the nonparametric Kruskal-Wallis H test was used. Non-metric multidimensional scaling (NMDS) was performed using the software R (version 4.0.4.).

3. Results and discussion

3.1. Crop yield and N use efficiency

No significant differences were observed in regards to fresh and dry maize yield at the time of harvest between the tested treatments, including the unfertilised control (Table 4). The assessment of soil properties by physico-chemical characterisation during pre-screening of the field gave an indication of the nutrients already present in the soil (Table 1). In industrialised farming regions such as Flanders, the soils are nutrient-rich due to the high levels of historic fertilisation, thus obscuring direct comparison of fertilised treatments with those unfertilised. The present study provides the status quo on agronomic output when fertilising an NVZ with potential RENURE materials. Additionally, it is emphasised that the identification of agronomic effects of tested RENURE materials would necessitate long-term trials in the fields with a variety of crops.

Although, there is a lack of significant difference between treatments, it needs to be noted that this trial resulted in overall lower yields, as compared to the field trials conducted with similar RENURE materials in previous studies. A single-year field trial studying AN and AS obtained from the same source exhibited ~0.5 - 0.6 (59 ± 6 for AN and 59 ± 4 tonnes ha^{-1} for AS) and ~0.6 - 0.7 times higher (17 ± 2 for AN and 17 ± 1 tonnes ha^{-1} for AS) fresh and dry yield, respectively [14]. Similarly, other field experiments testing scrubbing salts like AS demonstrated higher yield results in the range of ~0.5 for fresh (73 - 81 tonnes ha^{-1}) and ~1.5 times for dry yield (21 - 23 tonnes ha^{-1}), respectively, of maize [50,51].

There is some variability in crop growth that is visible in the yield results, and this could be attributed to weather-related effects on the growing period of maize. Availability of soil moisture became a defining parameter determining the crop growth, rather than the availability of N. The temperature and available moisture throughout the development, especially during key physiological growing periods, play a major role in final yield and grain quality in maize [52]. During the month of May 2019, the abnormally colder and dryer conditions were non-optimal for a crop like maize. This lower temperature period was followed by a drastic switch to extremely hot and dry conditions in July, thus causing the vegetative phase of maize to end at an early moment in crop development. Episodes of extreme temperature can lead to sterility, reduced productivity- both quantity and quality wise, and lethality for crops. Because of the unsustainable management of rivers, waterways and groundwater in the past, and high degree of urbanisation, compaction and sealing of soil, Northern Belgium is especially vulnerable in undergoing conditions of drought [53]. Additionally, sandy soils have low water retention and capacity to buffer the increasing variability in rainfall [54]. Research and field observations in the past have conclusively stated that climate impacts are leading to poorer harvests, thus affecting the quantity and quality of farmed products in Europe [55]. Although, positive effects on crop yields as a result of climatic changes have been witnessed in Belgium [55–57], climate change also tends to increase the occurrence of extreme events, resulting in lower inter-annual yield stability, and years with low yields [57]. While, climate change-induced mean yield increases have been calculated for crops like winter wheat, sugar beet etc., a mean yield decline by 5% has been calculated for maize [53]. Agriculture, in general, is expected to face higher interannual variability in yield and product quality, with increased risks of crop losses and the subsequent price volatility [58].

The lack of differences in yield between the chemical N fertiliser and the RENURE materials could also point to their comparable mineral nature. Among the tested products, AN and AS contain N entirely in the mineral form, similar to their synthetic counterpart. The concentrated form of N in these NH_4^+ salts are a result of the processing involved in their production. The addition of HNO_3 to the scrubber increases the N value in AN by contributing NO_3^--N to the product. In the case of AS, along with N, this BBF also contains S from the H_2SO_4 added into the scrubber. Due the source-based separation of manure, PU has a high mineral N:total N ratio of 84%, subsequently resulting in comparable yield with the synthetic reference.

