Changes in microbial utilisation and fate of soil carbon following the addition of different fractions of anaerobic digestate to soils

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Key words: digestate, soil nutrient status, carbon use efficiency, carbon cycle, microbial community, microbial respiration, carbon dioxide flux

Abstract

Applying digestate, the residue from anaerobic digestion, to soil as a replacement for inorganic fertiliser is of growing interest in agriculture. However, the impacts of different fractions of digestate on the soil carbon (C) cycle remain unclear and provide the focus for the research reported here. We examined the effects of applying whole digestate (WD) and solid digestate (SD) on carbon dioxide (CO_2-C) efflux, the concentrations of dissolved organic carbon (DOC), microbial biomass C (C_{micro}) and phospholipid fatty acids, alongside carbon use efficiency (CUE). A 21-day laboratory microcosm incubation was used to investigate the impacts of digestate when applied to two grassland soils of high versus low initial nutrient content. Application rates for SD and WD were based on recommended nitrogen (N) inputs to grassland soils for these organic materials. Compared to control treatments, cumulative CO_2-C efflux and the concentration of DOC increased significantly after WD and SD application, although only within the low nutrient soil. Both C_{micro} and the fungal to bacterial ratio increased significantly following SD application, regardless of the initial soil nutrient

content. These observations likely reflect the larger input of C, alongside the dominance of more strongly lignified compounds, associated with SD compared to WD to achieve a constant N application rate. Our results also indicate that the two digestate fractions generated significantly different CUE. The application of SD led to increases in C_{micro} and positive values of CUE, whilst decreases in C_{micro} and negative values of CUE were observed following WD application. These findings emphasise the need to carefully plan the management of digestate in agricultural production systems, to minimise negative impacts on C storage within soils whilst maximising the agronomic value derived from digestate.

Highlights

- Past research has not fully elucidated the impacts of digestate fractions on the soil C cycle
- Soil nutrient status + digestate fraction shown to impact microbial community and CO_2-C efflux.
- Solid digestate fraction has positive impacts on microbial biomass and carbon use efficiency.

1. Introduction

Agricultural soil is the largest active terrestrial reservoir in the global carbon (C) cycle. However, some agricultural practices, including deep tillage, over-application of inorganic fertilisers and intensification, have significantly impacted soil structural, chemical and biological conditions, increasing carbon dioxide (CO_2) emissions from soil and reducing soil organic matter (SOM) content (FAO, 2017). In contrast, soil C stocks may be increased by the promotion of agricultural practices that sequester soil organic C (FAO, 2017; Rumpel & Kögel-Knabner, 2011), through fixing atmospheric CO_2 within soil following plant photosynthesis and the transfer of CO_2 to plant biomass, or through the addition of allochthonous organic matter to soil. Additional practices may also help to

49 reduce the environmental impacts of agricultural production, including crop rotation, improved
50 nutrient and water application practices and the reduction of tillage intensity (IPCC, 2014). However,
51 due to microbial metabolism, the application of organic materials to agricultural soil may also result
52 in the release of significant quantities of CO_2, methane (CH_4) or nitrous oxide (N_2O) to the
53 atmosphere (WRAP, 2016).

