



Effect of different digestate-derived biochars on carbon mineralization and microbial activity: insights from ^{13}C tracing and calorespirometric analysis

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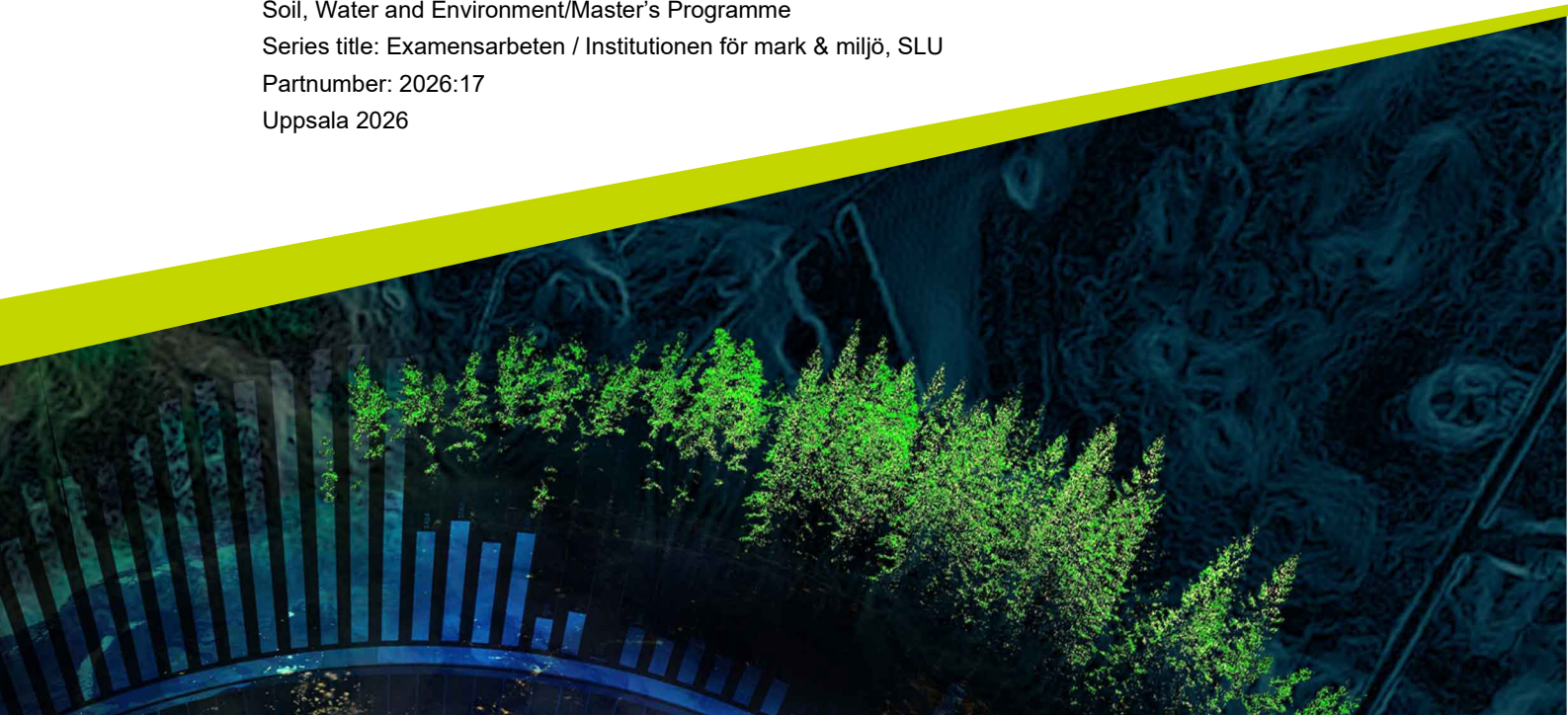
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Effect of digestate-derived biochars on carbon mineralization and microbial activity

Effekt av biokol från rötresten på kolmineralisering och mikrobiell aktivitet

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Abstract

Biochar has potential as a soil amendment and carbon storage material, but its effects on soil microorganisms can vary depending on feedstock and production conditions. This study investigated how biochars derived from manure digestate and produced from different solid fractions and at different pyrolysis temperatures affected soil carbon mineralization and microbial activity.

Two laboratory incubation experiments were conducted using a sandy soil. Experiment 1 compared biochars produced from centrifuge-recovered digestate solids at 400 and 550 °C (BCS400 and BCS550) and used ^{13}C -labelled glucose to distinguish glucose-derived from non-glucose-derived carbon dioxide (CO_2). Experiment 2 compared BCS400 with biochar produced from screw-press solids at 400 °C (BSS400) using unlabelled glucose. Carbon dioxide emission, cumulative heat dissipation, calorespirometric ratio (CR), and microbial biomass carbon (MBC) were evaluated.

The BCS400 biochar generally produced greater CO_2 emission and heat dissipation than BCS550, particularly without glucose addition and during the early incubation period, consistent with its less carbonized characteristics. The ^{13}C results showed that the pronounced early difference in respiration associated with BCS400 was largely related to the non-glucose-derived CO_2 fraction, whereas by day 65 the control showed greater glucose-derived CO_2 than both biochars. In Experiment 2, BCS400 generally produced greater respiration and heat dissipation than BSS400, despite both biochars being produced at 400 °C. Microbial biomass carbon responses varied with incubation time, with both biochars showing greater microbial biomass than the control at several sampling times. These results indicate that both pyrolysis temperature and digestate separation procedure influenced microbial responses to digestate-derived biochar.

Biochar amendment also altered the relationship between heat dissipation and CO_2 emission. However, many CR values approached or exceeded the theoretical reference value of 469 kJ mol^{-1} , and the difference-based CR represented the net metabolic response following glucose addition rather than glucose metabolism alone. Therefore, the CR results indicated changes in microbial carbon and energy metabolism but did not provide consistent evidence that biochar increased microbial carbon use efficiency (CUE). Overall, digestate-derived biochars should not be considered a uniform group of soil amendments because their microbial effects depend on both digestate processing and pyrolysis conditions.

Keywords: digestate-derived biochar; carbon mineralization; microbial activity; calorespirometry; ^{13}C -glucose; microbial biomass carbon; carbon use efficiency

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Abbreviations

Abbreviation	Description
AD	Anaerobic digestion
ANOVA	Analysis of variance
BCS400	Biochar produced from solids recovered by centrifugation of the screw-press liquid fraction and pyrolyzed at 400 °C
BCS550	Biochar produced from solids recovered by centrifugation of the screw-press liquid fraction and pyrolyzed at 550 °C
BSS400	Biochar produced from solids retained by screw-press separation and pyrolyzed at 400 °C
CO ₂	Carbon dioxide
CO ₂ -C	Carbon emitted as carbon dioxide
CR	Calorespirometric ratio
CUE	Carbon use efficiency
DOC	Dissolved organic carbon
MBC	Microbial biomass carbon
SD	Standard deviation
SE	Standard error
SSIM	Small Sample Isotope Model
WHC	Water hold capacity
Exp 1	Experiment 1
Exp 2	Experiment 2

1. Introduction

Large amounts of organic residues are produced from agriculture, livestock production, food systems, and other human activities. If these residues are not managed properly, they can contribute to environmental problems such as greenhouse gas emissions, nutrient losses, water pollution, and inefficient use of valuable resources (Subbarao et al., 2023; Singh et al., 2022). At the same time, these organic residues contain carbon and nutrients that can be recovered and reused instead of being lost (García-Prats et al., 2026; Sani et al., 2026). Anaerobic digestion (AD) is one important technology for this purpose because it can convert a wide range of organic residues, including wastewater sludge, municipal organic waste, manure, slurry, agricultural residues, and forestry residues, into biogas and digestate (García-Prats et al., 2026). The expansion of AD in Europe has also been supported by policies promoting a more circular and resource-efficient economy (Egene et al., 2021). Anaerobic digestion is a sustainable waste-to-energy and resource-recovery technology that converts organic residues into renewable biogas and a nutrient-rich digestate, as illustrated in Figure 1. By producing biogas from waste biomass, AD can reduce emissions compared with less controlled practices such as open dumping or burning (Subbarao et al., 2023). Digestate from AD contains organic matter, nutrients, and minerals and can be used as a fertilizer or soil conditioner, but its composition and fertilizer value can vary depending on the original feedstock (Basinas et al., 2023; Sani et al., 2026). Direct use of digestate may also create environmental and agronomic challenges, including odour emissions, greenhouse gas release, pathogen risks, nitrogen losses, and nitrate leaching, especially when application is not well matched with crop demand or soil conditions (Basinas et al., 2023; Sani et al., 2026).

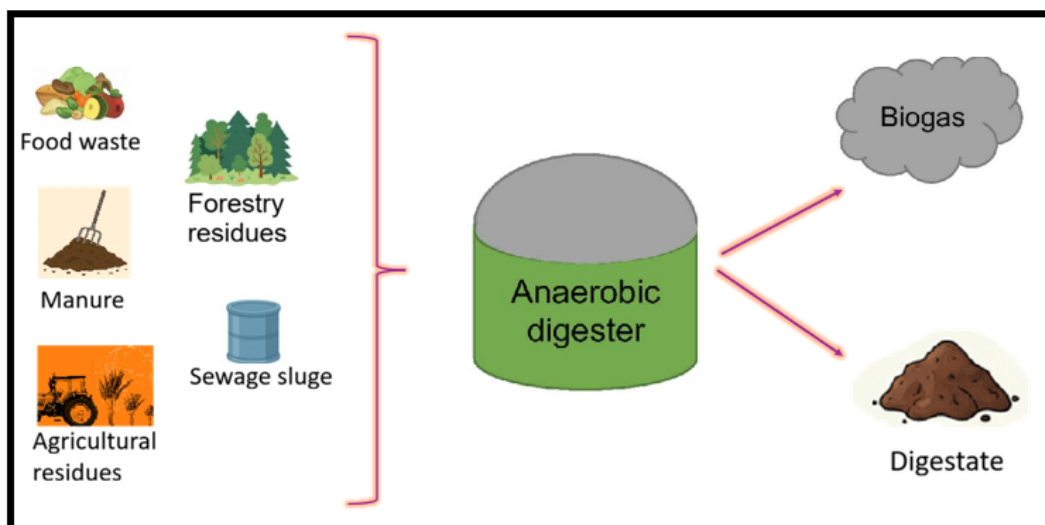


Figure 1. Simplified representation of anaerobic digestion of organic residues and the production of biogas and digestate.

Therefore, further treatment of digestate can be important for improving its stability, handling, and environmental value (Basinas et al., 2023; Sani et al., 2026). Solid–liquid separation is often used as a first step in digestate processing because it divides digestate into liquid and solid fractions with different nutrient and organic matter contents (Carraro et al., 2024; Cathcart et al., 2023; Lyons et al., 2021). The solid fraction contains more recalcitrant organic matter, lignocellulosic residues, and ash (Sani et al., 2026), and can be valuable because of its higher phosphorus and dry matter contents and lower water content (Nissen, 2022). However, further processing of the solid fraction can improve its valorisation and contribute to more sustainable recycling of digestate-derived resources (Dutta et al., 2021; Singh et al., 2022).

Pyrolysis has been proposed as a post-treatment for solid digestate. It is a thermochemical process in which organic material is heated under limited or no oxygen conditions, producing a solid carbon rich product, bio-oil, and non-condensable gases (Basinas et al., 2023; Cantrell et al., 2012). During pyrolysis, readily decomposable organic matter is transformed into more stable carbon structures, while mineral components, including nutrients such as P, K, Ca, and Mg, can remain concentrated in the resulting solid product (Sani et al., 2026). Nitrogen may behave differently because N-containing compounds can be volatilized during pyrolysis, and N retention generally decreases as pyrolysis temperature increases, particularly in manure-derived biochars (Ippolito et al., 2020; Rathnayake et al., 2023). In this way, pyrolysis can reduce the mass and volume of digestate solids, improve storage, increase product uniformity, and provide thermal sanitization compared with raw solid digestate (Sani et al., 2026).

The solid carbon-rich product formed during pyrolysis is referred to as biochar (Wei et al., 2019; Ippolito et al., 2020). Digestate-derived biochar may have potential as both a soil amendment and a carbon storage material (Basinas et al., 2023; Sani et al., 2026). Biochar has been widely studied as a soil amendment because it can influence soil fertility, nutrient retention, carbon storage, and greenhouse gas emissions (Tomczyk et al., 2020; Wei et al., 2019). Its ability to supply nutrients and contribute to carbon sequestration depends strongly on biochar composition, which in turn is influenced by feedstock type and pyrolysis conditions (El-Naggar et al., 2019). However, biochar properties are strongly affected by feedstock type and pyrolysis temperature (Tomczyk et al., 2020; Ippolito et al., 2020; Li et al., 2023). Higher pyrolysis temperatures generally increase biochar carbon stability, aromaticity, pH, ash content, and surface area, while reducing volatile matter and easily degradable carbon fractions (Tomczyk et al., 2020; Wei et al., 2019; Li et al., 2023). Because digestate-derived biochar properties are strongly influenced by the characteristics of the solid digestate used

as feedstock, differences created by the separation technology before pyrolysis may subsequently influence the properties of the resulting biochar (Hjorth et al., 2011; Carraro et al., 2024; Cathcart et al., 2023; Sani et al., 2026).

Two mechanical separation methods relevant to this study are screw pressing and centrifugation. These technologies operate through different separation mechanisms and can therefore produce solid fractions that differ in particle-size distribution, dry matter, carbon, nitrogen, phosphorus, and mineral composition (Hjorth et al., 2011; Lyons et al., 2021). Centrifugation generally recovers a greater proportion of fine particles, dry matter, and phosphorus in the solid fraction, whereas screw pressing tends to produce a drier solid fraction but with lower nutrient recovery (Carraro et al., 2024; Cathcart et al., 2023). The main digestate separation and pyrolysis pathways relevant to this study are illustrated in Figure 2.

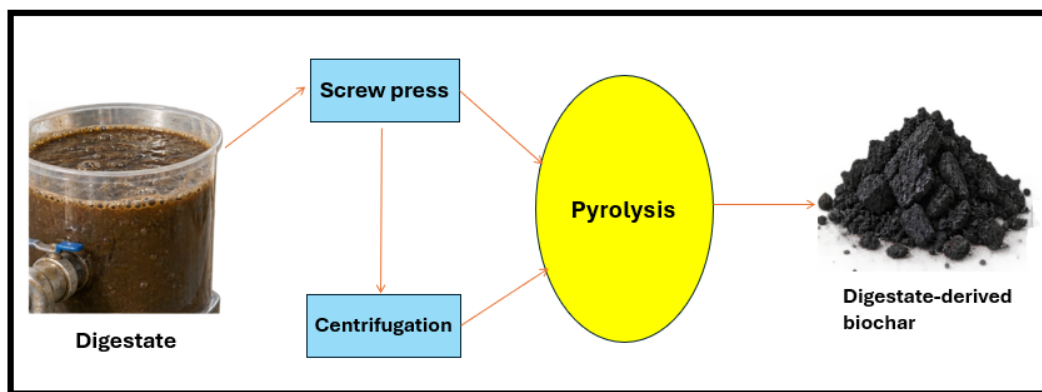


Figure 2. Simplified representation of digestate separation and pyrolysis pathways used to produce digestate-derived biochar.