Compared to the treatments with unfertilised control, PK control, and PU, plants treated with synthetic NPK had significantly higher N

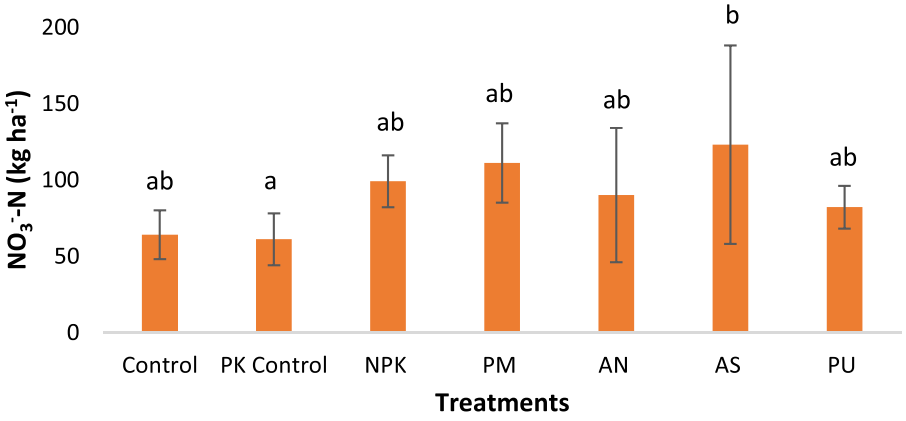

Figure 3. Mean residual NO_3^--N content in the 0-90 cm soil profile with standard deviation (n=8 : Control and PK Control; n=4 : other treatments), where the different lowercase letters indicate significant differences between treatments.
PK Control: Synthetic phosphorus and potassium; NPK: Synthetic nitrogen, phosphorus and potassium; PM: Pig manure; AN: ammonium nitrate; AS: Ammonium sulphate; PU: Pig urine.

uptake, whereas the performance of AN, AS and PM were comparable to the synthetic reference. In the case of ANR and NFRV, no significant differences between treatments were observed. The results indicate lower ANR values for all treatments in general, and analogous to the N uptake, the highest and lowest ANR was observed in treatments with NPK and PU, respectively. Treatment with AS displays the highest NFRV (0.89 ± 0.69), but it also shows a high standard deviation, indicating high variability among replicates of the treatment. Since NFRV values are calculated on the basis of ANR, the treatment with PU shows the lowest NFRV of 0.15. The treatments with PM (1.5 ± 0.28) demonstrated significantly higher NUE compared to PU (0.85 ± 0.10), but was comparable to the other treatments. A trend analogous to NUE was seen in the case of NRUE.

Estimation of N uptake and the agri-environmental indicators like ANR, NFRV, NUE, and NRUE differ in the inclusion (ANR, NFRV) or exclusion (NUE, NRUE) of the effect of the soil observed in the unfertilised control. There is a lack of consistency in literature regarding the use of unfertilised control treatment [59]. It is possible that in a short-term field trial, the unfertilised plot could still benefit from fertiliser application of previous years [60]. Hence, both sets of agri-environmental indicators, i.e., i) ANR & NFRV and ii) NUE & NRUE were determined to observe the effects of soil N on crop growth. Lack of differences in yield further reiterates the adequacy of the native soil N for crop requirements, in this scenario. In past studies with AN and AS conducted in controlled experimental conditions, the ANR in crops treated with these potential RENURE materials were higher than what was obtained in this trial (0.42-1.07 for AN and 0.55-0.73 for AS) [7,14]. The NUE results of treatments strengthen the assumption that most of the N uptake in crop could be from native soil N, rather than from the fertiliser application. Highest NUE was observed in treatment with PM, and this could be attributed to the lower N applied from the treatment. The N deficit, coupled with the lower water availability during the trial period ensured that the crops treated with PM scoured the soil for all available N, resulting in enhanced NUE, compared to the other treatments where N was provided in requisite amounts. This could imply that the N fertilisation doses should be reduced in nutrient surplus regions like Flanders with higher historic fertilisation rates. The highly variable results for ANR and NFRV as a result of the higher variability seen in yield and N uptake should be duly considered while interpreting these results.