54

55 Interest in the application of digestate, the residue remaining after anaerobic digestion, to
56 agricultural soil has grown substantially given the potential agronomic value of this material.
57 Digestate generally has a low C to N ratio, is rich in NH_4^+, P, K^+, Na^+, Mg^{2+} and other macronutrients,
58 can improve soil structure, water infiltration rate and water-holding capacity (García-Albacete et al.,
59 2014; Möller & Müller, 2012; Tambone et al., 2010). However, there are significant uncertainties
60 surrounding the impact of digestate application on the C cycle within agricultural soils. This is
61 particularly true following solid-liquid separation and the application of different factions of
62 digestate to soil. Separation allows for differentiation of the total nutrient content of digestate into
63 individual phases, enhancing the potential to match digestate application to crop nutrient
64 requirements when compared with the whole fraction of digestate without separation (Marcato et
65 al., 2008). Whole digestate is a mixture of fibre and liquid, with high viscosity and low infiltration
66 potential. It is generally rich in N, P, K^+ and other macronutrient elements that are present in plant-
67 available forms and usually has a C:N <10 (Tambone et al., 2010). In contrast, the solid fraction is rich
68 in total P (up to 90% of total P in whole digestate may be retained in the solid fraction), much
69 present as water extractable P, alongside Ca^{2+}, Mg^{2+}, S and Mn, usually with a C:N >10 (Bachmann et
70 al., 2016; Hjorth et al., 2010; Lukehurst et al., 2010; Marcato et al., 2008; Panuccio et al., 2016). The
71 forms of organic C present in the whole and solid fractions of digestate can also differ substantially.
72 The whole fraction has been shown to be a mixture of dissolved organic carbon (DOC), which is
73 readily available to microorganisms after application to land, and lignin compounds. In contrast, the

74 solid fraction is dominated by recalcitrant organic C compounds, including lignin, cutin, humic acids
75 and other complex compounds, considered as humus precursors with high biological stability (Nkoa,
76 2014; Tambone et al., 2009) that can promote SOM accumulation.

77

78 The application of digestate as a fertiliser in agriculture may influence C metabolism by the soil
79 microbial community, which biosynthesizes the C into compounds for growth and/or emits CO_2
80 through respiration. This balance dictates the Carbon Use Efficiency (CUE), which may be defined as
81 the efficiency of the biosynthesis of organic C from a source material relative to its respiration
82 (Manzoni et al., 2012). Usually, when CUE is positive and high the soil microbial community utilises a
83 C source for biosynthesis and growth, favouring the anabolic pathway, leading to C stabilization in
84 soil. In contrast, when CUE is low and/or negative, microbial utilisation of a C source for biosynthesis
85 is less efficient, the catabolic pathway is favoured, respiration rate and CO_2 production are enhanced
86 and C sequestration in soil is reduced (Geyer et al., 2016; Wang & Post, 2012; Wang et al., 2013).
87 Many factors influence the CUE, including temperature, moisture, quality of the C source (e.g. C:N)
88 and nutrient availability in soil. For example, Sinsabaugh et al. (2013) report that application of an
89 organic material to soil that is rich in recalcitrant C (often C:N>20), such as the solid fraction of
90 digestate, can increase bacterial catabolism in order to produce extracellular enzymes to hydrolyse C
91 compounds and, consequently, CO_2 is produced. In contrast, the addition of organic matter with
92 C:N<20 to soil, such as the whole fraction of digestate, can promote bacterial biosynthesis of C and,
93 consequently, reduced CO_2 production.

94

95 Soil nutrient availability, particularly the concentrations of N and P, may also influence CUE. When
96 soil is not N or P limited relative to C (e.g. low soil C:N), CUE tends to increase because bacteria seek
97 to maintain a balanced intracellular composition between C and nutrients (Roller & Schmidt, 2015;

98 Manzoni et al., 2012) and thus microbial biomass concentration tends to increase. However, when
99 an organic material containing liable C (e.g. the whole fraction of digestate) is applied to a low-
100 nutrient soil (high soil C:N ratio and, potentially, N limitation) (Blagodatskaya et al., 2014; Moorhead
101 & Sinsabaugh, 2006), bacteria tend to respire C that has been applied because maintenance
102 respiration is increased. This is also true after application of poor-quality resources (e.g. recalcitrant
103 compounds, such the solid fraction of digestate) to a stressed-environment (e.g. low nutrient
104 availability, high temperature or low water availability), because there is an increase in the cost of
105 producing intra/extracellular catabolism under these conditions and an increase in CO_2 production
106 (Malik et al., 2019; Sinsabaugh et al., 2009). Further, bacteria and fungi within the soil microbial
107 community have potentially different effects on CUE. For example, fungi are able to degrade organic
108 material with high C:N without emitting CO_2-C, thereby maintaining a high CUE, whilst bacteria are
109 less efficient at degrading organic material with high C:N (Blagodatskaya & Kuzyakov, 2008). For
110 bacteria, CUE also differs between r (growth strategists; high CUE) and K (competitive strategists;
111 low CUE) communities (Keiblinger et al., 2010; Roller & Schmidt, 2015).

