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Nitrogen dynamics and carbon sequestration in soil following application of digestates from one- and two-step anaerobic digestion

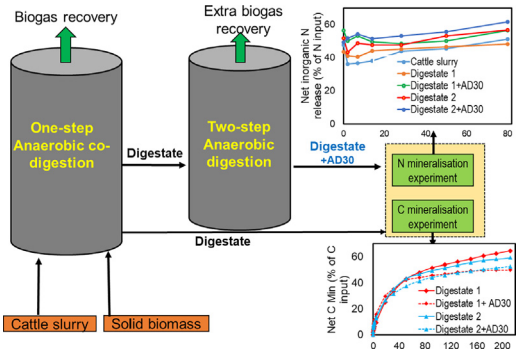
Jared Onyango Nyang'au [a,*], Henrik Bjarne Møller [b], Peter Sørensen [a]

[a] Department of Agroecology, Aarhus University, Blichers Allé 20, 8830 Tjele, Denmark
[b] Department of Biological and Chemical Engineering, Aarhus University, Blichers Allé 20, 8830 Tjele, Denmark

HIGHLIGHTS

- A secondary anaerobic digestion (AD) step increases net inorganic N release from digestates in soil.
- Two-step AD decreases digestate carbon mineralisation in soil due to extended decomposition in the AD process.
- The fertiliser value of digestates can be improved by prolonged digestion time without affecting the long-term soil C retention.
- Effects of a second step AD on C and N dynamics are dependent on hydraulic retention time utilized in the primary AD step.

GRAPHICAL ABSTRACT



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ABSTRACT

Anaerobic digestion (AD) is an important tool for reducing greenhouse gas emissions from agricultural production. A prolonged retention time by adding an extra anaerobic digestion step can be utilized to further degrade the digestates, contributing to increased nitrogen mineralisation and reducing decomposable organic matter. These modifications could influence the potential N fertiliser value of the digestate and soil carbon sequestration after field application. This study investigated the effects of prolonging retention time by implementing an additional anaerobic digestion step on carbon and nitrogen dynamics in the soil and soil carbon sequestration. Two digestates obtained from two biogas plants operating at contrasting hydraulic retention times, with and without an additional digestion step, were applied to a loamy sand soil. N mineralisation dynamics were measured during 80 days and C mineralisation during 212 days. After 80 days of incubation, the net inorganic N release from digestates obtained from a secondary AD step increased by 9–17 % (% of the N input) compared to corresponding digestates obtained from a primary AD step. A kinetic four-pool carbon model was used to fit C mineralisation data to estimate carbon sequestration in the soil. After 212 days of incubation, the net C mineralisation was highest in undigested solid biomass (68 %) and digestates obtained from the primary AD step (59–65 %). The model predicted that 26–54 % of C applied is sequestered in the soil in the long-term. The long-term soil C retention related to the C present before digestion was similar for one- and two-step AD at 12–16 %. We conclude that optimizing the anaerobic digestion configurations by including a secondary AD step could potentially replace more mineral N fertiliser due to an improved N fertiliser value of the resultant digestate without affecting carbon sequestration negatively.

* Corresponding author.
E-mail address: jn@agro.au.dk (J.O. Nyang'au).

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1. Introduction

Anaerobic digestion (AD) is a well-established biological process for the degradation and stabilisation of wastes, resulting in biogas production and a digestate as the by-product (Uludag-Demirer and Demirer, 2021). The biogas industry has received considerable attention in many European countries due to government incentives to promote carbon-neutral energy production as envisaged by the European Union (European Commission, 2018). The AD process can potentially reduce synthetic fertiliser utilization, greenhouse gas emissions (GHG) from biowastes, energy and nutrient leakage (Møller et al., 2022). Moreover, the digestate from the AD process receives much attention due to its agronomic benefits; it can be used as a bio-fertiliser, providing a sustainable substitute for mineral fertilizers and as a critical input in stockless organic farming (Zilio et al., 2022). During AD, organic nitrogen in the biowastes undergoes mineralisation to NH_4^+-N, increasing the NH_4^+-N/N ratio in the digestate. In addition, it reduces decomposable carbon in the digestate, improving the fertiliser value of the digestate as the nitrogen immobilisation potential is reduced (Sørensen et al., 2012).

Despite AD enhancing the utilization of nitrogen in the inputs in addition to the biogas yield, it faces limitations associated with the recalcitrant nature of the substrates, where only 40–70 % of the carbon in the agricultural substrates is converted into biogas during the AD process. This results in a digestate with high residual methane potential due to undecomposed organic matter (Romio et al., 2021). In addition, the agronomic benefits of the resultant digestate, storage, processing, and land application are associated with environmental concerns such as ammonia volatilization, GHG emission, and nutrient leaching (Møller et al., 2022; Moller and Muller, 2012). To alleviate these problems and improve the fertiliser value of the digestate, there is a need to develop a digestate management plan to maximise substrate degradation and digestate stabilisation (Zilio et al., 2022). Such an improved digestate management plan could include, e.g. a longer hydraulic retention time (HRT) in one or more digestion steps.

Several biogas plants have chosen a two-step configuration in Denmark (Møller and Nielsen, 2016). In a two-step AD process, biowastes are digested in a primary reactor (primary AD step) and then transferred to a second reactor, where they undergo a further AD process (secondary AD step). The serial continuous-flow stirred tank reactor (CSTR) configuration with one reactor having a long HRT and a second one having a short HRT can improve the biogas production and achieve a better digestate quality in comparison to a single CSTR reactor due to a better conversion of degradable matter (Boe et al., 2009). HRT is an important operating parameter for biogas reactors that affect the reactor's stability and performance. Increasing HRT can increase biogas yield and biowastes degradation, depending on the input quality (Shi et al., 2017).

Effects of HRT and digester's configuration, including a secondary AD step, have been widely studied, focusing on energy recovery from biowastes. However, the effects on digestate nitrogen mineralisation and organic carbon turnover in the soil are scarcely studied. Therefore, this study aims to evaluate the effects of a secondary AD step on C and N dynamics following the use of two contrasting primary AD step digestates as input. Additionally, the study aims to evaluate the relationship between the digestates' biochemical properties and the C and N mineralisation in soil and how the secondary AD step affects the carbon sequestration in soil. A secondary AD-step is hypothesized to significantly enhance the mineral N release from digestates without significantly affecting soil carbon sequestration.