When biochar or other organic amendments are added to soil, they can influence microbial activity and carbon mineralization. Soil microorganisms use available carbon sources for growth and respiration, and part of this carbon is released as carbon dioxide (CO₂) (Barros et al., 2016; Geyer et al., 2019; Wirsching et al., 2025). Biochar may increase soil respiration by increasing the availability of labile carbon, stimulating microbial activity, or promoting the mineralization of easily decomposable carbon fractions. In contrast, biochar can also reduce the mineralization of native soil organic carbon under some conditions (Chen et al., 2015), while changes in carbon availability and microbial activity may also contribute to lower soil respiration (Wu et al., 2021). However, digestate-derived biochars remain less extensively studied than conventional lignocellulosic biochars (Sani et al., 2026). Similarly, relatively little is known about whether digestate-derived biochars produced under different processing conditions alter microbial utilization of an easily available substrate such as glucose.

One approach to evaluate microbial metabolic responses is to add a readily available and chemically defined substrate such as glucose (Kalu et al., 2024; Pineiro et al., 2025). Because glucose is rapidly utilized by soil microorganisms, changes in CO₂ production and heat dissipation following its addition can be used to evaluate substrate utilization and microbial metabolic activity (Pineiro et al., 2025). Microbial responses to biochar and glucose can be evaluated by combining CO₂ measurements with calorimetric measurements. Carbon dioxide evolution reflects microbial respiration and carbon mineralization, while calorimetry measures heat released during microbial metabolism (Barros et al., 2016; Pineiro et al., 2025; Wirsching et al., 2025). These two measurements are complementary because CO₂ production describes carbon loss through respiration, whereas heat production describes energy dissipation during substrate use (Barros et al., 2016; Wirsching et al., 2025). Combining them allows calculation of the calorespirometric ratio (CR), which links heat release with CO₂ evolution and provides information about microbial metabolic responses (Barros et al., 2016; Geyer et al., 2019; Pineiro et al., 2025). The CR has been linked to microbial carbon use efficiency (CUE), which describes the proportion of carbon taken up by microorganisms that is allocated to microbial biomass growth rather than respiration, but this relationship requires caution in complex soil systems (Geyer et al., 2019; Pineiro et al., 2025; Wirsching et al., 2025). Interpretation based on the CR depends on assumptions about substrate type, oxygen availability, substrate oxidation state, CO₂ recovery, and microbial metabolic pathways (Geyer et al., 2019; Pineiro et al., 2025; Wirsching et al., 2025).

Biochar can also influence the fate of added glucose. In a study using hardwood-derived biochar, Kalu et al. (2024) observed reduced mineralization of ¹³C-glucose together with greater microbial retention of glucose-derived carbon. Biochar may affect glucose utilization through both physicochemical sorption and microbial processes; glucose can initially be retained on biochar surfaces and later become available for microbial uptake. However, it remains unclear whether digestate-derived biochars produced under different processing conditions affect glucose utilization in the same way.

Overall, digestate-derived biochars remain less systematically evaluated than conventional lignocellulosic biochars, despite their relevance to AD-based resource recovery (Sani et al., 2026). Their properties can vary substantially depending on feedstock characteristics and pyrolysis conditions (Basinas et al., 2023; Sani et al., 2026), and these differences may influence subsequent microbial responses in soil. Therefore, the aim of this study was to investigate how manure digestate-based biochars produced under different separation technologies and pyrolysis temperature influence soil carbon mineralization and microbial activity, as reflected by CO₂ emission, cumulative heat dissipation, CR, and microbial biomass carbon (MBC).

Two experiments were conducted. Experiment 1 investigated biochars produced from solids recovered by centrifugation of the liquid fraction obtained after screw-press separation, and pyrolyzed at two different temperatures, 400 and 550 °C. Glucose labelled with ^{13}C was used in this experiment, allowing total CO_2 to be separated into glucose-derived and non-glucose-derived fractions. This experiment was used to evaluate the effect of pyrolysis temperature on microbial carbon mineralization and glucose utilization. Experiment 2 compared biochars produced at the same pyrolysis temperature (400 °C) but from two different digestate fractions: solids retained by the screw press and solids recovered by centrifugation of the screw-press liquid fraction. This allowed the effect of the separation procedure on the resulting biochar response to be evaluated.

The following hypotheses were tested:

H1 – Carbon mineralization: Digestate-derived biochar amendments will alter overall carbon mineralization in the soil–biochar system compared with the control, as reflected by differences in CO_2 emissions with and without glucose addition.

H2 – Pyrolysis temperature: Biochars produced at different pyrolysis temperatures (400 and 550 °C) will differ in their effects on microbial respiration, glucose mineralization, and heat dissipation. Specifically, BCS400 will result in greater total CO_2 emission and heat dissipation than BCS550 because the lower-temperature biochar is expected to contain a greater proportion of readily decomposable carbon and have a less carbonized structure.

H3 – Digestate separation procedure: Biochars produced from solid fractions recovered at different stages of digestate separation will affect microbial activity differently. The screw press retained more cellulose- and hemicellulose-rich material, while the liquid fraction was further separated by centrifugation, producing a solid fraction that was relatively more lignin-rich. Thus, the screw-press solids will be more readily decomposable than the centrifuge-recovered solids. Based on these differences in the original feedstock, BSS400 will stimulate greater microbial respiration, heat dissipation, and microbial biomass than BCS400.

H4 – Calorespirometric response: Biochar amendments will alter the relationship between cumulative heat dissipation and CO_2 emission compared with the control, resulting in differences in the CR. Because CR depends on the relative changes in both heat dissipation and CO_2 production, no specific ranking among the biochars was predicted.

2. Materials and Methods

2.1 Soil and biochar materials

Soil was collected in May 2025 from SLU's experimental field at Ulleråker, Uppsala, Sweden, from a depth of 5-20 cm. The soil was partially air-dried until the moisture content reached approximately 6-7%, sieved to 2 mm, and stored frozen until use. The soil contained 2% organic matter, 9% clay, 11% silt, and 78% sand.

Three digestate-based biochars were used. The biochars were produced from manure-based digestate solids recovered either directly by screw-press separation or by centrifugation of the liquid fraction obtained after screw-press separation, and were then pyrolyzed at different temperatures under an inert atmosphere. Biochar abbreviations and descriptions are presented in Table 1, and characteristics of the biochars are presented in Table 2.

Table 1. Biochar treatments used in the incubation experiment

Symbol	Description
BSS400	Digestate solids separated by screw press and pyrolyzed at 400 °C
BCS400	Digestate solids separated by centrifugation of the screw-press liquid fraction and pyrolyzed at 400 °C
BCS550	Digestate solids separated by centrifugation of the screw-press liquid fraction and pyrolyzed at 550 °C

Table 2. Selected physicochemical characteristics of the digestate-derived biochars used in the incubation experiments. H/C and O/C are atomic ratios while C/N was calculated from the mean total C and total N.

Biochar	C (%)	H (%)	O (%)	N (%)	H/C (atomic ratio)	O/C (atomic ratio)	C/N ratio	DOC (mg kg ⁻¹)
BSS400	58.27	2.89	20.86	2.26	0.592	0.269	25.86	872.3
BCS400	37.70	2.57	26.02	2.31	0.813	0.518	16.34	2576.8
BCS550	35.57	1.08	22.33	1.85	0.361	0.471	19.26	100.1

2.2 Experimental design and incubation setup

Two laboratory incubation experiments were performed: Experiment 1 (Exp 1) and experiment 2 (Exp 2). Each experiment included three treatments: the Control

and two biochar-amended treatments. Each treatment was evaluated with and without glucose addition, resulting in six experimental conditions, with three replicates per condition. In the Exp1, the treatments were Control, BCS400 and BCS550, whereas in the Exp 2 they were Control, BCS400 and BSS400. Soil was thawed at room temperature before pre-incubation. Biochars were applied to provide an equivalent rate of 20 t C per ha. Assuming a soil depth of 20 cm and an average bulk density of 1.35 g cm⁻³, the soil mass was estimated as 2700 t soil ha⁻¹. Therefore, a biochar application rate of 20 t C ha⁻¹ corresponded to 7.4 mg C g⁻¹ dry soil. Based on this application rate, soil–biochar mixtures were prepared and placed in glass ampoules, each containing an amount equivalent to 4.1 g dry soil. This corresponded to approximately 30.4 mg biochar-C per ampoule, and the required mass of each biochar was calculated according to its carbon concentration. Control soil was mixed with talc to maintain similar physical conditions among treatments. The moisture content in all treatments was adjusted to 55% of soil water holding capacity (WHC) by adding ultrapure water, and samples were incubated in the dark at 20 °C. Moisture was checked and adjusted weekly when needed to maintain 55% WHC.

An overview of the incubation conditions for experiments 1 and 2 is presented in Table 3.

Table 3. Overview of the incubation experiments

Experiment	Biochar treatments	Pre-incubation	Incubation/sampling days	Glucose type
Exp 1	BCS400, BCS550, Control soil	1 week at 20 °C	3, 21, and 65 days*	¹³ C-labelled glucose (5 atom % ¹³ C)
Exp 2	BSS400, BCS400, Control soil	3 days at 20 °C	7, 14, and 28 days	Unlabelled glucose

**Note: Exp 1 included incubations on days 3, 21 and 65; however, only data from days 3 and 65 were included in the analyses and results presented in this thesis.*

The experimental design and analytical workflow for the Exp 1 and Exp 2 are presented in Figure 3 and 4, respectively.

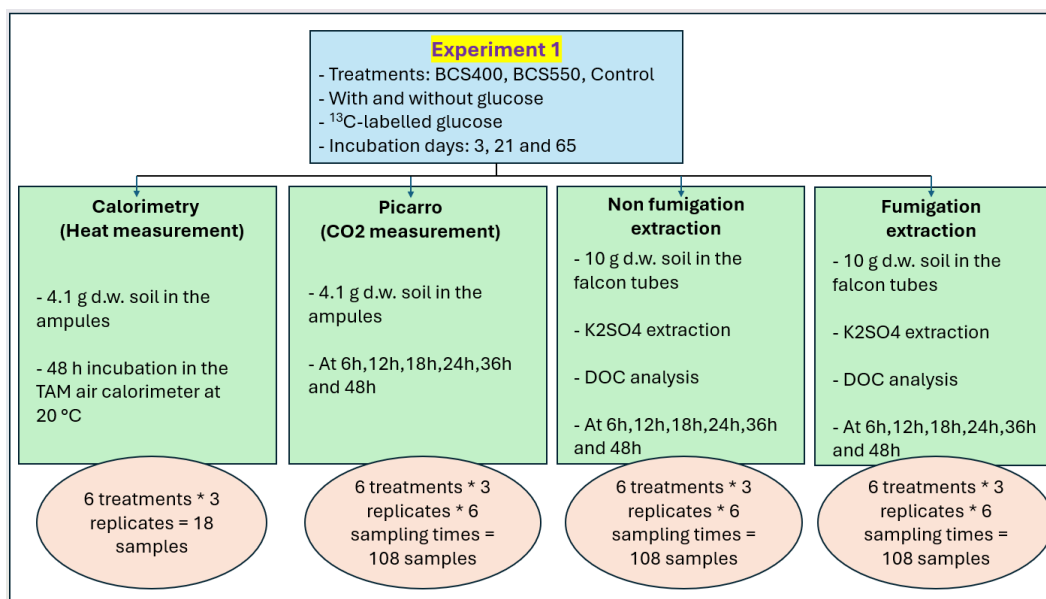


Figure 3. Experimental design and analytical workflow for Exp 1.

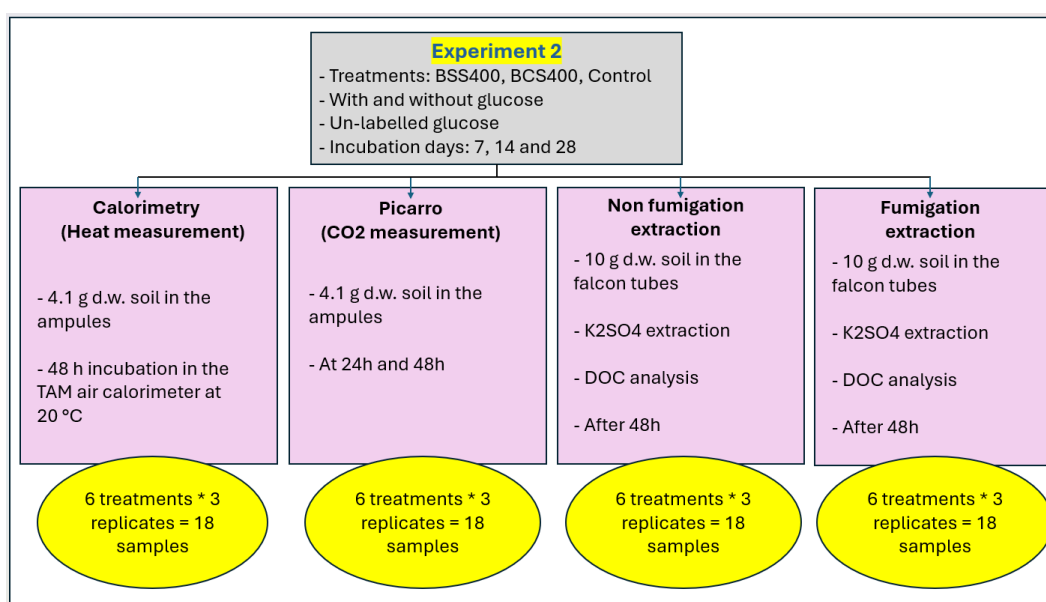


Figure 4. Experimental design and analytical workflow for Exp 2

2.3 Glucose and nutrient solution addition

After the end of the pre-incubation period, and before glucose addition, the moisture content of all samples was checked and adjusted to 55% WHC. Glucose-amended samples received 0.4 mg glucose-C g⁻¹ dry soil. Samples without glucose received the same volume of distilled water instead of glucose. In the Exp 1, glucose was added as ^{13}C -labelled glucose (5 atom % ^{13}C). In the Exp 2, unlabelled glucose was used.

Mineral nutrients were supplied uniformly to all treatments within each experiment to reduce the risk of nutrient limitation and to standardize nutrient inputs among treatments. Nutrient solutions were added before glucose addition. In the Exp 1, all samples received 0.057 mg N g⁻¹ dry soil as (NH₄)₂SO₄, 0.057 mg P g⁻¹ dry soil as K₂HPO₄, and 0.057 mg Mg g⁻¹ dry soil as MgSO₄. In the Exp 2, all samples received 0.057 mg N g⁻¹ dry soil as KNO₃.

2.4 Calorimetric measurements

Heat dissipation was measured using TAM Air calorimeters at 20 °C. Soil samples were placed in airtight glass ampoules with a total volume of 22.4 mL. Reference ampoules contained distilled water; the water volume was calculated to provide a heat capacity similar to that of the sample ampoules containing soil, water, nutrient solution, and glucose or distilled water.

After the ampoules were placed in the calorimeter, a thermal equilibration period of approximately 30–45 min was required before stable baseline conditions were reached. Heat flow was recorded for 48 h after glucose or water addition. In the Exp 1, heat dissipation was evaluated at 6, 12, 18, 24, 36, and 48 h after glucose addition. In the Exp 2, heat dissipation was evaluated at 24 and 48 h. Heat flow data were expressed as normalized heat dissipation rate, $P(t)$, in $\mu\text{W g}^{-1}$ dry soil. Cumulative heat dissipation, $Q(t)$, was calculated as the integral of heat flow over time (Yang et al., 2024):

$$Q(t) = \int_0^t P(t) dt$$

where $P(t)$ is the heat dissipation rate and $Q(t)$ is the cumulative heat released up to time t . In this study, cumulative heat dissipation was obtained from the normalized heat output provided by the calorimeter software.