3.2. Environmental impacts

3.2.1. Residual nitrate

Figure 3 presents data for residual NO_3^--N content in the 0-90 cm soil layer for all tested treatments. Both control treatments showed low residual NO_3^--N, whereas all fertilised treatments exhibited values higher than the maximum allowable legal limit of 80 kg NO_3^--N ha^{-1} [61]. Among the treatments with N fertilisation, AS (123 ± 65 kg ha^{-1}) and PU (82 ± 14 kg ha^{-1}) displayed the highest and lowest NO_3^--N, respectively. If there is excessive residual NO_3^--N in soil after harvest of crops, it can be flushed to the ground and surface water. Assessment of residual NO_3^--N in the 0-90 cm soil profile is done to understand the potential risk of NO_3^--N leaching by fertiliser application, which can saturate groundwater bodies and cause eutrophication. Since precipitation can flush out a significant portion of NO_3^--N, thus rendering the measurements performed after this period ineffective, the measurements are performed between 1st October and 15th November [61]. The sampling for NO_3^--N estimation in this trial also faced some marring weather-related effects. After the harvest of the crop in September 2019, the sampling of soil was done 19 days later in October, due to the frequent heavy precipitation. In the days ensuing harvest, the field received a total precipitation of 106.4 l m^{-2}, which is assumed to have led to significant NO_3^--N leaching in all plots. Lower NO_3^--N concentrations in the top soil layer (0-30 cm), and higher as well as more variable concentrations in the succeeding layers (30-60 cm and 60-90 cm), add strength to the assumption of leaching. As expected, the unfertilised plots show significantly lower NO_3^--N in comparison to the fertilised treatments. Also, all fertilised treatments demonstrate NO_3^--N higher than the legally stipulated value of 80 kg NO_3^--N ha^{-1}. Although, in general, all tested treatments exhibit high standard deviation owing to the weather-induced variability as well as the variable nature of soil NO_3^--N in NO_3^--rich fields, the RENURE materials behave comparably to the chemical N treatment.

3.2.2. Response of soil biota to BBF application

3.2.2.1. Community composition. The bacterial communities of all fertilised treatments were dominated by phyla consistent with the general composition of soil bacterial communities, based on a survey of 16S rRNA libraries [62], including Actinobacteriota (26.9%), Proteobacteria (18.5%), Firmicutes (18.3%) and Acidobacteriota (12.9%) (Figure S1). Communities remained relatively consistent in their abundances, with few exceptions (Table S2). The Proteobacteria, which are associated with a wide variety of functions involved in carbon , N and S cycling [63], significantly increased in abundance by approximately 4% in soil treated with PU compared to the synthetic NPK treatment. It is possible that PU application increased the abundance of NH_3-oxidising bacteria belonging to this phylum which aid in the nitrification process (e.g., genera such as Nitrosomonas, Nitrosococcus, Nitrobacter, etc.), as this has been noted previously in various studies upon application of animal urine [64]. Myxococcota were significantly enriched by application of AN and AS as the N source, increasing from 1% in the synthetic NPK treatment to 1.4% in both AN and AS treatments. These are a group of bacterial micropredators within soil ecosystems with diverse metabolic capabilities, including N cycling [65].

The soil fungal community was dominated by the phyla Ascomycota, Basidiomycota and Mortierellomycota, with average relative

Table 5
Mean ± stdev of alpha diversity indices for bacteria, fungi and nematodes in each treatment group (n=3 or n=4)