2. Materials and methods

2.1. Soil

The soil for this study was sampled from the top 20 cm plough layer from an arable field at Research Centre Foulum, Aarhus University, Denmark (56°30′N, 09°35′E). The soil is a loamy sand with dry matter containing 83 g clay kg^{-1}, 284 g silt kg^{-1}, 610 g sand kg^{-1}, 31 g organic matter kg^{-1}, 15.6 g total C kg^{-1}, 1.3 g total N kg^{-1} and pH (1,2.5 H_2O) of 6.51. The soil's water holding capacity (WHC) is 0.428 g g^{-1} dry soil. Air-dried soil was sieved through a < 4 mm sieve to remove stones and larger plant residues, homogenized and then stored at 10 °C. The soil has properties representing quite well the average of agricultural soil types in Denmark.

2.2. Substrates and digestates for incubation

Pre-digested digestates (AD1 and AD2) were obtained from Aarhus University, Foulum biogas plant (Tjele, Denmark) and Ausumgaard biogas plant (Hjerm, Denmark), respectively, two reactors running at different HRT. The Foulum biogas reactor was managed at a short HRT (14 days) at thermophilic conditions (51 °C). Prior to sampling, the reactor was fed 75 % cattle slurry and 25 % mixed grass-clover silage (solid biomass). The Ausumgaard biogas reactor was managed at a longer HRT (60 days) at thermophilic conditions (51 °C). The inputs comprised 55 % slurry, cattle slurry and pig slurry in a wet weight ratio of 7:3 and 45 % solid biomass, including deep litter, straw, chicken manure and grass.

Portions of digestates from Foulum (AD1) and Ausumgaard (AD2) biogas plants (from the primary AD step) were further anaerobically digested in a secondary AD step using 15 L laboratory-scale continuously stirred tank reactors (CSTR), resulting in digestates hereby referred to as $AD1^+$ and $AD2^+$, respectively. Before the start-up phase, all the reactors were filled with 1.5 L of inoculum and 13.5 L of materials from the first digestion steps and digested at 51 °C for 30 days. Weekly sub-samples were taken from the reactors to analyze VFA, ammonium nitrogen and pH to monitor the stability and possibility of inhibition in the AD process. Additionally, biogas yields were monitored weekly. After 30 days, the digestates were emptied manually and stored frozen at −18 °C. Table 1 provides an overview of the biochemical characteristics of the substrates and digestates used for the incubation experiment. Samples of substrates used at Ausumgaard biogas plant were not analysed as they were not available.

2.3. Soil incubation experiments

Two soil incubation experiments were set up based on different experimental protocols with the same input materials. The first experiment studied net N mineralisation after applying substrates and digestates to soil, while the second experiment studied carbon mineralisation after application to soil. The incubation experiments consisted of substrates obtained from the primary AD step (AD1 and AD2) from the two full-scale biogas plants and digestates obtained after a secondary AD step in a laboratory-scale CSTR with additional 30 days retention time ($AD1^+$ and $AD2^+$). Additionally, the undigested cattle slurry and the solid biomasses, key substrates for the primary stage AD in the Foulum biogas reactor, were included in this study.

The N mineralisation experiment involved weighing 50 g of the soil based on the dry matter weight into a 250 mL polyethylene bottle. The organic materials were added at the rate of 200 mg N Kg^{-1} soil (Table 1), followed by another 50 g of soil to cover the materials. The application rate of the treatments is equivalent to 180 kg N ha^{-1} assuming a 7-cm layer of soil is affected by the organic addition in the field (Sørensen et al., 2003). Based on the proportion of the solid biomass added to the digester (25 %), the solid biomass was only applied at a rate of 90 mg N Kg^{-1} soil (Table 1). Water was added to each bottle to achieve a moisture level equivalent to 55 % of the water holding capacity (WHC), optimal for microbial activity and low enough to limit denitrification. The bottles were covered with hole-pierced parafilm to prevent high water loss through evaporation while ensuring aeration during the incubation period. Each treatment was replicated 21 times to allow three replicates to be destructively sampled for analysis on days 0, 2, 7, 14, 28, 50 and 80. Reference treatments included a control soil and ammonium sulphate (200 mg N Kg^{-1} soil), giving a total of 168 samples. The samples were incubated at 10 °C in a dark room, representing the typical soil temperature in spring under field conditions in Denmark when manures are applied to fields so

Table 1
Biochemical characteristics of the substrates and digestates used for incubation experiment and amount of C and N applied to the soil. Values correspond to averages and standard deviations in brackets (n = 3).

Organic treatments	Concentrations in digestates and substrates											Applied to soil	
	DM	VS	pH	Total N	NH_4^+-N	Total C	C/N	Hemicellulose	Cellulose	Lignin	NDSF	C	N
	% of FM	% of DM	–	% of FM	% of total N	% of FM	ratio	% of DM	% of DM	% of DM	% of DM	mg C g^{-1} soil	μg N g^{-1} soil
Solid biomass[a]	32.1	93.9	–	0.66	7	16.54	25.1	23.9	30.8	8.6	36.9	2.26	90
	(0.22)	(0.43)		(0.021)		(0.21)		(0.80)	(0.98)	(0.41)	(0.32)		
Cattle slurry[a]	5.0	76.9	7.09	0.24	52	2.26	9.4	14.0	16.3	8.6	60.2	1.87	200
	(0.02)	(0.97)		(0.003)		(0.01)		(0.68)	(0.19)	(0.09)	(1.82)		
Solid biomass + cattle slurry[b]	11.8	81.2	–	0.35	41	5.85	13.3	16.4	20.0	8.6	54.4	1.97	200
	(0.04)	(0.62)		(0.006)		(0.05)		(0.38)	(0.37)	(0.35)	(1.44)		
AD1	7.6	80.3	7.52	0.37	46	2.35	6.4	13.8	24.6	15.5	46.4	1.29	200
	(0.12)	(0.14)		(0.002)		(0.01		(0.64)	(0.88)	(0.62)	(0.75)		
$AD1^+$	6.3	79.1	7.55	0.33	62	1.94	5.9	7.9	22.4	17.3	52.3	1.16	200
	(0.27)	(1.07)		(0.009)		(0.05)		(0.25)	(1.22)	(0.21)	(0.70)		
AD2	9.2	74.6	8.01	0.53	52	3.49	6.6	4.5	18.2	15.7	61.5	1.32	200
	(0.09)	(0.18)		(0.001)		(0.13)		(0.05)	(0.24)	(0.60)	(0.33)		
$AD2^+$	8.9	74.8	8.49	0.50	60	3.17	6.3	2.5	18.2	15.3	64.1	1.26	200
	(0.02)	(0.43)		(0.04)		(0.10)		(0.15)	(0.70)	(0.20)	(0.51)		