2.5 Carbon dioxide measurements

Carbon dioxide evolution from the samples was used as an indicator of microbial respiration and carbon mineralization. When microorganisms decompose organic substrates, part of the carbon is respired and released as CO₂. Carbon dioxide concentrations in the ampoule headspace gas were measured using a Picarro G2201-i analyzer equipped with a Small Sample Isotope Module (SSIM). The analyzer provided mean ¹²CO₂, mean ¹³CO₂, and an instrument dilution factor. Total CO₂ concentration was calculated as the sum of mean ¹²CO₂ and mean ¹³CO₂.

For non-glucose samples, headspace gas was sampled directly from the incubation ampoules and injected into the Picarro system. For glucose-amended samples, CO₂ concentrations were high; therefore, headspace gas was diluted before Picarro analysis. In the Exp 1, 2.5-3 mL headspace gas from glucose-amended ampoules was transferred into an N₂-flushed ampoule (22.4 mL). In the Exp 2, 3 mL headspace gas was transferred into an N₂-flushed ampoule (22.4 mL). Subsamples from the diluted ampoules were then injected into the Picarro system. In the Exp 1, CO₂ was measured at 6, 12, 18, 24, 36, and 48 h after glucose addition. In the Exp 2, CO₂ was measured at 24 and 48 h.

2.6 Dissolved organic carbon extraction and microbial biomass carbon

Dissolved organic carbon (DOC) was extracted from fumigated and non-fumigated samples. The difference in DOC between fumigated and non-fumigated samples was used to estimate MBC. Microbial biomass carbon was calculated from the difference between DOC concentrations in fumigated and non-fumigated samples using an extraction efficiency factor of 0.45.

$$MBC = \frac{DOC_{fumigated} - DOC_{non-fumigated}}{K_c}$$

The DOC extraction and MBC determination were included only in Exp 2; corresponding data from Exp 1 were not available for analysis.

For Exp 2, Falcon tubes containing an amount equivalent to 10 g dry soil were used for extraction. Potassium sulfate (K₂SO₄, 0.05 M) was added at a soil:extractant ratio of 1:4. Samples were shaken for 1 h, centrifuged at 3000 rpm for 5 min, and filtered using 0.7 µm syringe filters. The filtered extracts were stored at -20 °C until DOC analysis. For fumigated samples, soil was exposed to chloroform vapour in a desiccator for 24 h using 40 mL chloroform. After fumigation, the chloroform was removed before K₂SO₄ extraction. Non-fumigated samples were extracted directly without chloroform treatment. In Exp 2, extraction was performed 48 h after glucose or corresponding water addition. Dissolved organic carbon concentrations were measured using a carbon/nitrogen analyzer.

2.7 Calculations and data analysis

Carbon dioxide-carbon emission was calculated from the Picarro measurements by summing the measured ¹²CO₂ and ¹³CO₂ concentrations. Concentrations were corrected using the instrument dilution factor. For glucose-amended samples with

high CO₂ concentrations, headspace gas was diluted in an N₂-flushed ampoule before Picarro analysis. The dilution was accounted for using a manual dilution factor in the CO₂ calculations. Corrected CO₂ concentrations were converted to CO₂-C using the ideal gas law, accounting for the calculated headspace volume, and were normalized to dry soil mass ($\mu\text{g CO}_2\text{-C g}^{-1}$ dry soil). Detailed calculation procedures are provided in Appendix 1.

For glucose-amended samples, the CR was calculated as cumulative heat dissipation divided by accumulated CO₂ emission over the same incubation period and expressed as $\text{kJ mol}^{-1} \text{CO}_2\text{-C}$. The calorimetric response to glucose addition was evaluated using a difference-based CR, calculated as the difference in cumulative heat dissipation between the glucose-amended and corresponding non-glucose condition divided by the corresponding difference in CO₂ emission ($\Delta\text{heat}/\Delta\text{CO}_2$). This metric represents the net change in the heat–CO₂ relationship following glucose addition rather than a CR originating exclusively from glucose metabolism.

2.8 Statistical analysis

Statistical analyses were carried out in R using RStudio. Depending on the structure of the data, either ordinary ANOVA or linear mixed-effects models were used. Ordinary ANOVA was used when measurements at different sampling times were obtained from independent experimental units. Linear mixed-effects models were used for repeated calorimetric measurements obtained from the same ampoules over time, with sample identity included as a random effect. For CR, the statistical structure was less straightforward because cumulative heat was repeatedly measured from the same calorimeter ampoules, whereas CO₂ measurements at different sampling times were obtained from different ampoules. Model choice for CR was therefore based on the experimental structure of each dataset, and alternative model structures were examined where appropriate as a sensitivity check. For statistical analysis, treatment referred to the Control and biochar-amended groups, while glucose addition was included as a separate factor where both glucose conditions were analysed. Where relevant, the effects of sampling time, treatment, glucose addition, and their interactions were tested.

Model assumptions were checked using residual plots, tests of normality, and tests of homogeneity of variance where appropriate. Log transformation was tested when model assumptions were not sufficiently met. Total CO₂ emission data in Experiments 1 and 2 were log-transformed because transformation improved the model assumptions. The difference-based CR on day 3 of Exp 1 was also analysed after log transformation, whereas the corresponding day 65 data were analysed on the original scale. Other response variables were analysed on the original scale. Figures show the original, non-log-transformed observed means

$\pm$ standard deviation (SD), even when statistical inference was based on transformed data.

When treatment differences required further examination, model-estimated means were obtained using the emmeans package and pairwise comparisons were performed with Tukey adjustment. When a significant interaction was detected, treatments were compared separately within the relevant sampling time or experimental condition. When the interaction was not significant, treatment effects were compared across the corresponding levels of the other factor. Differences were considered significant at $p < 0.05$.

Figures were produced in R and generally show observed means $\pm$ SD. Different lowercase letters indicate significant differences among treatments based on Tukey-adjusted comparisons. Statistical tables present model-estimated means $\pm$ standard error (SE) where appropriate. For analyses performed on log-transformed data, model-estimated means were back-transformed to the original scale for presentation where applicable, while statistical tests were performed on the transformed scale.

Microsoft Excel was used for initial data organization and calculations before statistical analyses were performed in R.

3. Results

3.1 Overview of experimental results

This thesis presents the effect of digestate-based biochars on CO₂ emission, glucose-derived CO₂, heat dissipation, CR, and MBC. Results are presented separately for ¹³C-labelled glucose experiment and the unlabelled glucose experiment. The detailed Tukey-adjusted pairwise comparison tables are provided in Appendix 2 (for Exp 1) and Appendix 3 (for Exp 2).

3.2 Experiment 1: ¹³C-labelled glucose experiment

Experiment 1 concluded three incubations periods: day 3, day 21 and day 65. In this section, only results from day 3 and 65 are presented and analysed. Because ¹³C-labelled glucose was used, total CO₂ could be separated into glucose-derived and non-glucose-derived fractions. This made it possible to evaluate whether biochars produced at different pyrolysis temperatures influenced glucose mineralization, as proposed in Hypothesis 2.

3.2.1 Total carbon dioxide emissions

Carbon dioxide emissions increased over time in all treatments, and glucose addition resulted in substantially higher emissions than without glucose (Figure 5). The three-way ANOVA showed significant effects of hour, treatment, and glucose addition on both day 3 and day 65 ($p < 0.001$). The hour $\times$ treatment $\times$ glucose interaction was also significant on both days ($p < 0.001$), indicating that differences among treatments changed over time and depended on glucose addition.

On day 3 without glucose, BCS400 showed the highest CO₂ emission during the incubation. At 48 h, BCS400 emitted approximately 191% more CO₂ than BCS550 and 282% more than the control. All three treatments differed significantly, with BCS400 > BCS550 > control. The considerably higher CO₂ emission from BCS400 indicates greater respiratory C loss and is consistent with greater overall C mineralization in this treatment. With glucose addition, CO₂ emissions increased strongly during the first 24 h. At 48 h, BCS400 emitted approximately 29% more CO₂ than BCS550 and 17% more than the control. Carbon dioxide emission was significantly higher in BCS400 than both treatments, whereas the control and BCS550 did not differ significantly.

On day 65 without glucose, BCS400 again showed the highest CO₂ emission. At 48 h, BCS400 emitted approximately 79% more CO₂ than BCS550 and 45% more than the control. All three treatments differed significantly, with BCS400 > control > BCS550. With glucose addition on day 65, however, the treatment

pattern changed. At 48 h, the control showed the highest CO₂ emission and was significantly higher than BCS550, whereas BCS400 was intermediate and did not differ significantly from either treatment.

The higher CO₂ emission from BCS400 is consistent with greater overall C mineralization and respiratory C loss compared with the other treatments.

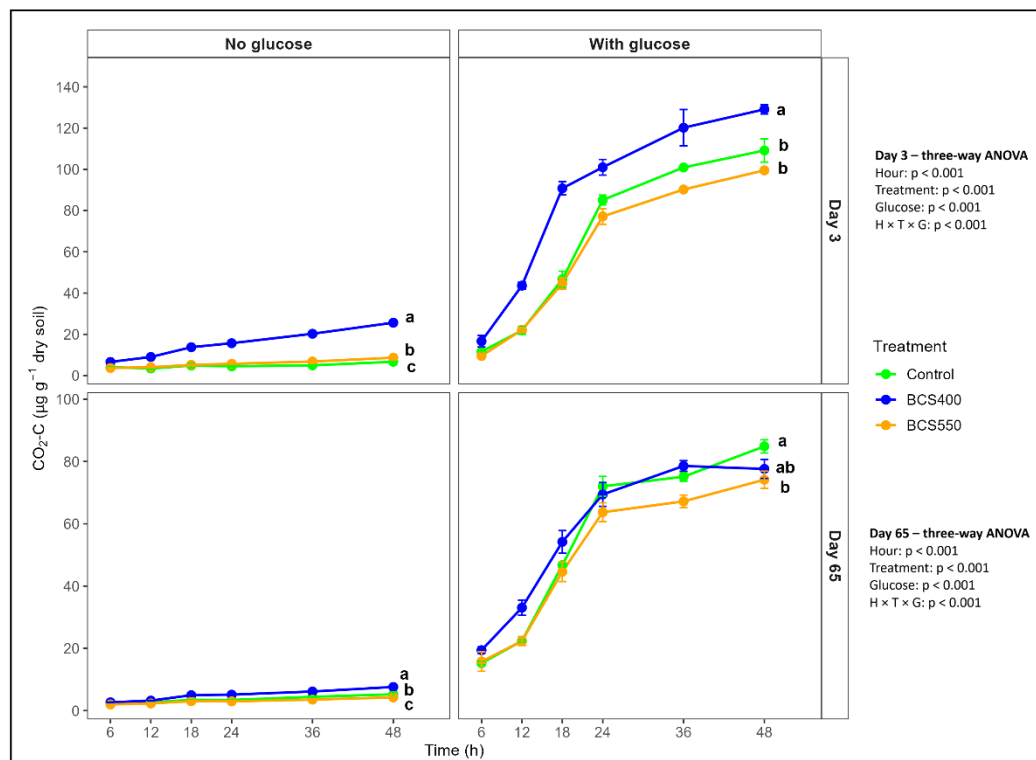


Figure 5. CO₂ emissions from control, BCS400 and BCS550 over 48 h on days 3 and 65, with and without glucose addition. Values represent means $\pm$ SD ($n = 3$).

3.2.2 Glucose- and non-glucose-derived carbon dioxide

Glucose-derived CO₂ represents the fraction of respired CO₂ originating from the added ¹³C-labelled glucose. Non-glucose-derived CO₂ represents the fraction not originating from the added glucose and may include CO₂ derived from native soil organic carbon, biochar-derived carbon, and microbial turnover.

Both glucose-derived and non-glucose-derived CO₂ increased over time, although their treatment patterns differed between day 3 and day 65 (Figure 6). On day 3, both CO₂ fractions were significantly affected by hour, treatment, and the hour $\times$ treatment interaction ($p < 0.001$). For glucose-derived CO₂ on day 3, BCS400 generally showed the highest respiration during the earlier part of the measurement period. At 48 h, however, BCS400 and the control did not differ significantly, and both had significantly greater glucose-derived CO₂ respiration than BCS550. At this time point, BCS400 respired approximately 18% more glucose-derived CO₂ than BCS550, while the control respired approximately 21%

more than BCS550. This indicates lower mineralization of the added glucose in BCS550 by the end of the 48 h measurement period. Treatment differences were much larger for non-glucose-derived CO₂ on day 3. At 48 h, all three treatments differed significantly, with BCS400 > BCS550 > control. The BCS400 treatment emitted approximately twice as much non-glucose-derived CO₂ as BCS550 and about 3.7 times as much as the control. The BCS550 treatment also emitted approximately 85% more non-glucose-derived CO₂ than the control. This shows that the high total CO₂ emission from BCS400 on day 3 was not caused only by respiration of the added glucose, but was also associated with substantially greater respiration of non-glucose C.

On day 65, glucose-derived CO₂ was significantly affected by hour, treatment, and their interaction ($p < 0.001$). At 48 h, the control had the highest glucose-derived CO₂ respiration and was significantly higher than both biochar treatments, whereas BCS400 and BCS550 did not differ significantly. The control respired approximately 23% more glucose-derived CO₂-C than BCS400 and 28% more than BCS550, indicating greater mineralization of the added glucose in the control at this stage of the incubation. In contrast, the hour $\times$ treatment interaction was not significant for non-glucose-derived CO₂ on day 65 ($p = 0.181$), although both hour and treatment had significant effects. Treatment differences were therefore evaluated overall across sampling times. The model-estimated mean was 10.45 $\mu\text{g CO}_2\text{-C g}^{-1}$ for BCS400, 10.94 $\mu\text{g CO}_2\text{-C g}^{-1}$ for BCS550, and 7.60 $\mu\text{g CO}_2\text{-C g}^{-1}$ for the control. Thus, averaged across sampling times, non-glucose-derived CO₂-C was approximately 38% higher in BCS400 and 44% higher in BCS550 than in the control. The two biochar treatments did not differ significantly from each other.

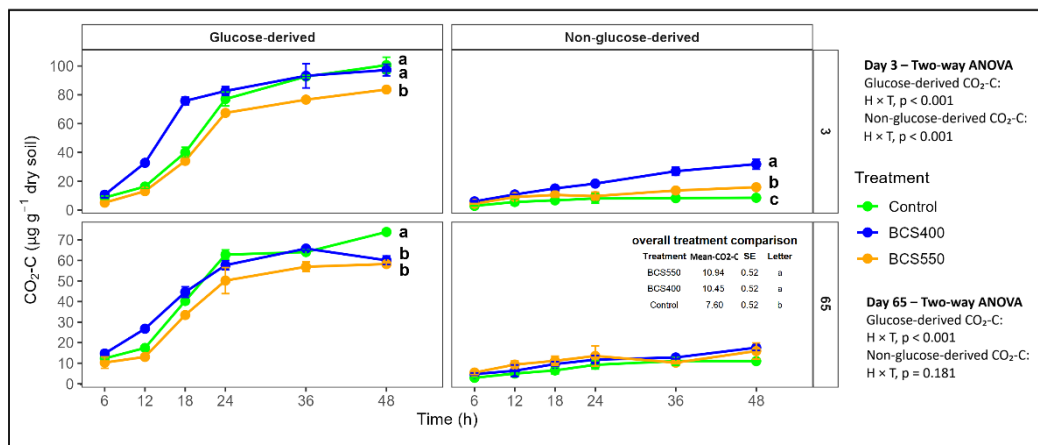


Figure 6. Glucose-derived and non-glucose-derived CO₂ from control, BCS400 and BCS550 over 48 h on days 3 and 65. Values represent means $\pm$ SD ($n = 3$).