	Bacteria		Fungi		Nematodes	
Treatment	Observed OTUs	Shannon index	Observed OTUs	Shannon index	Observed OTUs	Shannon index
PK	2310 ± 54^{a}	9.20 ± 0.05^{ab}	343 ± 97	3.38 ± 1.36^{a}	172 ± 17.2^{ab}	3.51 ± 0.67
NPK	2300 ± 61^{a}	9.10 ± 0.07^{a}	480 ± 33	5.50 ± 0.18^{b}	173 ± 5.7^{a}	3.63 ± 0.90
PM	2340 ± 15^{a}	9.20 ± 0.08^{ab}	446 ± 16	5.00 ± 0.21^{a}	146 ± 21.6^{abc}	2.59 ± 0.97
AN	2550 ± 95^{b}	9.38 ± 0.10^{c}	463 ± 78	5.32 ± 0.29^{ab}	133 ± 14.6^{c}	3.32 ± 0.74
AS	2507 ± 61^{b}	9.32 ± 0.03^{c}	475 ± 77	5.44 ± 0.26^{ab}	154 ± 1^{abc}	3.49 ± 0.57
PU	2515 ± 101^{b}	9.36 ± 0.16^{bc}	530 ± 43	5.62 ± 0.13^{b}	140 ± 17.5^{bc}	3.38 ± 0.59

PK Control: Synthetic phosphorus and potassium; NPK: Synthetic nitrogen, phosphorus and potassium; PM: Pig manure; AN: ammonium nitrate; AS: Ammonium sulphate; PU: Pig urine.
Values in columns followed by different lowercase letters are significantly different from each other at $P < 0.05$.

abundances among samples of 46.3%, 30.8% and 4.5%, respectively (Figure S2). These results are in line with a global study which examined the soil fungal communities across 365 different sites [66]. The remainder of sequences were either from unidentified phyla of the fungal kingdom or belonged to phyla present with very low abundances. Though, variation in the abundances of fungal taxa was evident between the different fertiliser treatments, no significant changes were identified, owing to the high standard errors among samples within the treatment groups. Variation in community data among fungal sample replicates is recognised and it is suggested that this variation can be reduced by increasing sample numbers or volume, or by sample pooling, though these solutions come at a higher cost [67–69].

The soil nematode community was dominated by the order Dorylaimida, followed by Rhabditida, Diplogasterida, Araeolaimida, Monhysterida, Tylenchida, Enoplida and Triplonchida, together accounting for 90% of the total nematode sequences in samples (Figure S3). The relative abundance of dorylaimids, which are sensitive to environmental disturbances, significantly decreased in treatment with synthetic NPK (Table S4) when compared with those of PM (P=0.02) and AS (P=0.04) treatments. Dorylaimida was the dominant nematode order in PM and AS accounting for 45.2% and 33.1% respectively, on average. These dorylaimid persisters favour undisturbed environments [32] and a decrease in their abundance may indicate disturbance or pollution of their habitat. However, there was no significant difference in terms of dorylaimids between the synthetic NPK treatment and the tested BBFs.

3.2.2.2. Alpha diversity. Diversity analysis was performed on bacterial, fungal and nematode samples rarefied to 22,698, 23,958 and 46,218 sequence reads, respectively. Numbers of observed bacterial OTUs were significantly higher ($P<0.05$) in the RENURE treatments compared to PK and both reference treatments (synthetic NPK and PM). The Shannon's index for bacteria, measuring species richness and evenness, was significantly higher in the AN and AS treatments, compared to unfertilised PK, synthetic NPK and PM treatments, and in the PU treatment, compared to the synthetic NPK. The environmental conditions immediately following the application of the RENURE materials may have resulted in the remarkable increase in bacterial diversity, and eventually reverted to background levels as a result of microbial cycling, abiotic chemistry and plant metabolism. The introduction of an external microbial community with fertiliser input cannot be ruled out as a factor increasing bacterial diversity. This is counterintuitive, however, given that increases were not observed in soil with the PM treatment, as it is widely accepted that organic inputs, such as manure, increase soil biodiversity [70]. Furthermore, total viable bacterial counts in a previous study found BBFs like AN to harbour little to no aerobic mesophiles [7].

The Shannon index for fungal diversity was significantly higher in NPK and PU treatments in comparison to both, the PK and PM treatments. Consistent with findings of [71], the application of N fertiliser to the soil appeared to stimulate fungal diversity when compared to the PK control treatment.