FM = Fresh matter, VS = Volatile solids, DM = dry matter, NDSF = Neutral detergent soluble Fibre, AD1 = digestate 1 from primary AD step, $AD1^+$ = digestate 1 after secondary AD step, AD2 = digestate 2 from primary AD step, $AD2^+$ = digestate 2 after secondary AD step for 30 days.
[a] Substrates for first-step digestion in Foulum biogas plant.
[b] Solid biomass + cattle slurry mixture properties calculated based on the proportions used in the Foulum biogas plant.

that the findings can be translated to practical scenarios. The soil moisture content was monitored every 2 weeks and adjusted based on the weight. For the N mineralisation experiment, the soil was removed from the polyethylene containers, homogenized, and 25 g of the soil sample was extracted by 100 mL of 1 M KCL solution by mechanical shaking for 1 h (end-over-end) on each sampling day. Extracts were frozen at −18 °C until analysis of soil inorganic N (NO_3^--N, NO_2^--N and exchangeable NH_4^+-N). Soil pH (in 1 M KCl extracts) and soil moisture content were also measured.

Carbon mineralisation from the same organic materials was evaluated by measuring CO_2 evolution from amended soil compared with a reference soil only receiving ammonium sulphate. First, 50 g of soil (oven-dry basis) was packed into stainless steel cylinders with a diameter of 6.3 cm, followed by organic materials or ammonium sulphate at the same rate as the first study, and then another 50 g of soil was added. The soil was then moistened to achieve soil moisture equivalent to 55 % WHC. The cylinders were weighed and placed in 0.5 L respirometric jars, then transferred to a RESPICOND VI respirometer (A. Nordgren innovations AG, Bygdev, Sweden) with a capacity of 48 respirometric jars. The jars were submerged in an insulated water bath set at 20 °C to maintain a constant temperature. The carbon dioxide evolved from the treatments was absorbed by 20 mL of 0.6 M KOH solution in a beaker within the jars. The captured CO_2 was monitored through the decrease in conductance of the KOH solution (Thomsen et al., 2013). The monitoring was done automatically with the respirometers' platinum electrodes for 212 days at the initial logging intervals of 1 h and later 6 h after the first 3 days. The moisture content of the soil was monitored regularly and adjusted accordingly. The KOH solution was replaced whenever one of the treatments reached 80 mg of accumulated CO_2. There were three replicates of each treatment, and empty jars were used as blanks.

2.4. Chemical analyses of digestates and soil

Total N in the substrates and digestates was analysed according to APHA (2005) by the Kjeldahl digestion method (Kjeltec™ 8400, Foss Analytical CO. LTD, Denmark). Ammonium-nitrogen was analysed using an automated distillation-titration method (Sommer et al., 1992) using a Gerhardt Vapodest 10s distillation apparatus (Bonn, Germany). Acid detergent fibre (ADF), acid detergent lignin (ADL) and neutral detergent fibre (NDF) in the substrates and digestates were analysed to determine the hemicellulose, cellulose and lignin contents (Van Soest et al., 1991) using a Foss Fibertec 2010 System (Foss Analytical Co. Ltd., Denmark). The inorganic N (NO_3^--N, NO_2^--N and exchangeable NH_4^+-N) in soil extracts was analysed by AA500 Auto Analyzer flow colourimetry (Seal analytical GmbH, Germany). Total carbon content was analysed by combustion elemental analysis (Vario Max Cube, Elementar Analysensteme GmbH). The dry matter content of the substrates and digestates was determined by drying at 80 °C for 24 h and of soil by drying at 105 °C.

3. Calculation and data analysis

The net inorganic N release in the soil as % of N input from the organic inputs was estimated as the difference between the total inorganic N (NH_4^+-N + NO_2^--N + NO_3^--N) in the amended soils and reference soils without any amendment divided by total N in the organic inputs. Denitrification and N_2O losses from the soils after incorporating digestates were assumed to be negligible or similar from all the treatments. The accumulated CO_2 emissions from the treatments incubated in the soil were used to calculate the net C mineralized as a percentage of C input from the treatments according to Eq. (1).

$$\text{Net C mineralisation } (\%\text{C added}) = \frac{0.273\left(CO_2t_{x-\text{Sample}} - CO_2t_{x-\text{control}}\right)\text{mg}}{C_{\text{added (mg)}}} \times 100 \quad (1)$$

where $CO_2t_{x\text{-sample}}$ is the accumulated CO_2 in the soil amended with the treatment after time x, $CO_2t_{x\text{-control}}$ is the accumulated CO_2 in the control reference soil without amendment after time x, 0.273 is the conversion factor from CO_2 to C and C_{added} is the amount of C added by the treatment in mg.

The C mineralisation kinetics of the substrates and digestates were fitted to a four-pool carbon (C_1, C_2, C_3, and C_4) model. Carbon pools defined by half-lives of 4, 20 and 100 days were chosen to represent the fastest turnover rate (C_1), medium turnover rate (C_2) and slow turnover rate (C_3) (Thomsen et al., 2013). Assuming the sum of three-carbon pools (C_1, C_2 and C_3) as the amount of C lost from the soil due to microbial respiration after incorporation of the treatments, the rest of the C proportion (C_4) is assumed to be stabilized and therefore sequestered in the soil for a prolonged period. The C mineralisation of a given pool size C_i was assumed to follow first-order kinetics, Eq. (2).

$$\frac{dC_I}{dt} = -k_i C_I \quad (2)$$

where K_i is the turnover rate of the ith carbon pool, and t is the time. Therefore, the net C mineralized from time 0 to t is described by Eq. (3);

$$\text{Net C mineralisation} = \Sigma_{i=1}^{3} Ci[1-\exp(-kt)] \quad (3)$$

where the Ci is the size of the carbon pool at time 0, the net C mineralized was modelled as a function of time, and the model parameters were estimated by a non-linear regression model with the aid of the solver function of Microsoft Excel® Tool Pak. The defined half-lives correspond to turnover rates with $k_1 = 0.173$, $k_2 = 0.0347$ and $k_3 = 0.0069$.