3.2.3 Heat dissipation

Cumulative heat dissipation increased over time in all treatments and was substantially greater following glucose addition (Figure 7). The three-way mixed-effects model showed significant effects of hour, treatment, and glucose addition on both day 3 and day 65 ($p < 0.001$). The hour $\times$ treatment $\times$ glucose interaction was also significant on both days ($p < 0.001$), indicating that differences among treatments changed over time and depended on glucose addition.

On day 3 without glucose addition, BCS400 showed a much greater increase in cumulative heat dissipation than BCS550 and the control. At 48 h, BCS400 reached approximately 0.9 J g^{-1} dry soil, compared with about 0.2 J g^{-1} for BCS550, while the control remained close to zero. The BCS400 treatment was significantly higher than both BCS550 and control, whereas BCS550 and control did not differ significantly. With glucose addition, heat dissipation increased strongly in all treatments. At 48 h, BCS400 reached approximately 5.9 J g^{-1} dry soil, compared with approximately 4.8 J g^{-1} for BCS550 and 4.5 J g^{-1} for the control. The BCS400 treatment was significantly higher than both treatments, while BCS550 and control did not differ significantly.

On day 65 without glucose, BCS400 again showed the highest cumulative heat dissipation, reaching approximately 0.36 J g^{-1} dry soil at 48 h. BCS550 was intermediate at approximately 0.19 J g^{-1} , while the control remained considerably lower. The BCS400 treatment was significantly higher than control, whereas BCS550 did not differ significantly from either treatment.

With glucose addition on day 65, differences among treatments were smaller than under the non-glucose condition. At 48 h, cumulative heat dissipation was approximately $4.6\text{--}4.7 \text{ J g}^{-1}$ dry soil in both biochar treatments and approximately 4.3 J g^{-1} in the control. Both BCS400 and BCS550 were significantly higher than control, whereas the two biochar treatments did not differ significantly from each other.

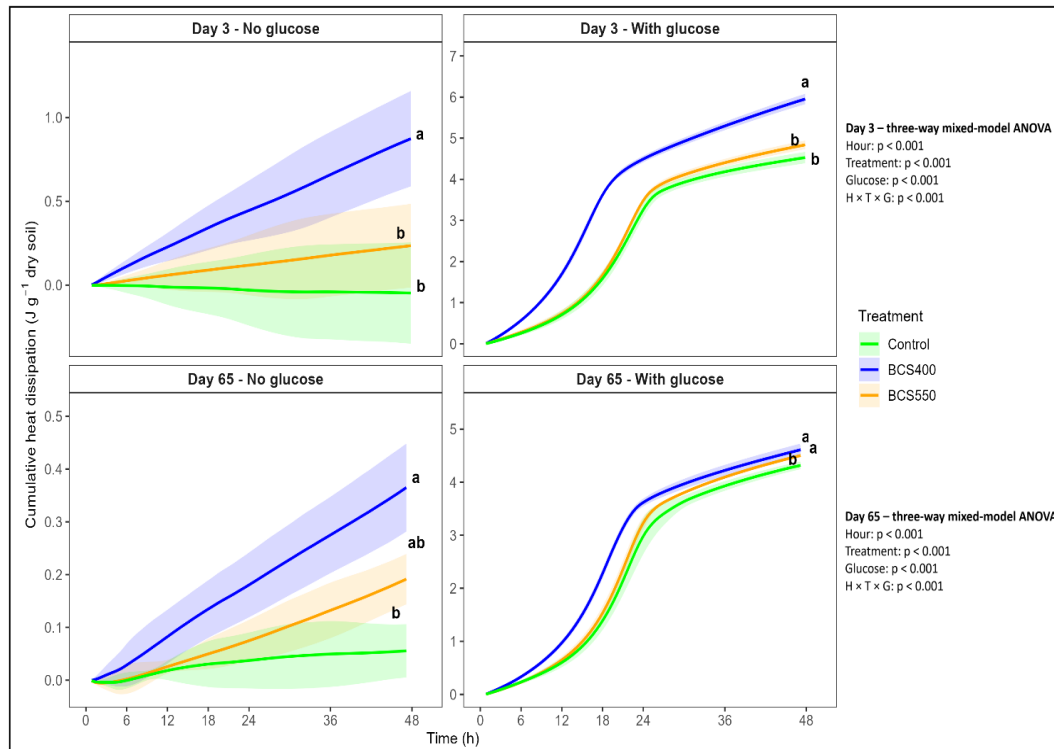


Figure 7. Cumulative heat dissipation from control, BCS400 and BCS550 over 48 h on days 3 and 65, with and without glucose addition. Lines represent mean cumulative heat dissipation, and shaded areas represent $\pm$ SD ($n = 3$).

3.2.4 Calorespirometric ratio

Calorespirometric ratio in glucose-amended soil increased with time on both incubation days (Figure 8).

On day 3, hour and treatment significantly affected CR ($p < 0.001$), whereas the hour $\times$ treatment interaction was not significant ($p = 0.346$). Treatments were therefore compared overall across sampling hours. The model-estimated mean CR was $497.9 \text{ kJ mol}^{-1}$ for BCS400 and $486.7 \text{ kJ mol}^{-1}$ for BCS550, compared with $420.6 \text{ kJ mol}^{-1}$ for the control. Thus, CR was approximately 18% higher in BCS400 and 16% higher in BCS550 than in control. Both biochar treatments had significantly higher CR than the control, while they did not differ significantly from each other.

On day 65, hour and treatment significantly affected CR ($p < 0.001$), and a significant hour $\times$ treatment interaction was observed ($p = 0.016$), indicating that treatment differences varied among sampling hours. No significant treatment differences occurred at 6 or 12 h. At 18 h, BCS400 had a significantly higher CR than both the control and BCS550. At 24 h, both BCS400 and BCS550 were significantly higher than the control and did not differ from each other. At 36 h, BCS550 was significantly higher than both BCS400 and control. By 48 h, BCS400 and BCS550 again showed similar CR values and were both significantly

higher than control. At this final sampling time, CR was approximately 715 kJ mol⁻¹ for BCS400, 730 kJ mol⁻¹ for BCS550, and 611 kJ mol⁻¹ for the control, corresponding to approximately 17% and 19% higher CR in BCS400 and BCS550, respectively, than in the control.

The dashed line in Figure 8 represents the reference value of 469 kJ mol⁻¹ used for glucose oxidation.

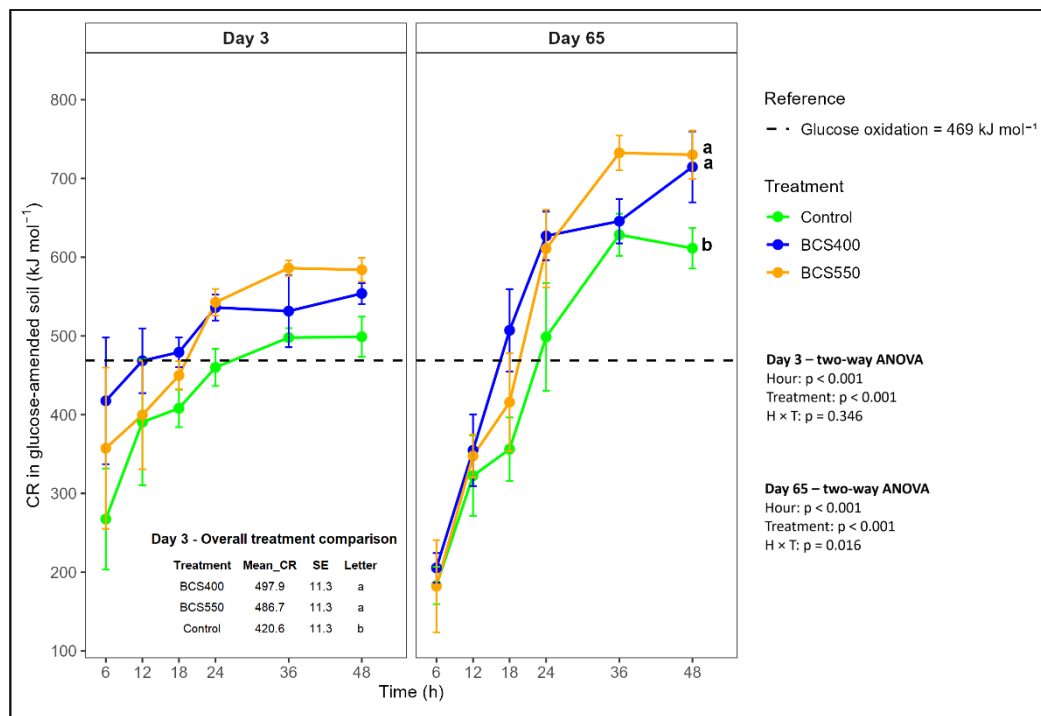


Figure 8. CR in glucose-amended control, BCS400 and BCS550 over 48 h on days 3 and 65. Values represent means $\pm$ SD ($n = 3$).

3.2.5 Calorespirometric response to glucose addition

The difference-based CR differed between incubation days and generally increased over the 48 h measurement period (Figure 9).

On day 3, hour ($p = 0.035$) and treatment ($p = 0.025$) significantly affected the difference-based CR, whereas the hour $\times$ treatment interaction was not significant ($p = 0.882$). Treatments were therefore compared overall across sampling hours. The back-transformed model-estimated CR was 549.7 kJ mol⁻¹ for BCS400, 534.8 kJ mol⁻¹ for BCS550, and 477.5 kJ mol⁻¹ for the control. The BCS400 treatment was approximately 15% higher than the control and was significantly different from it ($p = 0.028$), whereas BCS550 did not differ significantly from either BCS400 or the control.

On day 65, hour and treatment significantly affected the difference-based CR ($p < 0.001$), and the hour $\times$ treatment interaction was also significant ($p = 0.045$), indicating that treatment differences varied among sampling hours. No significant

treatment differences were detected at 6 or 12 h. At 18 h, BCS400 had a significantly higher CR than the control, while BCS550 was intermediate and did not differ significantly from either treatment. The model-estimated values at 18 h were 525.9 kJ mol⁻¹ for BCS400, 432.1 kJ mol⁻¹ for BCS550, and 376.3 kJ mol⁻¹ for the control, corresponding to approximately 40% higher CR in BCS400 than in the control. At 24 h, both BCS400 and BCS550 were significantly higher than the control and did not differ from each other. Their estimated CR values were 643.4 and 626.4 kJ mol⁻¹, respectively, compared with 517.4 kJ mol⁻¹ for the control. No significant treatment differences were detected at 36 or 48 h.

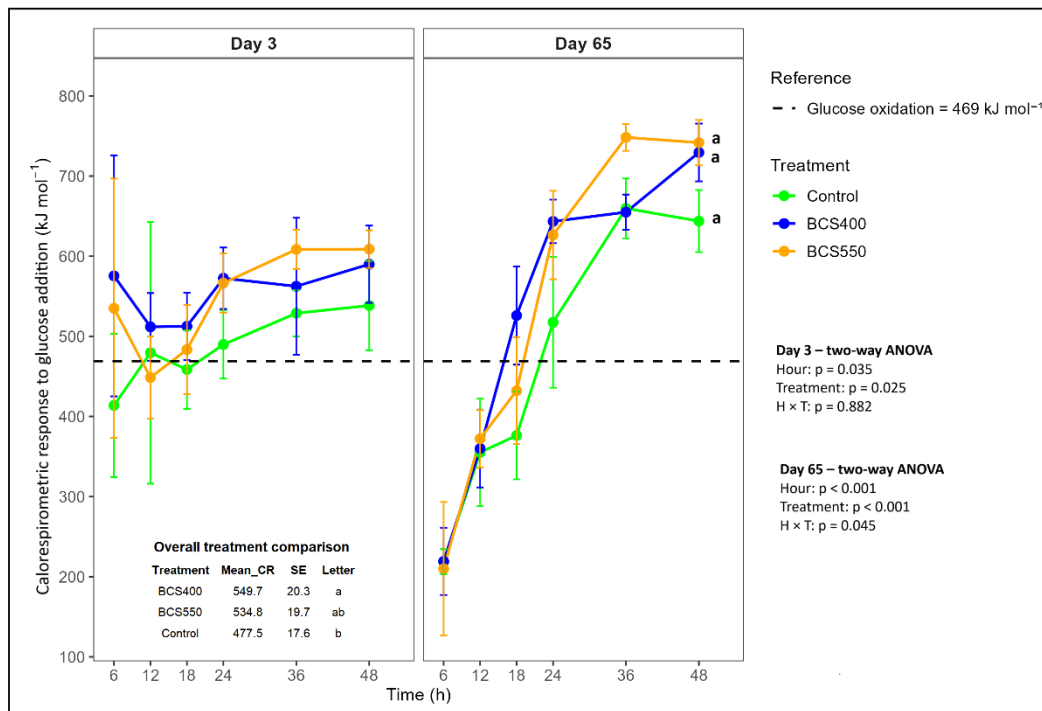


Figure 9. Difference-based CR following glucose addition in control, BCS400 and BCS550 over 48 h on days 3 and 65. Values represent means $\pm$ SD ($n = 3$).

3.3 Experiment 2: unlabelled glucose experiment

In the Exp 2, unlabelled glucose was used. Therefore, the results were interpreted based on total CO₂ emission, cumulative heat dissipation, CR, the difference-based CR, and MBC.

3.3.1 Total carbon dioxide emissions

In Exp 2, total CO₂ emission increased between 24 and 48 h, and glucose addition resulted in substantially greater CO₂ emission in all treatments (Figure 10).

Because unlabelled glucose was used in this experiment, the emitted CO₂ could not be separated into glucose-derived and non-glucose-derived fractions.

On day 7, the hour $\times$ treatment $\times$ glucose interaction was significant ($p < 0.001$), indicating that treatment differences depended on both sampling hour and glucose addition. Without glucose addition, BCS400 showed the highest CO₂ emission at both sampling times. At 48 h, BCS400 emitted approximately 17.4 $\mu\text{g CO}_2\text{-C g}^{-1}$ dry soil, compared with about 9.0 $\mu\text{g g}^{-1}$ for BSS400 and 4.4 $\mu\text{g g}^{-1}$ for the control. Thus, BCS400 emitted approximately 93% more CO₂ than BSS400 and almost four times as much as the control, and all three treatments differed significantly. With glucose addition at 24 h, BCS400 had significantly higher CO₂ emission than the control, while BSS400 was intermediate and did not differ significantly from either treatment. By 48 h, however, the treatment differences were no longer significant, with all treatments reaching approximately 95–106 $\mu\text{g CO}_2\text{-C g}^{-1}$ dry soil.