The number of observed nematode species in the AN treatment significantly decreased when compared with those in NPK (P=0.02) and PK (P=0.02) control treatments (Table 5). The PU treatment showed the lowest number of observed nematode species when compared with those in the NPK treatment. Despite the difference in numbers of nematode observed OTUs, there was no significant difference in the Shannon's index between the treatment groups. The species richness and evenness remained consistent among the treatment groups.

3.2.2.3. Beta diversity. The bacterial communities among the treatment groups were not significantly different based on Weighted Unifrac scores (P=0.108), but significant differences were detected based on Unweighted Unifrac scores (P=0.008). The BBF-treated plots were observed to have a significantly different bacterial community structure ($P<0.05$) compared to the synthetic NPK treatment (Table S3). While no tight clustering was apparent, separation between unfertilised PK, synthetic NPK and PM, and both the AN and AS treatments was evident in non-metric multidimensional scaling (NMDS), plotted based on unweighted Unifrac distances (Figure 4). Additionally, NMDS illustrated how phylogenetically variable the community of the PU treatment was. The physical state of the N fertilisers may have played a significant role in driving bacterial community structure. It is possible that the liquid nature of the BBFs allowed microbial populations to flourish due to the provision of rapidly available nutrients and additional moisture. One recent study found that soil biological properties of maize were enhanced under water stress where liquid organic fertilisers were applied in a pot experiment, while another found liquid organic fertilisers to significantly increase microbial diversity in the rhizosphere of chrysanthemum [72,73]. While the specific cause of this marked increase remains unclear, it is likely to be a combination of the above factors. Further study under regular weather conditions would be informative.

Dissimilarity of fungal communities was not significant when measured by either Weighted Unifrac (P=0.106) or Unweighted Unifrac (P=0.676). Due to the high level of sample variability within treatment groups, statistically, the response of fungal communities between the various fertilisation treatments were minor overall. Generally, N fertiliser is known to alter fungal communities [74]. However, other studies have shown fungal communities to be more responsive to crop species than fertilisation treatments [75,76]. The lack of significant differences among the treatments in the various analyses suggested that the resident fungal communities remained relatively stable under BBF treatments. However, a larger sample size may have provided deeper insight.

The nematode communities' structure in PU (Table S5) was significantly different to those in synthetic NPK treatment (P=0.039). This is likely due to the significantly increased relative abundance of bacterial feeding members of the order Diplogasterida in PU when compared with NPK (P=0.024). Diplogasterids, also known as enrichment opportunists [31], were more likely attracted by Proteobacteria, which significantly enriched the PU treatment when compared with mineral NPK (Table S3). The nematode food web becomes enriched when increased microbial activity enhances the bacteria feeding enrichment