To estimate the long-term C retention in the soil as a fraction of the initial C present before the first digestion step, the C recovered as biogas (CH_4 and CO_2) during primary and secondary AD steps are estimated based on the total C in the digestates. Then, using the proportions of the C mineralized during incubation of the treatments as modelled by the four-pool C model, the % of C retained in the soil was estimated (Table 6). The higher temperature for the C mineralisation experiment (20 °C), twice the average Danish spring temperatures, was selected so that the long-term C retention in the soil in the field set-up from the digestates could be estimated in accelerated controlled laboratory conditions over a shorter period, and under conditions similar to Thomsen et al. (2013).

The statistical software R version 4.0.3 (R Development Core Team, 2021) was used to analyze the data. The effect of treatments was evaluated by analysis of variance (ANOVA). In addition, a Tukey HSD test was performed to identify treatments significantly different. In addition to Shapiro-Wilk tests, a visual examination of the residuals against the fitted values was performed to confirm normality and homoscedasticity assumptions. The linear correlations between net C and N mineralisation and biochemical characteristics (i.e. lignin, cellulose, hemicellulose, C/N and NH_4^+-N/N ratio) were assessed by Pearson correlation coefficients using the R cor.test function. For all statistical tests, a significance level of 5 % is used.

4. Results

4.1. Biochemical properties of the substrates and digestates after one-and two-step AD

The biochemical properties of the substrates and digestates are summarized in Table 1. The two-step AD process of AD1 resulted in a 17 % decrease in the dry matter content in $AD1^+$, whereas the two-step AD process on AD2 resulted in only a slight decline of 3 % in the dry matter content. The decrease in dry matter content correlates with a decline in total carbon content at 17 % and 9 % in $AD1^+$ and $AD2^+$, respectively (Table 1). The primary AD step of the substrates (solid biomass and cattle slurry) and the secondary AD step of AD1 and AD2 resulted in a significant ($p < 0.001$) increase in NH_4^+-N/N ratio, with a more significant effect observed in AD1 compared to AD2. The hemicellulose fraction was the most affected in the two-step AD process, with a 43–45 % decrease (on a DM basis) in both digestates, whereas cellulose decreased by 9 % in $AD1^+$ and <1 % in $AD2^+$. The lignin content remained almost the same; in some cases, it slightly increased. The C/N ratio of the digestates ranged from 5.9 to 6.6, whereas that of the solid biomass was the highest at 25 (Table 1).

4.2. Nitrogen dynamics in the soil

All the treatments showed a maximum NH_4^+-N content on day 0 of incubation, with the lowest being in control soil (0.4 mg NH_4^+-N kg^{-1} soil) and the highest in $AD1^+$ (113.4 mg NH_4^+-N kg^{-1}). After 2 days of incubation, the concentration of inorganic N in the soil decreased with the largest decrease after the application of cattle slurry (Fig. 1). After day 2, there was a slow increase in inorganic N continuing to day 80, except after the application of solid biomass that continued to immobilize N until around day 50. The addition of the decomposable matter to the soil from the organic treatments induced microbial activity, as observed by subsequent intense microbial respiration (Fig. 2). Soil NO_3^--N increased gradually throughout the incubation, with the highest in soil amended with $AD2^+$ and the lowest in soil amended with solid biomass (Fig. S1).

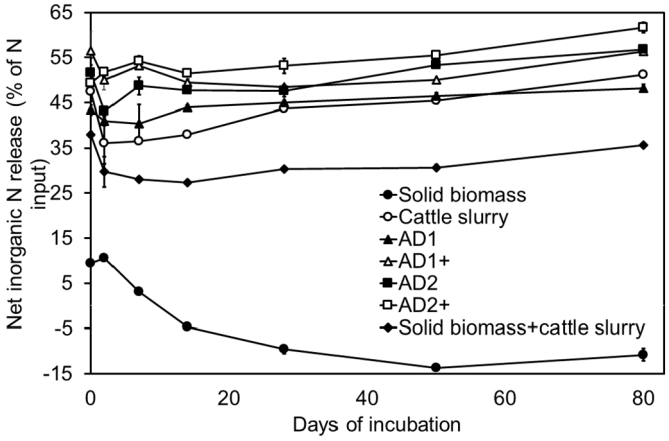

Fig. 1. Net inorganic N release (% of N input) in soil amended with substrates and digestates with different HRT in the primary AD stage and further digested in the secondary AD stage (+). Inorganic N in a control soil was subtracted to calculate the net N release. Bars indicate standard errors (n = 3). *The values for the solid biomass + cattle slurry mixture are calculated based on measurements in treatments with the single components using the proportions between inputs in the Foulum biogas plant (AD1, assuming no interaction).

The high C/N ratio in the untreated solid biomass significantly affected the net N release. Net N immobilisation occurred up to day 50 of incubation and was equivalent to around 20 % of the total N input, with a slightly positive net N release between days 50 and 80. After 80 days of incubation, there was a significant difference in the net inorganic N release among the treatments (Table 2), with the highest net N release after application of $AD2^+$ (62 % of N input) and the lowest from solid biomass (−11 % of N input). Based on the N release in soil measured separately from untreated solid biomass and cattle slurry and the proportion between these two inputs in the Foulum digester (for AD1), the net N release from the untreated mixture was estimated assuming no interaction between the two components. The estimated N release from the untreated mixture was equivalent to 36 % of the total N input (Fig. 1). The primary digestion step of co-digesting solid biomass and cattle slurry resulted in a significant increase ($p < 0.05$) of net inorganic N in AD1 by 13 % points related to N input. The secondary AD step with AD1 and AD2 as input resulted in a significant increase ($P < 0.05$) of net inorganic N release after 80 days of incubation

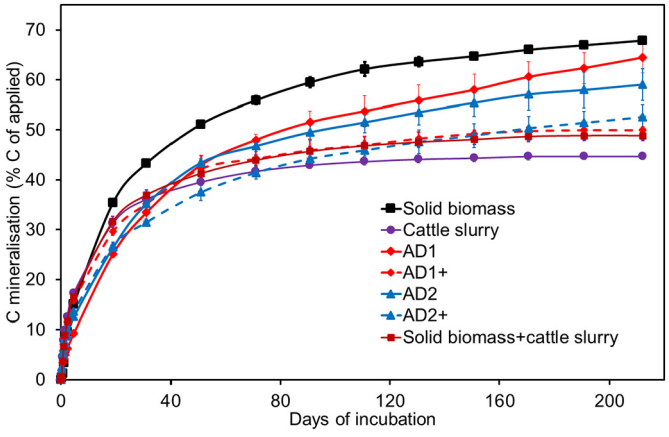

Fig. 2. Cumulated C mineralisation (% of the applied C) during 212 days of incubation of substrates used in primary digestion step and digestates from both primary (AD1 and AD2) and secondary ($AD1^+$ and $AD2^+$) digestion steps. Solid biomass + cattle slurry C mineralisation was estimated from the proportions of solid biomass and cattle slurry used in AD1. The background C mineralisation was subtracted using a reference soil. Bars indicate standard errors (n = 3).