On day 14, the hour $\times$ treatment $\times$ glucose interaction was also significant ($p = 0.009$). Without glucose addition, BCS400 again showed the highest CO₂ emission. At 48 h, BCS400 emitted approximately 11.0 $\mu\text{g CO}_2\text{-C g}^{-1}$, compared with around 7.3 $\mu\text{g g}^{-1}$ for BSS400 and 4.7 $\mu\text{g g}^{-1}$ for the control, and all three treatments differed significantly. The BCS400 treatment therefore emitted approximately 51% more CO₂ than BSS400 and more than twice as much as the control. With glucose addition at 24 h, BCS400 had significantly higher CO₂ emission than the control, while BSS400 was intermediate and did not differ significantly from either BCS400 or the control. At 48 h, BCS400 and BSS400 did not differ significantly from each other, but both biochar treatments had significantly higher CO₂ emission than the control.

On day 28, the three-way interaction was not significant ($p = 0.525$). Treatment differences were therefore evaluated overall across 24 and 48 h within each glucose condition. Without glucose addition, BCS400 had significantly higher CO₂ emission than BSS400, while BSS400 was significantly higher than the control. With glucose addition, no significant differences were found among the three treatments.

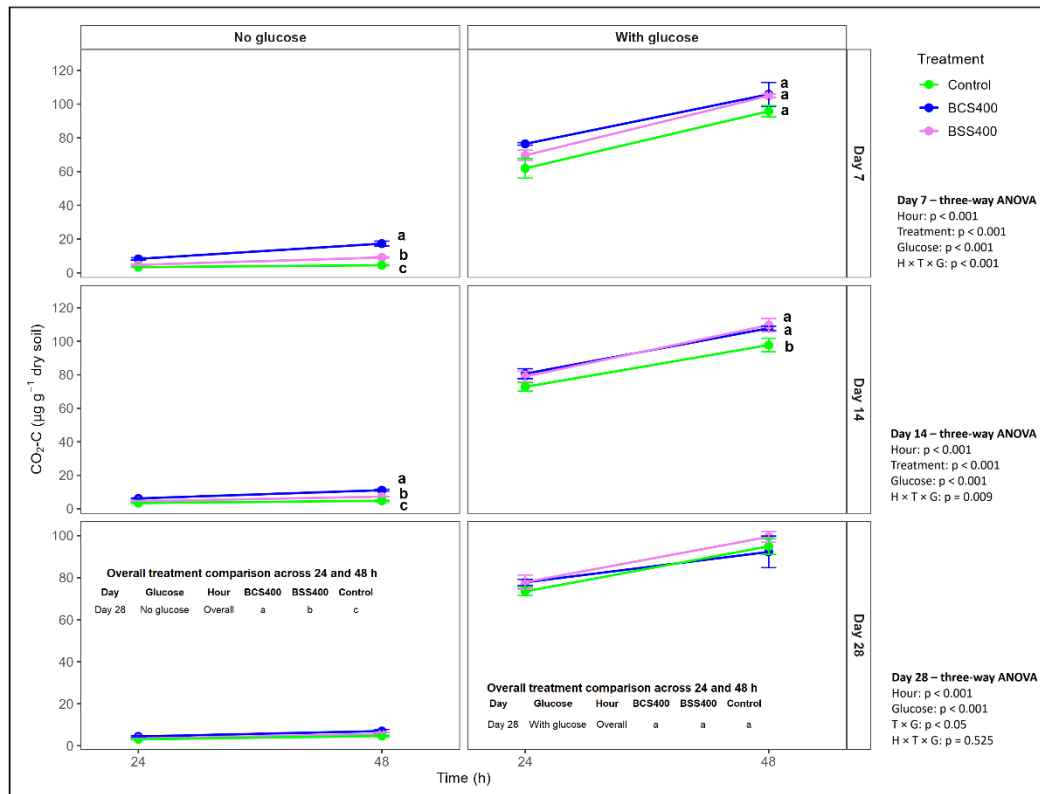


Figure 10. CO_2 emissions from control, BCS400 and BSS400 at 24 and 48 h on days 7, 14 and 28, with and without glucose addition. Values represent means $\pm$ SD ($n = 3$).

3.3.2 Heat dissipation

Cumulative heat dissipation increased over time, and glucose addition resulted in substantially greater heat dissipation in all treatments (Figure 11).

On day 7, calorimetric measurements were available only up to 24 h because the calorimeter measurement was interrupted. Therefore, statistical comparisons for day 7 were performed at 24 h. Treatment and glucose addition both had significant effects on cumulative heat dissipation, and the treatment $\times$ glucose interaction was also significant ($p < 0.001$). At 24 h without glucose addition, BCS400 showed the highest cumulative heat dissipation, reaching approximately 0.41 J g^{-1} dry soil, compared with 0.17 J g^{-1} for BSS400, while the control remained close to zero. The BCS400 treatment was significantly higher than both BSS400 and control, whereas BSS400 and control did not differ significantly. With glucose addition, cumulative heat dissipation increased to approximately 3.94 J g^{-1} in BCS400, 3.02 J g^{-1} in BSS400, and 2.53 J g^{-1} in the control. All three treatments differed significantly, with $\text{BCS400} > \text{BSS400} > \text{control}$. Therefore BCS400 dissipated approximately 31% more heat than BSS400 and 56% more than the control.

On day 14, hour, treatment, and glucose addition significantly affected cumulative heat dissipation, and the hour $\times$ treatment $\times$ glucose interaction was

significant ($p = 0.028$). Without glucose addition, no significant treatment differences were observed at 24 h. At 48 h, BCS400 had the highest cumulative heat dissipation at approximately 0.51 J g^{-1} , compared with 0.28 J g^{-1} in BSS400 and 0.07 J g^{-1} in the control. The BCS400 treatment was significantly higher than control, while BSS400 was intermediate and did not differ significantly from either treatment. With glucose addition on day 14, BCS400 was significantly higher than both BSS400 and the control at 24 h, whereas BSS400 and the control did not differ significantly. At 48 h, BCS400 and BSS400 reached approximately 4.73 and 4.68 J g^{-1} , respectively, compared with 4.27 J g^{-1} in the control. The two biochar treatments did not differ significantly from each other, but both were significantly higher than the control.

On day 28, the hour $\times$ treatment $\times$ glucose interaction was also significant ($p < 0.001$), indicating that treatment differences depended on both sampling time and glucose addition. Without glucose addition, no significant treatment differences were observed at 24 h. At 48 h, BCS400 had the highest cumulative heat dissipation at approximately 0.30 J g^{-1} , while BSS400 was intermediate at approximately 0.11 J g^{-1} and the control remained lower at approximately 0.05 J g^{-1} . The BCS400 treatment was significantly higher than control, whereas BSS400 did not differ significantly from either treatment. With glucose addition at 24 h, all three treatments differed significantly, with $\text{BCS400} > \text{BSS400} > \text{control}$. At 48 h, cumulative heat dissipation was approximately 4.73 J g^{-1} in BCS400, 4.57 J g^{-1} in BSS400, and 4.33 J g^{-1} in the control. Both BCS400 and BSS400 did not differ significantly from each other, but were significantly higher than the control.

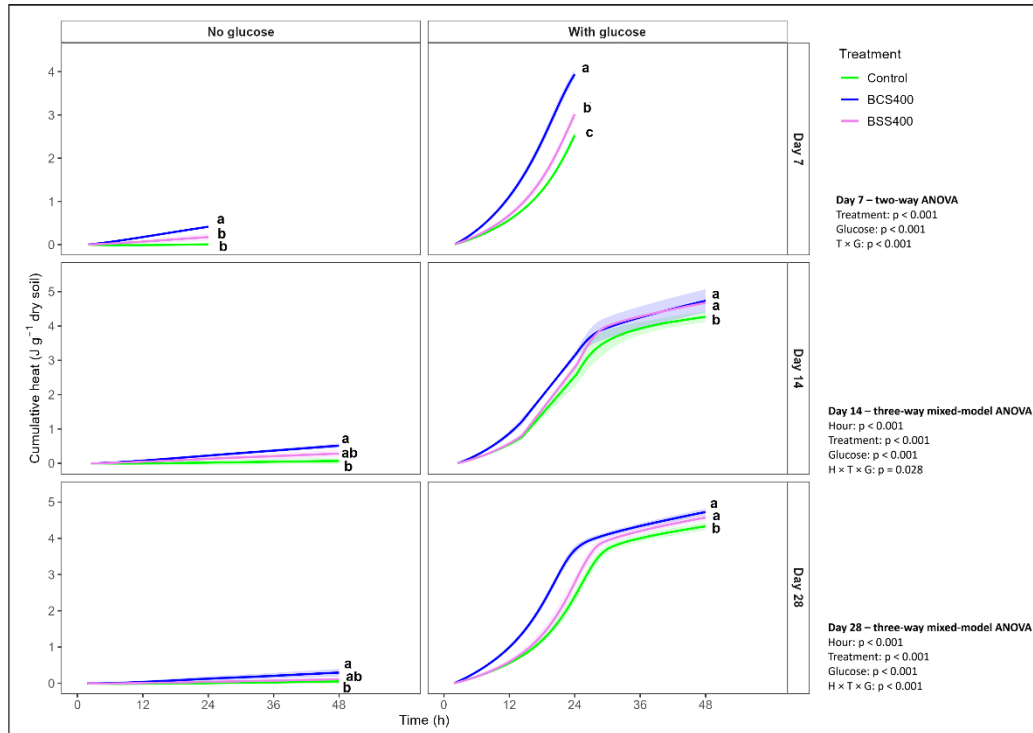


Figure 11. Cumulative heat dissipation from control, BCS400 and BSS400 on days 7, 14 and 28, with and without glucose addition. Lines represent mean cumulative heat dissipation, and shaded areas represent $\pm$ SD ($n = 3$).

3.3.3 Calorespirometric ratio

The pattern of CR in glucose-amended samples differed among the separately analysed incubation days (Figure 12).

On day 7, CR was evaluated only at 24 h because the calorimeter measurement was interrupted after this time point. Treatment had a significant effect on CR ($p = 0.003$). The BCS400 treatment showed the highest CR, approximately 625 kJ mol^{-1} , compared with approximately 529 kJ mol^{-1} in BSS400 and 502 kJ mol^{-1} in the control. Thus, CR in BCS400 was approximately 18% higher than BSS400 and 24% higher than the control. Mean CR values in all three treatments were above the 469 kJ mol^{-1} reference value.

On day 14, neither hour ($p = 0.804$) nor treatment ($p = 0.834$) significantly affected CR, and the hour $\times$ treatment interaction was also not significant ($p = 0.616$). Treatment comparisons were therefore evaluated overall across 24 and 48 h. The model-estimated mean CR values were $532.2 \text{ kJ mol}^{-1}$ for BCS400, $517.2 \text{ kJ mol}^{-1}$ for the control and $512.4 \text{ kJ mol}^{-1}$ for BSS400, and no significant differences were found among the three treatments. CR values were generally above the 469 kJ mol^{-1} reference value at both sampling times.

On day 28, both hour ($p = 0.003$) and treatment ($p = 0.005$) significantly affected CR, whereas the hour $\times$ treatment interaction was not significant ($p = 0.131$). Treatment differences were therefore evaluated overall across 24 and 48 h.

The model-estimated mean CR was 605.4 kJ mol⁻¹ for BCS400, 520.8 kJ mol⁻¹ for BSS400 and 499.8 kJ mol⁻¹ for the control. Therefore, BCS400 had an overall CR approximately 16% higher than BSS400 and 21% higher than the control. The BCS400 treatment was significantly higher than both the control ($p = 0.006$) and BSS400 ($p = 0.016$), whereas BSS400 and the control did not differ significantly ($p = 0.600$). At 24 h, the control mean was slightly below the 469 kJ mol⁻¹ reference value, while by 48 h all three treatments were above the reference line.

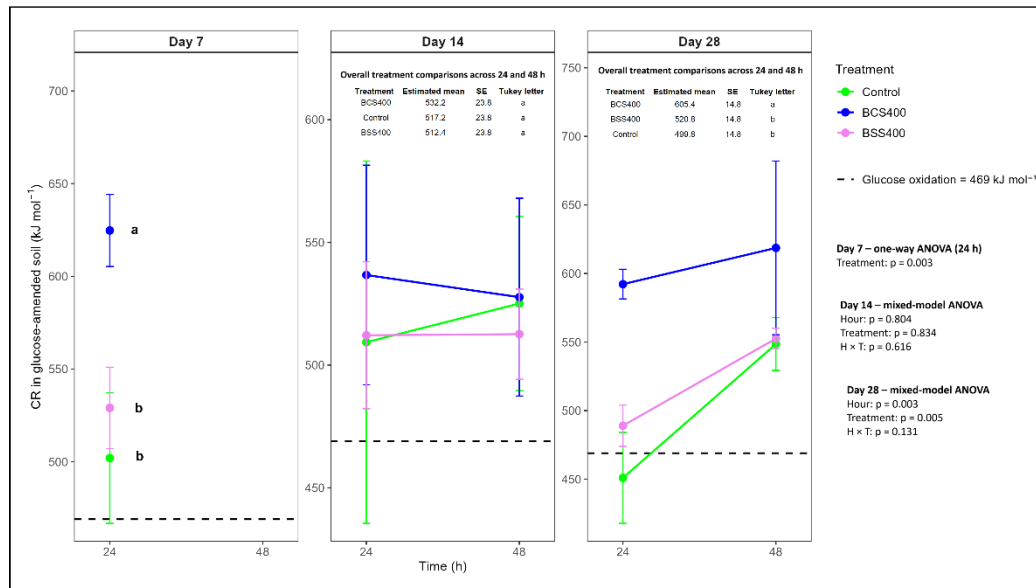


Figure 12. CR in glucose-amended control, BCS400 and BSS400 on days 7, 14 and 28. Values are means $\pm$ SD ($n = 3$).

3.3.4 Calorespirometric response to glucose addition

The pattern of the difference-based CR differed among the separately analysed incubation days (Figure 13).

On day 7, CR was evaluated only at 24 h because calorimetric measurements were available only up to this time point. Treatment had a significant effect on the difference-based CR ($p = 0.014$). The BCS400 treatment showed the highest CR, approximately 627 kJ mol⁻¹, compared with approximately 535 kJ mol⁻¹ for BSS400 and 530 kJ mol⁻¹ for the control. Thus, the difference-based CR in BCS400 was approximately 17% higher than in BSS400 and 18% higher than in the control. The BCS400 treatment was significantly higher than both treatments, whereas BSS400 and the control did not differ significantly. Mean difference-based CR values in all three treatments were above the theoretical reference value of 469 kJ mol⁻¹ for complete glucose oxidation.

On day 14, neither hour ($p = 0.843$) nor treatment ($p = 0.888$) significantly affected the difference-based CR, and the hour $\times$ treatment interaction was also not significant ($p = 0.567$). Treatment comparisons were therefore evaluated

overall across 24 and 48 h. The model-estimated mean difference-based CR values were 537.0 kJ mol⁻¹ for the control, 532.3 kJ mol⁻¹ for BCS400, and 517.7 kJ mol⁻¹ for BSS400, and no significant differences were found among the three treatments. Mean difference-based CR values remained above the theoretical 469 kJ mol⁻¹ reference value at both sampling times.