112

113 However, the impacts of digestate on the soil C cycle via microbial effects on CUE remain poorly
114 understood, especially when different physical fractions of digestate with varying nutrient form and
115 stoichiometry are applied to soils. The differing composition of whole and solid digestate may
116 influence soil bacterial and fungal communities differently, with potential effects on C cycling and
117 CUE. There has also been insufficient research focussed on the interactions between digestate
118 application and soil nutrient status, which has been considered as one of the main drivers
119 influencing bacterial and fungal activity and, subsequently, soil C stocks and other soil health
120 parameters. In this context, the research reported here tested the following hypotheses: i) for soil at
121 lower initial nutrient status, the application of either WD or SD stimulates microbial respiration and
122 reduces CUE to a greater extent than for soil at higher initial nutrient status; ii) at low or high soil

nutrient status, the application of WD will stimulate microbial respiration and reduce CUE compared to SD; and iii) the application of SD increases fungal:bacterial in soils at both low and high initial nutrient status, when compared to WD.

2. Materials and Methods

2.1. Soil sampling and initial characterization

Soils were sampled from two fields adjacent to a commercial biogas plant (Cockerham Green Energy Ltd, Northwest England, UK; latitude: 53.972, longitude: -2.822) on 17th September 2018. The two fields were selected to provide contrasting initial soil nutrient properties (Table 1) as driven by the management history of each field. Topsoil to 15 cm depth was sampled from each field using a gouge auger and following a 'W' sampling protocol (Natural England, 2008), in which samples from 20 points along a 'W' were combined into a single integrated soil sample for each field. High nutrient soil (HN) was under grass production at the time of sampling and used for grazing and silage production during previous years. This field receives liquid digestate four times per year, with the last application occurring at the end of July 2018. The low nutrient soil (LN) was fallow grassland at the time of soil sampling and had never previously received digestate. Following collection and homogenisation, soils were sieved through a 2 mm mesh and stored in sealed plastic bags at 4 °C until the incubations began.

ADD TABLE 1 HERE

2.2. Digestate sampling and characterization

On 24th September 2018, whole and solid fractions of anaerobic digestate were collected from Cockerham Green Energy Ltd, following sampling protocols detailed by Agriculture and Horticulture Development Board (2017), and stored at 4°C prior to the start of the incubations. Digestate from Cockerham Green Energy Ltd is fermented in a mesophilic, single stage digester with a retention time of 50 days. The feedstock is livestock and poultry manure, co-digested with food waste including wheat, potatoes, tea bags and whey. Whole digestate is unpasteurised and separated into liquid and solid fractions using a screw-press. The liquid fraction is collected in covered lagoons, whilst the solid fraction is stored in an uncovered open-space. Whole digestate was sampled directly from the anaerobic digester before separation, whilst the solid fraction was sampled from material that had been stored for seven days prior to collection. The two fractions of digestate were chosen to provide contrasting properties for the experiment (Table 2).

ADD TABLE 2 HERE

2.3. Experimental design

A microcosm incubation was carried out between 8th – 30th October 2018, involving control (Ctr), whole digestate (WD) and solid digestate (SD) treatments. Each amendment was conducted in triplicate for both HN and LN soil types, with soil × amendment combinations placed randomly in amber and Duran bottles inside a temperature-, pressure- and moisture-controlled room in the dark. The WD and SD amendments were added to soils inside separate glass containers in order to achieve the same N application rate (170 kg N (as NH_4^+-N) ha^{-1} $year^{-1}$), after Agriculture and Horticulture Development Board (2017). This resulted in the addition of c.12,500 mg kg^{-1} dry weight (DW) soil of C for SD and 625 mg kg^{-1} DW soil of C for WD treatments to both soils. Digestate fractions were

mixed thoroughly with soil and then sub-divided into Duran (for respirometry) or amber bottles (destructive samples) prior to the incubation.