Table 2
Total inorganic N (mg N kg^{-1} soil) in soil amended with substrates and digestates from primary and secondary ($^+$) AD steps and in reference soil with no amendment (n = 3).

Treatments	Days after application						
	0	2	7	14	28	50	80
Solid biomass	19.7^{d}	20.8^{d}	15.3^{e}	9.2^{g}	6.4^{f}	7.7^{f}	13.5^{g}
Cattle slurry	106.2^{abc}	83.5^{d}	85.4^{c}	89.5^{d}	102.4^{c}	111.0^{c}	125.6^{c}
Solid biomass + cattle slurry*	84.5^{c}	67.8^{c}	67.9^{d}	69.4^{e}	78.4^{d}	85.2^{d}	97.6^{e}
AD1	97.9^{bc}	93.3^{ab}	93.3^{bc}	101.4^{c}	105.2^{bc}	113.0^{c}	117.3^{d}
AD1$^+$	124.0^{a}	111.4^{a}	119.0^{a}	112.4^{b}	112.0^{b}	120.0^{b}	135.9^{b}
AD2	114.5^{ab}	97.6^{b}	109.9^{ab}	108.9^{b}	110.2^{b}	126.6^{a}	136.7^{b}
AD2$^+$	109.8^{ab}	114.7^{a}	120.8^{a}	116.5^{a}	121.3^{a}	131.1^{a}	146.5^{a}
Control soil	11.2^{d}	11.3^{d}	12.5^{f}	13.5^{f}	15.0^{e}	20.0^{e}	23.2^{f}

Means followed by different letters within each column are significantly different ($p < 0.05$).
* The values for the solid biomass + cattle slurry mixture are calculated based on measurements in treatments with the single components using the proportions between inputs in the AD1 (assuming no interaction).

(Table 2). The highest increase of inorganic N was observed after two-step digestion of AD1 to obtain AD1$^+$ by 17 % versus a 9 % increase in AD2$^+$ after two-step digestion of AD2 (Fig. 1, Table 2). The net inorganic N release from digestates after 80 days in soil was significantly influenced ($p < 0.001$) by both the source of the digestate and the AD step (Table S1).

4.3. Net inorganic N release from digestates related to biochemical properties

The net inorganic N release from the treatments after 80 days of incubation was negatively and strongly correlated with the C/N ratio of the substrates and digestates ($R^2 = 0.98$, $p < 0.0001$) (Fig. S2). Similar strong negative relationships between the C/N ratio and net N mineralisation were observed at different incubation periods except between days 0 to 7 and 51 to 80, where the relationship was insignificant (Table S2). Hemicellulose and cellulose were negatively correlated with the net inorganic N release after 80 days with correlation coefficients of $R^2 = 0.89$, $p < 0.0001$ and $R^2 = 0.85$, $p < 0.0001$, respectively, while NDSF was strongly and positively correlated with Net inorganic N release ($R^2 = 0.84$, $p < 0.0001$) (Fig. S2).

Lignin and hemicellulose concentrations in the treatments positively correlated with net N mineralisation at different stages of incubation, except between days 0 to 7 and 51 to 80, where the relationship was negative but insignificant. Using a multiple component linear model with hemicellulose, cellulose and lignin as parameters, cellulose content was negatively correlated with net N mineralisation at various stages of incubation except for periods 0 to 7 and 51 to 80 days, where it was positively correlated but insignificant (Table 4). On day 80 of incubation, cellulose was the only digestate characteristic that negatively influenced net N mineralisation in the soil (Table 4). The combined effect of lignin, hemicellulose and cellulose characteristics of the substrates and digestates was best related to the net N mineralisation between days 15 to 28 of incubation, as exhibited by the highest correlation coefficient for this period (Table 4). The net inorganic N release after 80 days of incubation was strongly and significantly correlated ($R^2 = 0.98$, $p < 0.0001$) with the NH_4^+-N/N ratio in the organic treatments (Fig. S2).

4.4. Carbon mineralisation and sequestration in soil

Amendment of the substrates and digestates to the soil caused a rapid increase in microbial activity, resulting in high CO_2 evolution during the first 10 days of incubation (Fig. 2). The average net CO_2 evolved differed significantly between some substrates and digestates in the first 10 days (Table 3), with the highest net CO_2 evolving from solid biomass. The two-step AD significantly ($P < 0.05$) affected the net C mineralisation among the digestates during this period, with the highest net C mineralized observed in AD1$^+$ (Table 3). Digestates obtained from the primary AD step had a lower CO_2 evolution in the first 20 days of incubation than digestates

Table 3
Mean fractions of C mineralized as a percentage of C added during different periods after application of different organic materials to soil kept at 20 °C.

	Net C mineralized (% of applied C)			
Treatments	0–10	11–80	81–212	0–212
	Days			
Solid biomass	25.7^{a}	31.9^{a}	10.2^{a}	67.8^{a}
Cattle slurry	24.7^{ab}	17.5^{c}	1.6^{a}	43.9^{d}
Solid biomass + cattle slurry*	24.5^{ab}	20.2^{c}	3.8^{a}	48.6^{cd}
AD1	16.5^{e}	33.5^{a}	14.7^{a}	64.5^{ab}
AD1$^+$	23.0^{bc}	21.9^{bc}	4.8^{a}	49.7^{bcd}
AD2	19.3^{d}	28.7^{ab}	11.0^{a}	59.1^{abc}
AD2$^+$	20.9^{cd}	21.9^{bc}	9.8^{a}	52.6^{abcd}

Means with different letters within a column are significantly different ($p < 0.05$).
* The values for the solid biomass + cattle slurry mixture are calculated based on measurements in treatments with the single components using the proportions between inputs in the AD1 (assuming no interaction).

obtained from the secondary AD step, but after 20 days, C mineralisation was higher than in the digestates obtained from the secondary AD step (Fig. 2). After 100 days of incubation, the rate of C mineralisation decreased in all the treatments until the end of the incubation, where they nearly became constant. The C mineralisation from the solid biomass had a lag phase in the first 2 days, after which the rate of CO_2 evolution increased and remained highest throughout the incubation period (Fig. 2).