On day 28, both hour ($p = 0.002$) and treatment ($p = 0.013$) significantly affected the difference-based CR, whereas the hour $\times$ treatment interaction was not significant ($p = 0.108$). The difference-based CR increased overall between 24 and 48 h, and treatment differences were therefore evaluated overall across both sampling times. The model-estimated mean difference-based CR was 616.7 kJ mol⁻¹ for BCS400, 537.6 kJ mol⁻¹ for BSS400, and 519.4 kJ mol⁻¹ for the control. Thus, BCS400 had an overall difference-based CR approximately 15% higher than BSS400 and 19% higher than the control. The BCS400 treatment was significantly higher than both BSS400 and the control, whereas BSS400 and the control did not differ significantly. At 24 h, the control mean was close to the theoretical 469 kJ mol⁻¹ reference value, while by 48 h mean difference-based CR values in all treatments were above the reference.

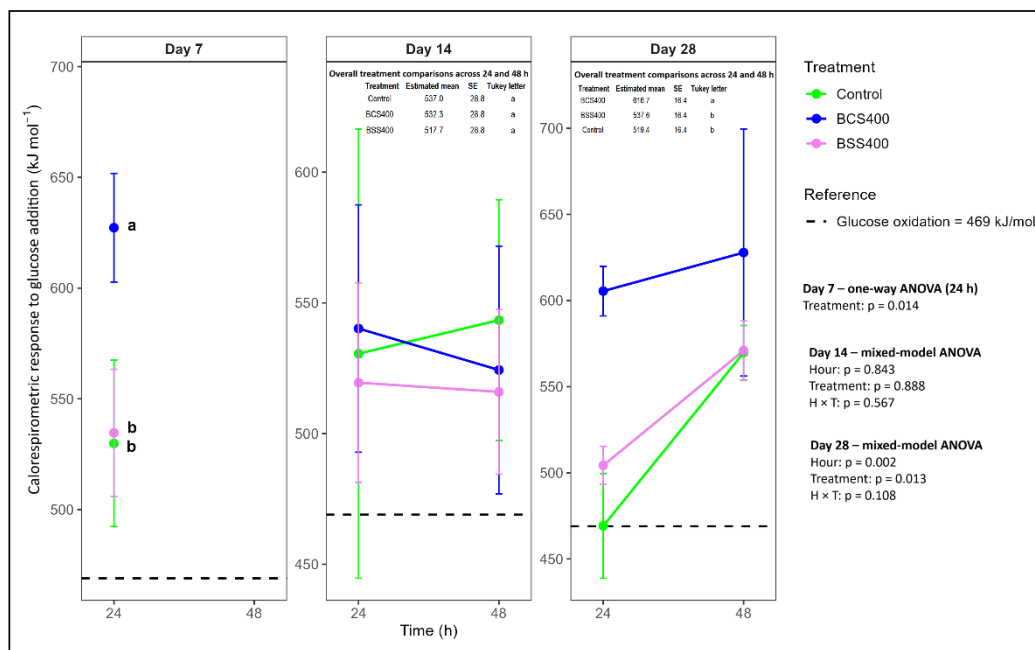


Figure 13. Difference-based CR following glucose addition in the control, BCS400 and BSS400 on days 7, 14 and 28. Values represent means $\pm$ SD ($n = 3$).

3.3.5 Microbial biomass carbon

In the glucose-amended samples, MBC was significantly affected by day and treatment ($p < 0.001$), and the day $\times$ treatment interaction was also significant ($p = 0.020$) (Figure 14). This interaction indicates that the effect of treatment on MBC

differed among incubation days. Therefore, treatments were compared separately within each day.

On day 7, BCS400 showed the highest MBC, at approximately $340 \mu\text{g C g}^{-1}$ dry soil, compared with approximately $260 \mu\text{g C g}^{-1}$ in BSS400 and $110 \mu\text{g C g}^{-1}$ in the control. The BCS400 treatment had significantly higher MBC than control, while BSS400 was intermediate and did not differ significantly from either BCS400 or control.

On day 14, MBC increased markedly in both BCS400 and BSS400. The BCS400 treatment reached approximately $610 \mu\text{g C g}^{-1}$ dry soil and BSS400 approximately $550 \mu\text{g C g}^{-1}$, whereas the control remained close to $120 \mu\text{g C g}^{-1}$. Both BCS400 and BSS400 had significantly higher MBC than control, while the two treatments with biochar did not differ significantly from each other.

On day 28, BSS400 showed the highest MBC, at approximately $550 \mu\text{g C g}^{-1}$ dry soil, followed by BCS400 at approximately $510 \mu\text{g C g}^{-1}$ and the control at approximately $350 \mu\text{g C g}^{-1}$. BSS400 had significantly higher MBC than control, whereas BCS400 was intermediate and did not differ significantly from either treatment.

Microbial biomass carbon was also measured without glucose on day 28. The BCS400 treatment showed a considerably higher value, approximately $370 \mu\text{g C g}^{-1}$ dry soil, compared with approximately $145 \mu\text{g C g}^{-1}$ in both BSS400 and the control. However, only one measurement was available for each treatment ($n=1$); therefore, these values are presented descriptively and were not included in the statistical analysis.

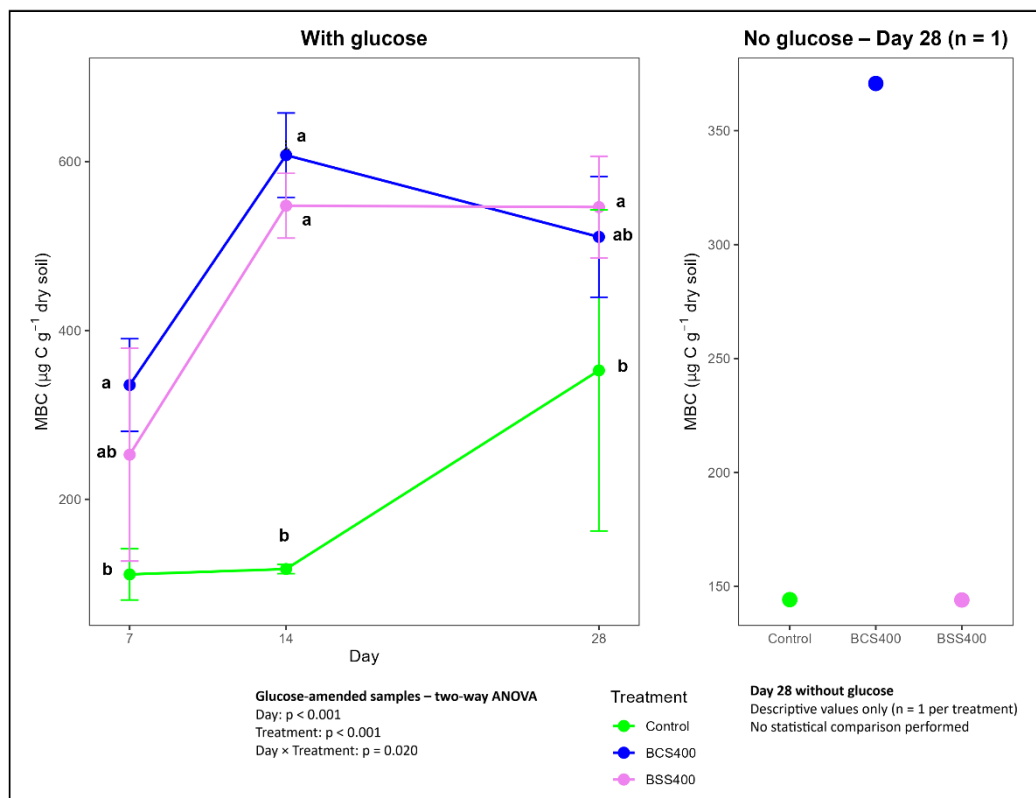


Figure 14. MBC in control, BCS400 and BSS400 on days 7, 14 and 28. Values represent means $\pm$ SD ($n = 3$) for glucose-amended samples. Non-glucose samples on day 28 are individual observations ($n = 1$ per treatment).

4. Discussion

4.1 Effect of pyrolysis temperature on carbon mineralization and microbial activity

Experiment 1 primarily addressed H2, while also providing evidence relevant to H1. Overall, the evidence was generally consistent with H2, although the magnitude and direction of the effect depended on incubation time and glucose addition. The BCS400 treatment generally showed greater CO₂ emission and heat dissipation than BCS550, particularly on day 3 and in the absence of glucose. This indicates greater overall carbon mineralization and microbial metabolic activity in soil amended with the lower-temperature biochar.

The difference between BCS400 and BCS550 may partly be explained by their degree of carbonization and carbon availability. Compared with BCS550, BCS400 had a substantially higher H/C ratio (0.813 compared with 0.361), while its O/C ratio was also slightly higher (0.518 compared with 0.471). The lower H/C ratio of BCS550 is consistent with a more carbonized and chemically stable structure. In addition, BCS400 had a substantially higher DOC concentration than BCS550 (2576.8 compared with 100.1 mg kg⁻¹), indicating a larger dissolved organic carbon pool in the lower-temperature biochar. This agrees with previous studies showing that increasing pyrolysis temperature generally decreases H/C and O/C ratios and increases biochar carbon stability (Li et al., 2023; Wei et al., 2019). Wei et al. (2019) also reported that lower-temperature biochars contained more dissolved and unstable organic carbon than biochars produced at higher temperatures. Therefore, the greater CO₂ emission and heat dissipation from BCS400 may partly reflect its less carbonized structure and greater availability of potentially accessible carbon.

The two biochars also differed in total N content, with 2.31% N in BCS400 compared with 1.85% in BCS550. This difference may partly reflect N loss during pyrolysis, as N can be volatilized in gaseous forms and N retention generally decreases with increasing pyrolysis temperature, including for manure-derived biochars (Ippolito et al., 2020; Rathnayake et al., 2023). The biochars also differed in C/N ratio, with values of 16.34 for BCS400 and 19.26 for BCS550. Differences in biochar C/N ratio and carbon availability could potentially influence microbial N availability through N immobilization, although total elemental C/N ratio alone is not necessarily a reliable predictor of this response (Phillips et al., 2022; Bekchanova et al., 2024). In the present study, mineral N was added to all samples to reduce the possibility of N limitation. However, inorganic N was not measured during the incubation, so it is not possible to determine whether differences in N immobilization or inorganic N availability contributed to the contrasting microbial responses of BCS400 and BCS550.

The ^{13}C -labelled glucose results provided further information about the source of the respired carbon (Figure 6). On day 3, the large respiratory response of BCS400 was particularly evident in the non-glucose-derived CO_2 fraction. At 48 h, BCS400 emitted approximately twice as much non-glucose-derived CO_2 as BCS550 and about 3.7 times as much as the control. In contrast, differences in glucose-derived CO_2 were smaller, although BCS400 and the control both showed greater glucose-derived respiration than BCS550 at 48 h. Thus, the high total respiration of BCS400 on day 3 was not explained only by greater glucose mineralization but also by greater mineralization of non-glucose carbon. By day 65, the pattern had changed. The control showed significantly greater glucose-derived CO_2 than both biochar treatments at 48 h, whereas non-glucose-derived CO_2 was, on average, higher in both biochar treatments than in the control. This shows that the influence of biochar differed between the added glucose and the non-glucose carbon pool and also changed as the incubation progressed. However, the non-glucose-derived fraction includes potential contributions from native soil organic carbon, biochar-derived carbon, and microbial turnover. Therefore, the present experiment cannot determine which of these sources was responsible for the higher non-glucose-derived respiration.

The BCS400 treatment generally dissipated more heat than BCS550, particularly on day 3 and in the absence of glucose, indicating greater microbial metabolic activity. The higher H/C ratio and substantially higher DOC concentration of BCS400 provide a possible explanation for this stronger early response. Lower-temperature biochars have also been associated with greater microbial activity and cumulative CO_2 production in previous studies (Kerner et al., 2023). As incubation progressed, however, the difference between BCS400 and BCS550 became smaller, particularly following glucose addition, suggesting that the influence of the initial differences in biochar carbon availability became less pronounced when a readily available external substrate was supplied.

Overall, the evidence was generally consistent with H2, and H2 was therefore not rejected. As predicted, BCS400 generally produced greater total CO_2 emission and heat dissipation than BCS550, particularly during the early incubation period, consistent with the less carbonized characteristics and substantially higher DOC concentration. However, the predicted difference was not observed under all conditions. By day 65, BCS400 and BCS550 did not differ significantly in glucose-derived CO_2 , and their heat responses following glucose addition were also similar. The evidence was also consistent with H1, and H1 was not rejected, because biochar amendment altered overall carbon mineralization relative to the control under several experimental conditions. However, the direction and magnitude of this effect depended on biochar type, glucose addition, and incubation time.

4.2 Effect of digestate separation procedure on microbial activity

Experiment 2 addressed H3 by comparing BCS400 and BSS400, which were both produced at 400 °C but from solid fractions recovered at different stages of digestate separation. Hypothesis 3 predicted that BSS400 would stimulate greater microbial activity because the screw-press solids were expected to contain more readily decomposable cellulose- and hemicellulose-rich material than the centrifuge-recovered solids. However, the evidence was not consistent with the predicted direction of H3. Contrary to the prediction, BCS400 generally resulted in greater CO₂ emission and heat dissipation than BSS400, particularly in the absence of glucose.

The difference between the two biochars may partly be related to the different characteristics of the fractions produced during mechanical separation. Screw pressing mainly retains larger particles, while smaller and finer particles remain in the liquid fraction and can subsequently be recovered by centrifugation. Centrifugation can also concentrate a greater proportion of nutrients and mineral-associated material in the recovered solid fraction (Hjorth et al., 2011; Carraro et al., 2024; Cathcart et al., 2023; Lyons et al., 2021). Thus, although both biochars were produced from the same manure-based digestate and at the same pyrolysis temperature, the feedstocks entering pyrolysis were compositionally different. These differences remained visible after pyrolysis. The BSS400 contained more total carbon than BCS400 (58.27% compared with 37.70%) and had lower H/C and O/C ratios (0.592 and 0.269 compared with 0.813 and 0.518, respectively). The lower H/C and O/C ratios of BSS400 are consistent with a more carbonized and chemically stable structure. In contrast, BCS400 had a much higher DOC concentration than BSS400 (2576.8 compared with 872.3 mg kg⁻¹). This indicates that, despite both materials being pyrolyzed at 400 °C, the separation procedure before pyrolysis resulted in biochars with substantially different carbon characteristics.

The two biochars had similar total N concentrations, with 2.26% N in BSS400 and 2.31% in BCS400, but their C/N ratios differed substantially (25.86 and 16.34, respectively) because BSS400 contained more total C. Differences in biochar C/N ratio and carbon availability could potentially influence microbial N availability through N immobilization (Phillips et al., 2022; Bekchanova et al., 2024). Therefore, differences in N dynamics could have contributed to the contrasting microbial responses of BSS400 and BCS400. However, because inorganic N was not measured, this mechanism cannot be confirmed.

The microbial responses may partly be related to these differences in biochar properties. Without glucose addition, BCS400 consistently showed greater CO₂ emission than BSS400 during Exp 2. The same general pattern was observed for

heat dissipation, with BCS400 showing the stronger response particularly during the earlier measurements. The substantially higher DOC concentration of BCS400 suggests that it contained a larger dissolved carbon pool that may have been more accessible to microorganisms, potentially contributing to its greater respiration and heat dissipation. However, DOC was measured as a biochar characteristic rather than traced as a carbon source during the incubation. Therefore, the present study cannot determine how much of the additional CO₂ or heat originated specifically from this dissolved carbon, and other differences in nutrient composition, mineral content, or biochar properties may also have contributed.