The moisture content of the soils was set at 50% water holding capacity (WHC) using milliQ water (>18.2 MΩ.cm at 25°C). Control soils were left un-amended without any digestate addition and only received milliQ water in order to maintain 50% WHC. Respirometry measurements were carried out using a Micro-Oxymax Respirometer (Columbus Instruments International Corp. Columbus, USA), with an automated 20-channel closed-circuit and with two empty bottles used as analytical blanks. For respirometry samples, the respirometer maintained a constant moisture content throughout the incubation. The concentration of CO_2 in the headspace of each Duran bottle was monitored at a partial pressure of 1063.9125 hPa and a temperature of 23 ±1°C, via a specialised GL 45 three-port connection at 2 hr intervals, with emission rates of CO_2-C and cumulative CO_2-C expressed as a rate (mg C h^{-1}) and as a mass (mg C) respectively. In addition, a parallel set of destructive samples was prepared using amber bottles in order to monitor changes in soil properties through time. These destructive samples were analysed at 0, 1, 2, 3, 4, 7, 14 and 21 days (for the 21-day time point, respirometry samples were destructively sampled). The moisture content of the destructive samples was checked daily by weighing the amber bottles without lids and adding milliQ water to maintain 50% WHC. The destructive samples were placed inside the same dark controlled room as the respirometry samples.

2.4. Soil analyses

Destructive soil samples were analysed for microbial biomass C (C_{micro}) and dissolved organic carbon (DOC). Additional samples were taken at 0 and 21 days for analysis of phospholipid fatty acid (PLFA) content. Extraction for C_{micro} was carried out following the chloroform fumigation method (Brookes

et al., 1985; Vance et al., 1987). Duplicate, fresh soils were extracted with and without chloroform fumigation according to Brookes et al (1985) and Vance et al. (1987) (1:5 w/v, 0.5 M K_2SO_4, pH~7, filtered Whatman No 42). The determination of TC for the two set of extracts was carried out using a TOC-L/TN Series Analyser (Shimadzu, Japan) based on a combustion-reduction method. Microbial biomass C was calculated as the difference in concentration between fumigated and unfumigated samples, with subsequent correction by K_{ec} for C evolved as CO_2 (Brookes et al., 1985; Joergensen, 1995, 1996).

Fresh soil samples were extracted in milliQ water (1:10 w/v; 15 minutes shaking) for DOC analyses (Jones & Willett, 2006), filtered (Whatman No 42) and the extract was analysed using a TOC-L/TN Series Analyser (Shimadzu, Japan) after sample acidification to remove inorganic C.

The PLFA extraction was carried out as described by Quideau et al. (2016), using a three-stage extraction. Frozen soil (-80° C) was freeze-dried and between 1-1.5 g of soil was used for the extraction. Extracted samples were analysed using a Gas Chromatograph-FID (Agilent Technology 6890N, USA). A C13 (Methyl tridecanoate) and C19 (Methyl nonadecanoate) mixed standard was used as an internal standard in order to identify the range of the retention time of the PLFAs of interest.