In the period between day 11 to 80 days of incubation, there was a significant difference ($p < 0.001$) in net C mineralisation (% of C input) among the treatments, with the secondary AD step lowering the net C mineralized significantly by 12 % and 7 % points (based on % C input) in AD1$^+$ and AD2$^+$ respectively (Table 3). For the period between 81 and 212 days of incubation, net C mineralized (% of C input) from the treatments was nearly the same ($p > 0.05$). At the end of incubation, the soil amended with solid biomass showed the highest accumulated net C mineralisation, while the soil amended with a cattle slurry had the lowest C mineralisation. The high N immobilisation observed in the soils that received solid biomass (Fig. 1) is consistent with the high CO_2 evolution and microbial activity requiring high N assimilation in soil microflora. Moreover, the highest C mineralisation in solid biomass correlated with the highest hemicellulose and cellulose content among all the treatments (Table 1).

Among the digestates, the highest cumulative net C mineralisation was from the digestates sourced from the primary AD step at 65 % and 59 % (based on C input) for AD1 and AD2, respectively (Fig. 2, Table 3). The secondary AD step reduced the net C mineralisation by 15 % and 7 % points, respectively, in AD1$^+$ and AD2$^+$. These reductions corroborated with the reductions in hemicellulose and cellulose contents (Table 1) after the secondary AD step, possibly influencing their C mineralisation dynamics. The C mineralisation from the cattle slurry was intermediate for the first 40 days; then, it stabilized afterwards. At the end of 212 days of incubation, there was a slight difference in the cumulative net CO_2 release among the treatments, with only cellulose correlating positively with C mineralisation (Table 3, Table 4). The C mineralisation correlated negatively with both hemicellulose and lignin concentrations, with the most significant effect ($p < 0.05$) observed in the period between days 8 to 14 (Table 4).

A kinetic four-pool model generally fitted the experimental C mineralisation data well with R^2 values >0.95 and low RMSE values (Table 5). The distinct biochemical differences among the substrates and digestates were reflected in the carbon pools estimated by the four-pool carbon model (Table 5). This model gives the proportions of the labile and recalcitrant carbon in the organic materials. Cattle slurry had the highest proportion of easily decomposable C, while AD1 had the lowest. The secondary AD step increased the fast decomposable C pool proportion in AD1$^+$ and AD2$^+$ by 12 % points and 6 % points, respectively. The increased portion of decomposable C is in line with the fast and highest evolution of CO_2 from the digestates after the secondary AD step in the first 10 days of incubation (Fig. 2). The prolonged digestion step by a secondary AD step reduced the medium (C_2) and slow turnover (C_3) carbon pools in AD1$^+$. In contrast, for AD2$^+$, the slow C pool was relatively large, but the medium

Table 4
Multiple component linear models relating net N and net C mineralisation during different periods after application in the soil to lignin, cellulose and hemicellulose contents (% of DM) of the substrates and digestates.

	Time	Intercept	Slope			R^2
			Lignin	Cellulose	Hemicellulose	
Net N mineralisation	0–7	−5.56	-0.86^{ns}	1.38 ns	-1.47^{ns}	0.37
	8–14	−4.94	1.65*	−1.52**	1.26*	0.46
	15–28	11.03	0.51^{ns}	−1.10**	0.59*	0.73
	29–50	4.99	0.50^{ns}	−0.53*	0.12^{ns}	0.71
	51–80	11.21	-0.33^{ns}	0.02^{ns}	-0.26^{ns}	0.25
	0–80	16.73	1.45^{ns}	−1.75*	0.24^{ns}	0.72
Net C mineralisation	0–7	28.8	-0.89^{ns}	0.20^{ns}	-0.31^{ns}	0.41
	8–14	3.8	−0.22***	0.17***	−0.08*	0.94
	15–28	1.1	0.31^{ns}	0.06^{ns}	0.20^{ns}	0.33
	29–50	−4.29	0.51^{ns}	0.23^{ns}	0.07^{ns}	0.41
	51–80	1.1	-0.18^{ns}	0.39^{ns}	-0.19^{ns}	0.30
	0–80	32.7	-0.47^{ns}	1.12*	-0.26^{ns}	0.73
	81–212	−6.3	0.21^{ns}	0.73^{ns}	-0.32^{ns}	0.16
	0–212	26.3	-0.26^{ns}	1.85^{ns}	-0.58^{ns}	0.41

* $p < 0.05$ significant level.
** $p < 0.01$ significant level.
*** $p < 0.0001$ significant level.
ns = not significant.

turnover portion was also reduced (Table 5). The solid biomass had a relatively large C proportion in the C_2 and C_3 pools. As predicted by the kinetic model, the amount of applied C retained in the soil ranged from 26 % to 54 % (C_4 pool), with the lowest proportion being in the solid biomass. The two-step AD process increased the amount of carbon sequestered in the soil by 18 % and 6 % points in $AD1^+$ and $AD2^+$, respectively, when related to the C input to soil (Table 5). The long-term C retained in the soil by incorporating digestates from one- and two-step AD related to C before primary digestion was similar and ranged from 12 to 16 % (Table 6).

5. Discussion

5.1. Effects of one- and two-step AD on biochemical composition of the digestates

Variations in digestate characteristics after one- and two-step AD are primarily the consequence of differences in substrate nature, composition, and operating conditions of the biogas reactors (Uludag-Demirer and Demirer, 2021). Our findings show that one- and two-step AD of the substrates and digestates significantly enriched the NH_4^+-N content in the digestates, attributed to enhanced mineralisation of organically bound N during the digestion (Moller and Muller, 2012). The 35 % increase in NH_4^+-N/N ratio after the two-step AD of AD1 compared to the 17 % increase in $AD2^+$

Table 5
Estimated carbon pools (C_1, C_2, C_3 and C_4) in percentage of total C for the digestates and substrates used in the experiments as predicted by a four-pool model, including a stable pool (C_4).

Treatments	Carbon pool proportions (%)					
	C_1	C_2	C_3	C_4	RMSE	R^2
Solid biomass	17.3	32.0	24.3	26.4	0.27	1.000
Cattle slurry	24.1	16.6	5.8	53.5	2.06	0.997
Solid biomass + cattle slurry*	22.4	19.0	10.4	48.2	1.12	0.998
AD1	7.3	29.0	34.9	28.7	0.77	0.998
$AD1^+$	19.2	22.0	12.2	46.6	3.04	0.995
AD2	12.3	26.5	26.0	35.2	2.01	0.997
$AD2^+$	18.2	13.3	27.5	41.0	2.63	0.997

C_1 = fastest turnover carbon pool, C_2 = medium turnover carbon pool, C_3 = slow turnover carbon pool, C_4 = stable turnover carbon pool, RMSE = root mean square error, R^2 = coefficient of determination.