After glucose addition, the differences between BCS400 and BSS400 often became smaller or disappeared at later sampling times, suggesting that the addition of a readily metabolizable external carbon source reduced the relative importance of differences associated with the biochars themselves. Because glucose was unlabelled in Exp 2, however, glucose-derived respiration could not be separated from respiration of soil- or biochar-derived carbon.

The MBC results provided additional evidence relevant to H3, although they did not show a consistent difference between the two biochars. The BCS400 treatment had the highest MBC on day 7, the two biochars did not differ significantly on day 14, and BSS400 showed the highest MBC on day 28. Thus, the predicted greater microbial biomass response in BSS400 was not consistently observed, and the effect of the separation procedure on microbial biomass changed during incubation. The generally greater MBC in the biochar-amended soils compared with the control is consistent with previous studies reporting increased microbial biomass following biochar addition (Kerner et al., 2023; Kalu et al., 2024).

Overall, the evidence was not consistent with the specific prediction of H3, and H3 was therefore rejected. BSS400 was expected to stimulate greater microbial activity than BCS400 because the screw-press fraction was assumed to contain more cellulose- and hemicellulose-rich material. However, this expectation based on the original digestate fractions did not correspond to the measured characteristics of the biochars after pyrolysis. BSS400 had lower H/C and O/C ratios, consistent with a more carbonized structure, whereas BCS400 had almost three times higher DOC concentration. The larger dissolved carbon pool in BCS400 may therefore partly explain why it generally produced greater CO₂ emission and heat dissipation than BSS400. These findings indicate that differences in the original digestate fractions can be modified during pyrolysis and that the properties of the final biochar, rather than the presumed composition of the original feedstock alone, are important for determining microbial responses.

4.3 Calorespirometric response, implications for carbon use efficiency, and study limitations

The calorespirometric results addressed H4, which predicted that biochar amendment would alter the relationship between heat dissipation and CO₂ emission compared with the control. The evidence was generally consistent with this hypothesis, although the magnitude of the response depended on biochar type, incubation day, and sampling time. Two related CR metrics were evaluated: the CR in glucose-amended samples and the difference-based CR representing the net calorespirometric response to glucose addition. The difference-based CR does not represent glucose metabolism exclusively because glucose addition may alter mineralization of other carbon sources, while calorimetry cannot distinguish heat originating from different carbon sources or metabolic pathways.

A value of approximately 469 kJ mol⁻¹ C is commonly used as the theoretical reference for complete aerobic oxidation of glucose (Geyer et al., 2019). Under the assumptions of the calorespirometric model, values below this reference can indicate that a greater proportion of substrate carbon is allocated to microbial biomass rather than completely oxidized to CO₂, and CR is therefore inversely related to CUE within this valid range. However, values above 469 kJ mol⁻¹ cannot be directly converted into CUE and may reflect the use of substrates with different oxidation states, incomplete oxidation, changes in metabolic pathways, or limitations in CO₂ recovery (Geyer et al., 2019; Pineiro et al., 2025). In the present study, this limitation is particularly important because neither the total CR nor the difference-based CR represents glucose metabolism alone. The 469 kJ mol⁻¹ value should therefore be considered a theoretical reference rather than a direct threshold for calculating CUE.

In Exp 1, both CR calculations showed that the biochars changed the relationship between heat dissipation and respiration. On day 3, the CR calculated from total heat and total CO₂ in glucose-amended samples was significantly higher in both BCS400 and BCS550 than in the control when averaged across sampling times, while the two biochars did not differ significantly. The difference-based CR showed a similar pattern, with BCS400 having a significantly higher mean value than the control, while BCS550 was intermediate. On day 65, the response became more dependent on sampling time. Both biochars showed higher total CR than the control at several later sampling times, while differences in the response to glucose addition occurred mainly at 18 and 24 h and were no longer significant at 36 and 48 h. These results indicate that pyrolysis temperature influenced the balance between microbial heat dissipation and CO₂ production, but the difference between BCS400 and BCS550 was not consistent throughout the incubation.

The relationship with CUE should therefore be interpreted cautiously. Some early CR values were below the theoretical glucose-oxidation value and, under the assumptions of the calorimetric model, could be consistent with greater allocation of carbon to microbial growth rather than respiration. However, most CR values approached or exceeded 469 kJ mol^{-1} as the measurements progressed. Values above this reference cannot be interpreted simply as evidence of lower CUE. In particular, the higher CR observed in the biochar treatments indicates that the relative amount of heat dissipated per unit of $\text{CO}_2\text{-C}$ differed from the control, but it does not by itself show whether microbial carbon retention was greater or smaller.

Experiment 2 provided further evidence that biochar characteristics influenced the calorimetric response. The BCS400 treatment generally showed the highest CR. On day 7, both the total CR and the difference-based response to glucose addition were significantly higher in BCS400 than in BSS400 and the control. No significant treatment differences were observed on day 14, whereas on day 28 BCS400 again had significantly higher total and difference-based CR than both BSS400 and the control. In contrast, BSS400 generally remained closer to the control. This shows that the digestate separation procedure affected not only respiration and heat dissipation separately, but also the relationship between these two metabolic outputs. The CR results therefore further demonstrate that BCS400 and BSS400 behaved differently despite being produced at the same pyrolysis temperature.

The higher CR of BCS400 in Exp 2 should not, however, be interpreted as evidence of lower CUE because most values were above the theoretical glucose-oxidation reference. Rather, these results indicate that the heat-to- CO_2 balance differed between the two biochars. This is consistent with the differences in their elemental characteristics and with the respiration and heat-dissipation results discussed above. BSS400 had lower H/C and O/C ratios than BCS400, indicating a more carbonized and chemically stable structure, whereas BCS400 generally supported greater respiration and heat dissipation. These differences suggest that the separation procedure before pyrolysis affected the composition of the resulting biochars sufficiently to influence microbial carbon and energy metabolism.

Comparison with previous studies also illustrates why a direct CUE conclusion is not possible here. Kalu et al. (2024) found that biochar reduced mineralization of ^{13}C -labelled glucose while increasing microbial incorporation and retention of glucose-derived carbon, allowing them to conclude that biochar increased microbial CUE. In the present study, the information required for such a calculation was not obtained within the same experiment. Experiment 1 provided isotope-resolved CO_2 measurements, while MBC was evaluated in Exp 2 using unlabelled glucose. Consequently, substrate-derived carbon incorporated into microbial biomass and substrate-derived carbon lost through respiration could not

be quantified simultaneously. The CR results can therefore provide qualitative information related to microbial carbon and energy use, but they cannot provide a direct quantitative estimate of CUE. Calorespirometric estimates of CUE require specific assumptions and become difficult to interpret when several processes, including substrate mineralization, priming, microbial turnover, and maintenance respiration, occur simultaneously.

Another limitation is that oxygen concentration was not measured. The calorimetric ampoules were closed during the 48 h measurements, while glucose addition strongly stimulated microbial activity. Oxygen availability may therefore have changed during incubation, particularly during periods of high activity. Because oxygen availability can influence microbial metabolic pathways and the relationship between heat production and CO₂ evolution, it may also affect CR interpretation (Pineiro et al., 2025). However, because oxygen was not monitored, neither the onset nor the magnitude of possible oxygen limitation can be determined from the present data, and it is therefore not possible to attribute the observed CR patterns specifically to oxygen limitation.

CR also generally changed during the 48 h measurement periods, showing that the relationship between cumulative heat dissipation and CO₂ production was not constant over time. This may reflect changes in substrate availability and microbial metabolism as incubation progressed. Because cumulative heat and CO₂ do not necessarily increase at the same rate, the calculated CR also depends on the measurement interval. Therefore, differences among early and later CR values should be interpreted as changes in the overall heat–CO₂ relationship rather than as direct changes in CUE.

Overall, the evidence was consistent with H4, and H4 was not rejected. Biochar amendment altered the relationship between heat dissipation and CO₂ emission compared with the control, and differences were also observed among the biochars. Among the treatments, BCS400 most consistently showed higher CR than the control and, in Exp 2, than BSS400, although these differences depended on incubation day and sampling time. The results therefore indicate that pyrolysis temperature and digestate separation procedure influenced microbial metabolic responses. However, because many CR values were above the theoretical range that can be directly related to glucose-based CUE, and because the difference-based CR did not isolate heat and CO₂ produced exclusively from glucose metabolism, the CR results should be interpreted as indicators of changes in microbial carbon and energy metabolism rather than as direct quantitative measurements of CUE.

5. Conclusions

This study showed that digestate-derived biochars influenced carbon mineralization and microbial activity in soil, and that these responses depended on both pyrolysis conditions and digestate separation procedure.

Pyrolysis temperature influenced microbial responses, with BCS400 generally producing greater CO₂ emission and heat dissipation than BCS550, particularly during the early incubation period and without glucose addition. The ¹³C-labelled glucose experiment further showed that the strong early respiratory response of BCS400 was not driven only by mineralization of the added glucose.

Digestate separation procedure also mattered. Although BCS400 and BSS400 were produced at the same pyrolysis temperature, they caused different microbial responses, with BCS400 generally producing greater respiration and heat dissipation. Thus, biochars derived from different fractions of the same digestate should not necessarily be considered equivalent soil amendments.

Biochar amendment also altered the relationship between heat dissipation and CO₂ emission. However, the CR results did not provide direct evidence that biochar increased microbial CUE.

Overall, digestate-derived biochars should not be considered a uniform group of soil amendments, because their microbial effects depend on both pyrolysis conditions and digestate processing.

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Popular science summary

Anaerobic digestion is a process that can treat many types of organic materials, including manure, wastewater sludge, municipal organic waste, and agricultural residues. During anaerobic digestion, microorganisms break down organic material in the absence of oxygen and produce two main products: biogas and digestate. Biogas can be used as a renewable energy source, while digestate contains organic matter and plant nutrients and can therefore be used as a fertilizer or soil conditioner. However, direct use of digestate can also create challenges related to storage, transport, nutrient losses, odour, and hygiene. For this reason, further treatment of digestate can be useful. One possible post-treatment is pyrolysis, in which organic material is heated under little or no oxygen. Pyrolysis converts the solid fraction of digestate into biochar, a carbon-rich material that can be used as a soil amendment. The high temperatures used during pyrolysis can also provide thermal sanitisation of the digestate material.

Biochar has attracted interest because it may contribute to carbon storage and can influence soil properties, nutrient retention, and biological activity. Not all biochars behave in the same way. Their properties depend both on the original material used to produce them and on the conditions during production. For example, pyrolysis temperature can influence the stability and chemical structure of biochar. The way digestate is processed before pyrolysis may also be important. Solid–liquid separation methods such as screw pressing and centrifugation can produce solid fractions with different amounts of organic matter, nutrients, and fine particles. These differences may remain important even after the separated material has been converted into biochar.

This study investigated how different biochars produced from manure-based digestate affected microbial activity and carbon mineralization in soil. Two laboratory experiments were carried out. In the first experiment, the liquid fraction obtained after screw-press separation was further separated by centrifugation, and the centrifuge-recovered solids were used to produce two biochars: one pyrolyzed at 400 °C (BCS400) and the other at 550 °C (BCS550). Comparing these two biochars made it possible to study the influence of pyrolysis temperature. In the second experiment, two biochars produced at the same temperature of 400 °C were compared. One was produced from solids retained directly by the screw press (BSS400), while the other was produced from solids recovered by centrifugation of the screw-press liquid fraction (BCS400). This comparison was used to investigate whether the digestate separation procedure before pyrolysis could influence the response of soil microorganisms.

Soil microorganisms are important because they decompose organic materials, recycle nutrients, and release carbon dioxide during respiration. To study their response to the biochars, microbial activity was evaluated by measuring both

carbon dioxide production and heat release. Carbon dioxide provides information about microbial respiration and carbon mineralization, while heat production reflects the energy released during microbial metabolism. Glucose was also added as an easily available source of carbon to stimulate microbial activity. In the first experiment, glucose labelled with the stable carbon isotope ^{13}C was used. This made it possible to distinguish carbon dioxide produced from the added glucose from carbon dioxide originating from other carbon sources in the soil–biochar system.

The results showed that pyrolysis temperature influenced the microbial response. The biochar produced at 400 °C generally resulted in greater carbon dioxide emission and heat production than the biochar produced at 550 °C, particularly during the earlier stage of incubation and when glucose was not added. The lower-temperature biochar also had chemical characteristics consistent with a less carbonized material and contained substantially more dissolved organic carbon. These characteristics may have contributed to the stronger microbial response. The ^{13}C -labelled glucose experiment also showed that the greater respiration observed with BCS400 was not simply due to greater use of the added glucose. A substantial part of the difference was associated with carbon sources other than the added glucose.

The way the digestate was separated before pyrolysis was also important. Although BCS400 and BSS400 were both produced at 400 °C, they did not cause the same microbial response. Overall, BCS400 generally produced greater respiration and heat dissipation than BSS400, particularly without glucose. This biochar also contained considerably more dissolved organic carbon than BSS400, which may partly explain its stronger microbial response. This shows that differences created during digestate separation can influence the characteristics of the resulting biochar and its interaction with soil microorganisms. When glucose was added, differences between the biochars often became smaller, suggesting that the readily available external carbon source reduced the relative importance of differences between the biochars.

Biochar also affected microbial biomass, although the response changed during the incubation. At several sampling times, microbial biomass was higher in biochar-amended soil than in the control soil. The study also combined measurements of heat release and carbon dioxide production using the calorimetric ratio. This provided additional information about changes in microbial metabolism. However, this ratio can be influenced by several factors and should therefore not be interpreted as a direct measurement of how efficiently microorganisms retain carbon.

Overall, the results show that digestate-derived biochars should not be considered a uniform type of soil amendment. Both the temperature used during pyrolysis and the way digestate is separated before pyrolysis can influence how

the resulting biochar affects soil microorganisms and carbon mineralization. Understanding these differences is important when evaluating digestate-derived biochars for future use as soil amendments and as part of more sustainable management of organic residues.

Appendix 1

Detailed calculation:

Manual dilution factor = 22.4 mL / transferred headspace volume gas

Corrected CO₂ concentrations were converted to CO₂-C using the ideal gas law, assuming a pressure of 1 atm and a temperature of 293.15 K (20 °C):

$$\text{CO}_2\text{-C } (\mu\text{g C L}^{-1} \text{ headspace}) = [\text{CO}_2 \text{ (ppm)} \times \text{dilution factor} \times 12.011] / [0.0820575 \times T]$$

CO₂-C was then converted to $\mu\text{g C ampoule}^{-1}$ using the calculated headspace volume and finally normalized to dry soil mass ($\mu\text{g CO}_2\text{-C g}^{-1}$ dry soil).

Headspace volume was calculated by subtracting the volume occupied by soil, biochar or talc, and water from the total ampoule volume:

$$V_{\text{headspace}} = V_{\text{ampoule}} - V_{\text{solid}} - V_{\text{water}}$$

Solid volume was estimated using a particle density of 2.65 g cm⁻³, while water volume included both the water already present in the wet soil and all added liquid volumes.