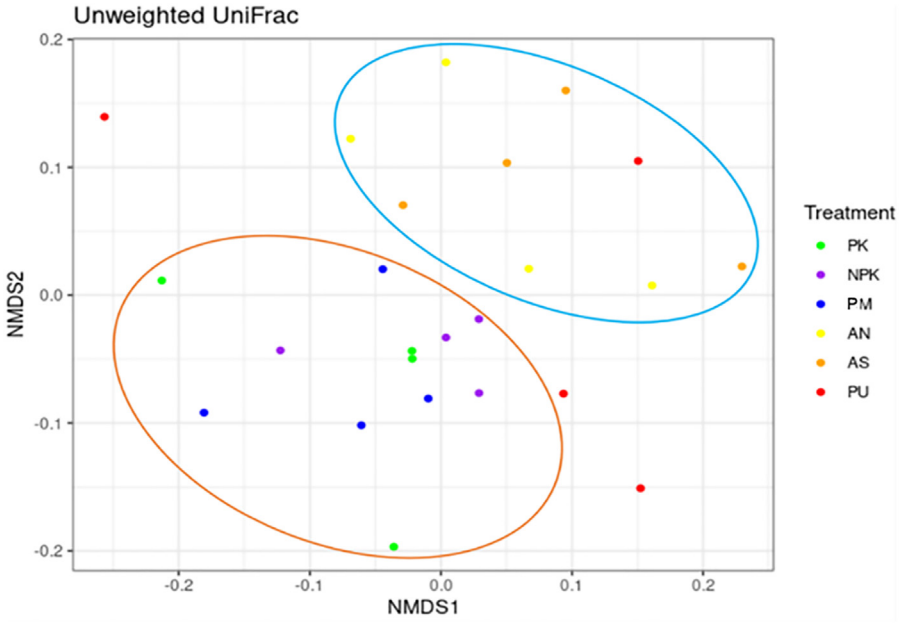

Figure 4. NMDS plot based on Unweighted Unifrac distances of bacterial communities. Samples are coloured by treatment.
PK: zero N control; NPK, synthetic nitrogen, phosphorus and potassium; PM, pig manure; AN, ammonium nitrate; AS, ammonium sulphate; PU, pig urine. The orange circle represents clustering of control treatments, and the blue circle represents clustering of the AN and AS treatments. Stress=0.166.

opportunists [19]. Based on Unweighted Unifrac distances, the nematode communities in the three tested RENURE materials were significantly different to those in synthetic NPK treatment ($P<0.05$) (Table S6). This could be related to the alpha diversity indices and a significant reduction of observed nematode species in the AN and PU treatments when compared to those in synthetic NPK.

Frequent and/or long-term droughts can change the structure of nematode communities and weaken their role in the agricultural soil food web systems [77]. In addition to dry soil conditions, temperature was reported as an important environmental factor affecting nematode communities [77–80]. A high air temperature, drought, and dry soil condition that prevailed during this field trial could have affected nematode diversity and community composition. Previous long-term N fertilisation studies showed that the nematode abundance, diversity and community composition fluctuated among different fertilisation treatments [81–83]. Moreover, the responses of soil nematode communities to the addition of N often varied with the time of the growing season.

5. Conclusion

This study assessed the short-term effects of three N-based BBFs, which can potentially replace the Haber-Bosch derived chemical N fertilisers as RENURE materials, once the regulation comes to force. This trial outcome expounds on crop response to RENURE material fertilisation in NVZs under changing climatic scenarios. The results of the trial exhibited no significant differences in the context of crop yield and residual NO_3^--N content between the tested RENURE materials and the synthetic fertiliser reference. The significantly lower N uptake in treatment with PU, which could be attributed to the weather-related impacts faced by the crops during their growth, compared to the synthetic NPK treatment, did not affect the maize yield. The authors emphasise on multi-year trials with RENURE materials to corroborate the effects of weather impacts observed in this study. The results of agri-environmental parameters like ANR and NFRV indicate the overall nutrient richness of soil which is a result of the historic fertilisation practices.

The study on soil biota demonstrated that microbial communities responded well to BBFs, i.e. the communities either remained stable, or displayed increased levels of diversity, which is indicative of healthy, productive soils. Nematode community diversity significantly changed under the various fertilisation treatments of this trial. After the application of AS, the nematode communities were more structured and less disturbed when compared with the communities in the synthetic NPK treatment. This is concluded due to the high relative abundance of persistent and long-lived dorylaimids in the AS treatments. Additionally, variations in nematode community composition and diversity could be due to exceptionally dry weather conditions and N addition. Long-term studies with diverse crop varieties are necessary to draw definite conclusions and to validate the findings of this short-term trial.

Data availability statement

Sequence information available at NCBI Sequence Read Archive database, bioproject PRJNA762644. Bacterial sequence accession numbers SRR20821390–SRR20821413. Fungal sequence accession numbers SRR20824911-SRR20824934. Nematode sequence accession numbers SSR20824275–SSR20824298.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.clcb.2023.100043.

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Erratum

Erratum to "Exploring the short-term in-field performance of Recovered Nitrogen from Manure (RENURE) materials to substitute synthetic nitrogen fertilisers" [Cleaner and Circular Bioeconomy Volume 5, August 2023, 100043]

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