* The values for the solid biomass + cattle slurry mixture are calculated based on measurements in treatments with the single components using the proportions between inputs in the AD1 (assuming no interaction).

Table 6
Estimated long-term C retention in the soil based on % C before primary AD of the digestates obtained from primary and secondary AD steps.

Treatment	C before primary AD	C after primary AD[a]	C after secondary AD[b]	Estimated C mineralisation in soil[c]	Long-term C retention in soil
	% of C before primary AD			% of applied C	% of C before primary AD
AD1	100	40	–	71	12
$AD1^+$	100	40	33	53	16
AD2	100	40	–	65	14
$AD2^+$	100	40	36	59	15

[a] Based on 60 % reduction of C in AD1 by primary AD step of substrates (solid biomass + cattle slurry) in Foulum biogas reactor (Table 1) and this was assumed applicable to AD2 sourced from Ausumgaard biogas plant.
[b] Based on 17 % and 9 % reduction of C in $AD1^+$ and $AD2^+$ after the secondary AD step of AD1 and AD2 (Table 1).
[c] $C_1 + C_2 + C_3$ in Table 5.

could be linked to the differences in the HRT in the primary AD step at the two biogas plants. AD1 was sourced from a short HRT biogas reactor, whereas AD2 was sourced from a long HRT biogas reactor. A prolonged retention time of the substrates in the AD biogas reactors allows more time for interactions between substrates and microbes, enhancing the hydrolysis step. This improves degradation of the substrates compared to less interaction time in a short HRT reactor where substrates have a short residence time in the reactor resulting in an incompletely degraded digestate. Moreover, the extension of HRT by a two-step AD enhances the nutrient solubilisation from the unstable digestates by an improved degradation efficiency of the organic matter. In continuous digesters with one daily emptying, a part of the input only stays in the digester for 1 day and this part is equal to 1/HRT. Thus, by prolonged HRT a smaller part of the input stays in the digester for only one or a few days. In a two-step AD all parts of the substrate have a residence time of at least 2 days, and if step two (secondary AD step) is a batch digester, like in this study, all the substrate is exposed to a long digestion period.

After the secondary AD step, the slight decrease in C and dry matter content in AD2 compared to AD1 could be associated with a larger proportion of unstable compounds in AD1 due to a shorter residence time in the reactor. This is in accordance with Thygesen et al. (2014), who recovered higher residual biochemical methane potential from digestates sourced from a short HRT biogas plant than those from a longer HRT. The changes in the NH_4^+-N and C content after two-step AD were reflected in the C/N ratio, which primarily influences C and N dynamics. Alongside the HRT, the variation in the biochemical properties of the digestates after two-step AD could be attributed to the degradability of the feedstock used in the primary AD step (Moller and Muller, 2012). For instance, AD1 was obtained from a digester with cattle slurry and solid biomass (grass-clover) as the primary inputs, whereas AD2 was sourced from a reactor whose inputs were diverse, i.e. a mixture of cattle manure and pig slurry, deep litter, straw, chicken manure and grass. As a result, the fibre components of the treatments degraded differently, and more recalcitrant materials such as lignin remained unchanged or slightly increased due to their enrichment in the digester. In contrast, hemicellulose and cellulose are significantly reduced as they are easily degradable by AD. The increase in pH after two-step AD could be ascribed to the decomposition of the VFAs and the formation of ammonium carbonate (Moller and Muller, 2012).

5.2. N turnover and availability in the soil

Reconfiguration of the biogas digesters by including a two-step AD to prolong the retention time of the substrates in the digester could modify the digestates' characteristics, contributing to increased N mineralisation and reducing the decomposable organic matter (Guilayn et al., 2020). The short-term net N immobilisation between days 0 to 2 after application to soil was probably due to microbial assimilation of N due to C mineralisation

associated with decomposition of VFA (Kirchmann and Lundvall, 1993; Sørensen, 1998). The gradual increase of inorganic N after the immobilisation period indicates a partial re-mineralisation of the immobilized N. The inorganic N release in the soil from the added digestates differed significantly after 80 days of incubation based on the digestion step. Some fractions of the applied NH_4^+-N may have been clay fixed as only 92 % of the applied NH_4^+-N in ammonium sulphate was recovered in exchangeable form shortly after application (Fig. S1). The significant increase of inorganic N in soil amended with digestates from the two-step AD is attributed to the increased NH_4^+-N due to the enhanced mineralisation of organically bound N during digestion. The extended retention time by two-step AD increased NH_4^+-N in the digestates and decreased organic matter content, thus reducing N immobilisation. The negative inorganic N release in solid biomass is probably due to microbial N immobilisation, whereby the undecomposed solid biomass also led to the highest C mineralisation in the soil.

Microorganisms play a crucial role in N and C mineralisation and applying organic amendments of different characteristics induce variable microbial activities. The organic amendments can induce changes in microbial diversity in the soil to influence biogeochemical processes that make nutrients available through complex microbial interactions. Measuring relationships between mineralisation processes and the microbial community is very complex and was not the scope of our study. Future studies could consider how digestates influence microbial diversity in soil. However, a study by Johansen et al. (2013) indicated that digested materials only induce small and transient changes in soil microbial community compared to undigested materials.

The residence time of substrates in the biogas reactors significantly affects the biowastes' degradation efficiency (Kaparaju et al., 2009). A more significant increase in inorganic N release in $AD1^+$ compared to $AD2^+$ could be linked to a shorter HRT in the primary AD step of AD1. AD1 was sourced from a biogas plant running on a short HRT, where substrates take a limited time to be decomposed by microorganisms, as discussed above. Additionally, the high inorganic N release in the soil from the digestates compared to the substrates could also be associated with a reduced dry matter content and C/N ratio after the primary and secondary AD steps (Sørensen et al., 2003). These findings are consistent with Reuland et al. (2022) and Cavalli et al. (2017).