Appendix 2

Tukey-adjusted pairwise comparisons- Exp 1

Table 4. Tukey grouping for CO₂ emission in Exp 1

Tukey grouping of CO ₂ -C emission treatments					
Day	Glucose	Hour	BCS400	Control	BCS550
Day 3	No glucose	6 h	a	b	b
Day 3	No glucose	12 h	a	c	b
Day 3	No glucose	18 h	a	b	b
Day 3	No glucose	24 h	a	c	b
Day 3	No glucose	36 h	a	c	b
Day 3	No glucose	48 h	a	c	b
Day 3	With glucose	6 h	a	b	c
Day 3	With glucose	12 h	a	b	b
Day 3	With glucose	18 h	a	b	b
Day 3	With glucose	24 h	a	b	b
Day 3	With glucose	36 h	a	b	b
Day 3	With glucose	48 h	a	b	b
Day 65	No glucose	6 h	a	a	b
Day 65	No glucose	12 h	a	b	c
Day 65	No glucose	18 h	a	b	c
Day 65	No glucose	24 h	a	b	c
Day 65	No glucose	36 h	a	b	c
Day 65	No glucose	48 h	a	b	c
Day 65	With glucose	6 h	a	b	b
Day 65	With glucose	12 h	a	b	b
Day 65	With glucose	18 h	a	b	b
Day 65	With glucose	24 h	a	a	a
Day 65	With glucose	36 h	a	ab	b
Day 65	With glucose	48 h	ab	a	b

Different lowercase letters indicate significant differences among treatments within the same day, hour and glucose condition (Tukey-adjusted comparisons, $p < 0.05$).

Table 4. Tukey grouping of control, BCS400 and BCS550 for CO₂ emissions at each sampling time on day 3 and day 65, with and without glucose addition. Different lowercase letters indicate significant differences among treatments within the same sampling time and glucose condition ($p < 0.05$).

Table 5. Tukey grouping for glucose-derived and non-glucose-derived CO₂ in Exp 1.

Treatment comparisons within each sampling hour					
Day	CO2_source	Hour	BCS400	Control	BCS550
Day 3	Glucose-derived	6 h	a	a	a
Day 3	Glucose-derived	12 h	a	b	b
Day 3	Glucose-derived	18 h	a	b	b
Day 3	Glucose-derived	24 h	a	a	b
Day 3	Glucose-derived	36 h	a	a	b
Day 3	Glucose-derived	48 h	a	a	b
Day 3	Non-glucose-derived	6 h	a	a	a
Day 3	Non-glucose-derived	12 h	a	b	a
Day 3	Non-glucose-derived	18 h	a	c	b
Day 3	Non-glucose-derived	24 h	a	b	b
Day 3	Non-glucose-derived	36 h	a	c	b
Day 3	Non-glucose-derived	48 h	a	c	b
Day 65	Glucose-derived	6 h	a	ab	b
Day 65	Glucose-derived	12 h	a	b	b
Day 65	Glucose-derived	18 h	a	a	b
Day 65	Glucose-derived	24 h	b	a	c
Day 65	Glucose-derived	36 h	a	a	b
Day 65	Glucose-derived	48 h	b	a	b

Day 65 - Non-glucose-derived CO2-C: overall treatment comparison

Treatment	Mean-CO2-C	SE	Letter
BCS550	10.94	0.52	a
BCS400	10.45	0.52	a
Control	7.60	0.52	b

Different lowercase letters indicate significant differences among treatments (Tukey-adjusted $p < 0.05$).

Table 5. Different lowercase letters indicate significant differences among treatments within the same sampling time ($p < 0.05$). For day 65 non-glucose-derived CO₂, letters represent the overall treatment comparison across sampling times because the hour $\times$ treatment interaction was not significant.

Table 6. Tukey grouping of control, BCS400 and BCS550 for cumulative heat dissipation in Exp 1.

Tukey grouping of cumulative heat treatments					
Day	Glucose	TimePoint	BCS400	BCS550	Control
Day 3	No glucose	6 h	a	a	a
Day 3	No glucose	12 h	a	a	a
Day 3	No glucose	18 h	a	ab	b
Day 3	No glucose	24 h	a	b	b
Day 3	No glucose	36 h	a	b	b
Day 3	No glucose	48 h	a	b	b
Day 3	With glucose	6 h	a	a	a
Day 3	With glucose	12 h	a	b	b
Day 3	With glucose	18 h	a	b	b
Day 3	With glucose	24 h	a	b	b
Day 3	With glucose	36 h	a	b	b
Day 3	With glucose	48 h	a	b	b
Day 65	No glucose	6 h	a	a	a
Day 65	No glucose	12 h	a	a	a
Day 65	No glucose	18 h	a	a	a
Day 65	No glucose	24 h	a	a	a
Day 65	No glucose	36 h	a	ab	b
Day 65	No glucose	48 h	a	ab	b
Day 65	With glucose	6 h	a	a	a
Day 65	With glucose	12 h	a	b	b
Day 65	With glucose	18 h	a	b	b
Day 65	With glucose	24 h	a	b	c
Day 65	With glucose	36 h	a	ab	b
Day 65	With glucose	48 h	a	a	b

Different lowercase letters indicate significant differences among treatments within the same day, time point and glucose condition (Tukey-adjusted $p < 0.05$).

Table 6. Values show Tukey grouping of treatments at the selected sampling times. Different lowercase letters indicate significant differences among treatments based on Tukey-adjusted pairwise comparisons from the mixed-effects model ($p < 0.05$).

Table 7. Tukey grouping of control, BCS400 and BCS550 for the CR in glucose-amended soil in Exp 1.

Day 3 - Overall treatment comparison			
Treatment	Mean_CR	SE	Letter
BCS400	497.9	11.3	a
BCS550	486.7	11.3	a
Control	420.6	11.3	b

Day 65 - Treatment comparisons within each sampling hour			
Hour	BCS400	Control	BCS550
6 h	a	a	a
12 h	a	a	a
18 h	a	b	b
24 h	a	b	a
36 h	b	b	a
48 h	a	b	a

Different lowercase letters indicate significant differences among treatments (Tukey-adjusted $p < 0.05$).

Table 7. On day 3, Tukey-adjusted comparisons represent overall treatment differences across sampling times because the hour $\times$ treatment interaction was not significant. On day 65, treatment comparisons were performed within each sampling hour because the hour $\times$ treatment interaction was significant. Different lowercase letters indicate significant differences among treatments ($p < 0.05$); treatments sharing a letter are not significantly different.

Table 8. Tukey grouping of control, BCS400 and BCS550 for calorespirometric response to glucose addition in Exp 1.

Day 3 - Overall treatment comparison			
Treatment	Mean_CR	SE	Letter
BCS400	549.7	20.3	a
BCS550	534.8	19.7	ab
Control	477.5	17.6	b

Day 65 - Treatment comparisons within each sampling hour			
Hour	BCS400	Control	BCS550
6 h	a	a	a
12 h	a	a	a
18 h	a	b	ab
24 h	a	b	a
36 h	a	a	a
48 h	a	a	a

Day 3 values are back-transformed model-estimated means; tests were performed on log-transformed CR. Different lowercase letters indicate significant differences among treatments (Tukey-adjusted $p < 0.05$).

Table 8. On day 3, treatment comparisons were made overall across sampling times because the hour $\times$ treatment interaction was not significant. On day 65, treatments were compared within each sampling hour because the hour $\times$ treatment interaction was significant. Different lowercase letters indicate significant differences among treatments ($p < 0.05$); treatments sharing the same letter are not significantly different.

Appendix 3

Tukey-adjusted pairwise comparisons- Exp 2

Table 9. Tukey grouping of control, BCS400 and BSS400 for CO₂ emissions in Exp 2.

Day 7 and Day 14 - Treatment comparisons within each sampling hour					
Day	Glucose	Hour	BCS400	BSS400	Control
Day 7	No glucose	24 h	a	b	c
Day 7	With glucose	24 h	a	ab	b
Day 7	No glucose	48 h	a	b	c
Day 7	With glucose	48 h	a	a	a
Day 14	No glucose	24 h	a	b	c
Day 14	With glucose	24 h	a	ab	b
Day 14	No glucose	48 h	a	b	c
Day 14	With glucose	48 h	a	a	b
Day 28 - Overall treatment comparison across 24 and 48 h					
Day	Glucose	Hour	BCS400	BSS400	Control
Day 28	No glucose	Overall	a	b	c
Day 28	With glucose	Overall	a	a	a

Different lowercase letters indicate significant differences among treatments within the same comparison group (Tukey-adjusted $p < 0.05$).

Table 9. On days 7 and 14, treatment comparisons were performed within each sampling hour and glucose condition because the hour $\times$ treatment $\times$ glucose interaction was significant. On day 28, treatments were compared overall across sampling hours within each glucose condition because the three-way interaction was not significant. Different lowercase letters indicate significant differences among treatments based on Tukey-adjusted pairwise comparisons ($p < 0.05$); treatments sharing the same letter are not significantly different.

Table 10. Tukey grouping of control, BCS400 and BSS400 for cumulative heat dissipation in Exp 2.

Experiment 2 – Cumulative heat dissipation						
Treatment comparisons within sampling hour and glucose condition						
Day	Hour	Glucose	Treatment	Estimated mean	SE	Tukey letter
Day 7	24	No	BCS400	0.414	0.046	a
Day 7	24	No	BSS400	0.174	0.046	b
Day 7	24	No	Control	0.005	0.046	b
Day 7	24	Yes	BCS400	3.942	0.046	a
Day 7	24	Yes	BSS400	3.017	0.046	b
Day 7	24	Yes	Control	2.533	0.046	c
Day 14	24	No	BCS400	0.224	0.088	a
Day 14	24	No	BSS400	0.130	0.088	a
Day 14	24	No	Control	0.017	0.088	a
Day 14	24	Yes	BCS400	3.140	0.088	a
Day 14	24	Yes	BSS400	2.795	0.088	b
Day 14	24	Yes	Control	2.533	0.088	b
Day 14	48	No	BCS400	0.513	0.088	a
Day 14	48	No	BSS400	0.283	0.088	ab
Day 14	48	No	Control	0.068	0.088	b
Day 14	48	Yes	BCS400	4.733	0.088	a
Day 14	48	Yes	BSS400	4.677	0.088	a
Day 14	48	Yes	Control	4.266	0.088	b
Day 28	24	No	BCS400	0.127	0.059	a
Day 28	24	No	BSS400	0.039	0.059	a
Day 28	24	No	Control	0.005	0.059	a
Day 28	24	Yes	BCS400	3.675	0.059	a
Day 28	24	Yes	BSS400	2.777	0.059	b
Day 28	24	Yes	Control	2.399	0.059	c
Day 28	48	No	BCS400	0.295	0.059	a
Day 28	48	No	BSS400	0.114	0.059	ab
Day 28	48	No	Control	0.052	0.059	b
Day 28	48	Yes	BCS400	4.726	0.059	a
Day 28	48	Yes	BSS400	4.574	0.059	a
Day 28	48	Yes	Control	4.331	0.059	b

Table 10. Values are model-estimated means $\pm$ SE. On day 7, treatments were compared within each glucose condition at 24 h. On days 14 and 28, treatments were compared within each sampling hour and glucose condition because the hour $\times$ treatment $\times$ glucose interaction was significant. Different lowercase letters indicate significant differences among treatments based on Tukey-adjusted pairwise comparisons ($p < 0.05$); treatments sharing the same letter are not significantly different.

Table 11. Tukey grouping of control, BCS400 and BSS400 for the CR in glucose-amended soil in Exp 2.

Experiment 2 – CR in glucose-amended soil					
Day 7: treatment comparisons at 24 h; Days 14 and 28: overall treatment comparisons across 24 and 48 h.					
Day	Comparison	Treatment	Estimated mean	SE	Tukey letter
Day 7	24 h	BCS400	624.7	15.3	a
Day 7	24 h	BSS400	529.0	15.3	b
Day 7	24 h	Control	502.0	15.3	b
Day 14	Overall across 24 and 48 h	BCS400	532.2	23.8	a
Day 14	Overall across 24 and 48 h	Control	517.2	23.8	a
Day 14	Overall across 24 and 48 h	BSS400	512.4	23.8	a
Day 28	Overall across 24 and 48 h	BCS400	605.4	14.8	a
Day 28	Overall across 24 and 48 h	BSS400	520.8	14.8	b
Day 28	Overall across 24 and 48 h	Control	499.8	14.8	b

Different lowercase letters indicate significant differences among treatments based on Tukey-adjusted pairwise comparisons ($p < 0.05$). Treatments sharing the same letter are not significantly different.

Table 11. Values are model-estimated means $\pm$ SE. On day 7, treatments were compared at 24 h. On days 14 and 28, treatment comparisons were performed overall across 24 and 48 h because the hour $\times$ treatment interaction was not significant. Different lowercase letters indicate significant differences among treatments based on Tukey-adjusted pairwise comparisons ($p < 0.05$); treatments sharing the same letter are not significantly different.

Table 12. Tukey grouping of control, BCS400 and BSS400 for the calorespirometric response to glucose addition in Exp 2.

Experiment 2 – Glucose-induced CR					
Day 7: treatment comparison at 24 h; Day 14 and Day 28: Overall treatment comparison across 24 and 48 h					
Day	Comparison	Treatment	Estimated mean	SE	Tukey letter
Day 7	24 h	BCS400	627.3	17.8	a
Day 7	24 h	BSS400	534.7	17.8	b
Day 7	24 h	Control	529.9	17.8	b
Day 14	Overall across 24 and 48 h	Control	537.0	28.8	a
Day 14	Overall across 24 and 48 h	BCS400	532.3	28.8	a
Day 14	Overall across 24 and 48 h	BSS400	517.7	28.8	a
Day 28	Overall across 24 and 48 h	BCS400	616.7	16.4	a
Day 28	Overall across 24 and 48 h	BSS400	537.6	16.4	b
Day 28	Overall across 24 and 48 h	Control	519.4	16.4	b

Different lowercase letters indicate significant differences among treatments based on Tukey-adjusted pairwise comparisons ($p < 0.05$). Treatments sharing the same letter are not significantly different.

Table 12. Values are model-estimated means $\pm$ SE. On day 7, treatments were compared at 24 h. On days 14 and 28, treatment comparisons were performed overall across 24 and 48 h because the hour $\times$ treatment interaction was not significant. Different lowercase letters indicate significant differences

among treatments based on Tukey-adjusted pairwise comparisons ($p < 0.05$); treatments sharing the same letter are not significantly different.

Table 13. Tukey grouping of control, BCS400 and BSS400 for MBC in glucose-amended samples in Exp 2.

Experiment 2 – Microbial biomass carbon				
Treatment comparisons within each sampling day; glucose-amended samples only				
Day	Treatment	Estimated mean	SE	Tukey letter
Day 7	BCS400	335.4	50.5	a
Day 7	BSS400	252.9	50.5	ab
Day 7	Control	111.1	50.5	b
Day 14	BCS400	607.6	50.5	a
Day 14	BSS400	547.8	50.5	a
Day 14	Control	117.4	50.5	b
Day 28	BSS400	546.1	50.5	a
Day 28	BCS400	510.8	50.5	ab
Day 28	Control	352.7	50.5	b

Table 13. Values are model-estimated means $\pm$ SE. Because the day $\times$ treatment interaction was significant, treatments were compared separately within each incubation day. Different lowercase letters indicate significant differences among treatments based on Tukey-adjusted pairwise comparisons ($p < 0.05$); treatments sharing at least one letter are not significantly different

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