The cellulose content was the only parameter that had a significant negative relationship to the net N mineralisation after 80 days of incubation, and this could be ascribed to the fact that cellulose is the only primary source of carbon for microbial biomass that affect net inorganic N release after approximately 50 days of incubation (Bending et al., 1998). The insignificant relationship between net N mineralisation and C/N between the period 51 to 80 days of incubation (Table S2) is linked to the availability of C from the recalcitrant portions in the digestates and the re-mineralisation of the immobilized N (Li et al., 2020). The strong correlations between the net inorganic N release and C/N and NH_4^+-N/N ratio indicate that they are good predictors of the net N mineralisation and N available for plant uptake in the treatments, and this is consistent with previous studies (Cavalli et al., 2016; Fontaine et al., 2020; Li et al., 2020). It is noteworthy to mention that despite the two-step digestion process hypothesized to enhance digestion performance and process stability, it is only sensitive to the substrate with easily decomposable organic matter (Boe and Angelidaki, 2009), as seen in this study. Therefore, optimization of the operations conditions of the digestion by adding a second AD step could have less influence if the first digestion step has a long HRT.

5.3. Carbon turnover and C sequestration

The CO_2 evolution from the organic treatments followed an exponential pattern linked to the treatments, which is characterized by different decomposability rates (Cavalli et al., 2017). A lag phase in CO_2 evolution from solid biomass and digestates sourced from the primary AD step at the initial incubation period is probably due to the potential reduced microbial activities and inadequate contact between decomposers and the organic treatments (Thomsen et al., 2013). The subsequent C mineralisation from the primary AD step exceeded those sourced from the secondary AD step by 6–15 % points, and the differences are attributed to the varying biochemical properties dependent on the digestion steps of the digestates.

The two-fold larger decrease of C in $AD1^+$ compared to $AD2^+$ is due to the differences in the HRT in the primary AD step and substrate characteristics whose effects are explained earlier. The extended HRT results in more metabolism of easily decomposable C by microorganisms during the AD process, resulting in proportionally more stable C in the digestate. The higher C loss by the two-step AD process, in the form of CH_4 and CO_2 in biogas, is compensated by low C mineralisation after the soil incorporation of the digestates (Moller, 2015). The similar soil C sequestration, when relating to the initial C input in the system, combined with a reduced potential of methane emission during digestate storage and increased biogas yield recovered, gives an overall reduced GHG emission from digestates (Møller et al., 2022) as with the two-step AD evaluated in this study. The lower mineralisation in the soil of applied cattle slurry C compared to C applied in digestates (Table 3) was unexpected. A possible explanation for this observation could be that the digestates originated from a mixture of manure and undecomposed plant materials (solid biomass). The cattle slurry had all passed the digestive system of cows, whereas the solid biomass had only been digested by AD. Our results indicate that the digestion in the biogas plant was less effective in decomposing the materials than the digestion in a cow. This resulted in digestates containing more mineralisable C than found in the untreated cattle slurry.

The soil organic carbon has a critical role in maintaining soil structure and improving soil fertility to secure food production. Also, it is regarded as a way to mitigate CO_2 concentrations increase in the atmosphere (Beghin-Tanneau et al., 2019). The carbon sequestration in the soil entirely relies on the balance between the formation of the soil organic matter from the microbial decomposition of organic inputs and the emission of CO_2 (Cotrufo et al., 2015). Therefore, the addition of organic material to the soil is crucial for maintaining the soil's organic carbon (SOC) content. However, the extent of C sequestration is influenced by several explanatory variables such as climate, application rate, and management system, i.e. extent of stabilisation, among other factors (Maillard and Angers, 2014). Therefore, properly assessing the C sequestration potential from organic amendment is crucial in designing digestate management practices and strategies to improve agricultural production and control greenhouse gas emissions (Wijesekara et al., 2021). The use of kinetic models describing the temporal C mineralisation in the soil can estimate the amount of C stabilized in soil (Thomsen et al., 2013). This study's four-pool carbon kinetic model indicates that a prolonged retention time by a two-step AD process could increase C retention in the soil related to the soil C input in the range of 6–18 % points from the digestates based on the HRT in the primary stage. Increasing HRT increases C retention related to the C applied to soil due to a higher proportion of the recalcitrant components such as lignin. The proportion of long-term C retention from solid biomass (grass-clover silage) of 26 % is somewhat higher than the C retention of 14 % from a feed mixture applied directly to soil reported by Thomsen et al. (2013). The feed mixture applied by Thomsen et al. (2013) consisted of maize silage, alfalfa and rape-seed cake with high digestibility, and this could possibly explain the difference to the present results.

The two-step AD process on digestates limits the soil microbial activity after applying digestates due to their stability and consequently influences the soil's C and N dynamics. Despite the C retention in soil based on the C input being higher from the digestates sourced from the two-step AD than those from one-step AD, the estimated C retention based on the C present before digestion was similar. The estimated long-term C sequestration related to the original C input before AD was in this study, 12–16 % among the digestates from one- and two-step AD, and within the range of the carbon retention efficiency of 12 ± 4 % from manure reported by Maillard and Angers (2014) in a global meta-analysis in an average study of 18 years. Additionally, our results are concurrent with those of Jensen et al. (2022), who found a 11 % retention of C (% of applied C) from slurry in a long-term organic dairy crop rotation experiment spanning 32 years. However, this study shows very contrasting results compared to Thomsen et al. (2013).

They found that regardless of the fate of the feed, either ruminant digestion or anaerobic digestion, the long-term C retention after soil application, when related to the original input of C in fresh biomass, is similar at 12–14 %. The estimated C retention by direct soil application of solid biomass of 26 % is higher than that found by Thomsen et al. (2013) but consistent with the estimated average C retention of 23 % reported by Bhogal et al. (2007).

6. Conclusions

This study gives insights into the effects of one- and two-step AD processes on carbon and nitrogen dynamics before and after the application of digestates to the soil. The two-step AD process significantly increased net inorganic N release in the soil without negatively affecting the long-term C retention. The increase in inorganic N release in the soil after the two-step AD process is dependent on the HRT in the primary AD step. The C mineralisation related to the C input to soil was lowest in digestates sourced from a two-step AD process due to recalcitrance of the components, with the highest mineralisation observed in the undigested solid biomass. The long-term C sequestration in soil, related to the C input before the first digestion step, was not significantly influenced by the second AD step. The high correlations between the biochemical characteristics of the digestates, such as C/N, NH_4^+-N/N ratio and hemicellulose content with C and N mineralisation, indicate their possible use to predict C and N dynamics after the incorporation of digestates in the soil.

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CRediT authorship contribution statement

Jared Onyango Nyang'au: Conceptualization, Investigation, Methodology, Formal analysis, Visualization, Writing – original draft. **Henrik Bjarne Møller:** Conceptualization, Methodology, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Peter Sørensen:** Conceptualization, Methodology, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Data availability

Data will be made available on request.

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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Appendix A. Supplementary data

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