Soil pH was determined on fresh soil samples (1:5 w/v; 30 minutes shaking) using milliQ water. Air-dried soil samples were analysed for Olsen P as described by Murphy & Rilely (1962) and Olsen et al. (1954). Samples were extracted (1:20 w/v; 30 minutes shaking) with a 0.5 M $NaHCO_3$ solution, with pH adjusted to 8.5, and subsequently filtered (Whatman No 42). The extracted samples were analysed using a SEAL Autoanalyzer AA3 (Seal Analytical, UK;Method No G-103-92 Rev1; Multitest Mt7/MT8) based on the molybdenum blue colorimetric reaction. Soil dry matter (DM) and loss-on-ignition (LOI) were determined using a gravimetric method (Allen, 1989; Gardner, 1986). Approximately 12g of fresh soil was oven-dried at 105°C for 48 h to constant weight to determine DW. Subsequently, around 1.5g of oven-dried soil was heated at 550°C for 6h in a muffle furnace,

left to cool overnight and subsequently weighed to determine LOI. The TC and TN content of soils was determined using an automated Dumas procedure on a Carbo Erba NA 1500 analyser (Erba Science, UK), working with 30±1 mg of oven-dried and ball-milled soil. Fresh soil samples were also extracted for available N using 1 M KCl (1:5 w/v, 1 hour shaking) (Bremmer, 1965; McTaggart & Smith, 1993) and filtered (Whatman No 42). The filtrate was subsequently analysed for NH_4^+ and NO_3^- content using a SEAL Autoanalyzer AA3 (Seal Analytical, UK; Method No G-102-93 Rev 2; Multitest MT7/MT8) with two different colorimetric reactions (ISO 11732, 1997 and ISO 13395, 1996 respectively).

2.5. Calculations for % TC respired, CUE and statistical analysis

The % TC respired from soils after the addition of digestate was calculated as:

% TC respired at each time point = (cumulative CO_2-C produced at each time point/(TC present in the soil at day 0 + TC applied in digestate amendment)) * 100

where all C terms were expressed in mg.

The CUE was estimated as described by Frey et al. (2001) and Tiemann & Billings (2011), using the following equation:

$$CUE = dBc/(dBc + \Sigma CO_2\text{-}C)$$

where dBc is the change in C_{micro} and ΣCO_2-C is the cumulative C lost through microbial respiration during the incubation, both expressed in mg C. For both WD and SD treatments, C_{micro} and ΣCO_2-C were standardised by the Ctr treatment, in order to focus on the fate of C that was added to the soil with digestate, following Tiemann & Billings, (2011). The CUE of Ctr treatments was not calculated, because no C was added to soils.

Statistical analyses were performed in R version 3.6.1 (R Core Team, 2019). One-way and two-way ANOVA was employed to assess the significance of the factors 'soil' (HN, LN) and 'digestate amendment' (Ctr, WD, SD) and their interaction. Levene's tests were used to check the homogeneity of variance assumption of ANOVA, with $\log_{10}$ or square root transformations applied to data where necessary. A Tukey-test (HDS) was employed to compare individual levels where a significant factor was identified in ANOVA. For CUE, a Kruskal-Wallis test was used to assess the significance of the factors soil type and digestate amendment.

Due to the non-linear nature of many response variables across the incubations, multivariate polynomial regression was used to model time × soil type × digestate amendment interactions. Time was treated as a numerical variable and expressed from 0 to 21 days. For C_{micro} and DOC, in order to fully capture the nonlinear nature of changes through time, a cubic polynomial regression was used, whilst for cumulative CO_2-C efflux, %TC respired and fungal:bacterial linear regression models were applied. Where significant regression models were identified, T-tests were performed on cumulative CO_2-C efflux, %TC respired and fungal:bacterial data in order to determine the nature of the time × soil type × digestate amendment interaction.

In all statistical analyses, p-values < 0.05 were deemed as significant, whilst p-values between 0.05 and 0.06 were marked as borderline significant after Hofmann & Meyer-Nieberg (2018). Residual plots (S-L, Q-Q, Residual-Leverage and Cook's distance - leverage) were employed to assess the quality of the model fits and the assumption of normally distributed residuals for ANOVA, as well as the presence of leverage points or outliers. Missing observations were excluded from the analysis and no data imputation was performed. Clear outliers, assumed to represent sample error or contamination, were removed from the datasets prior to analysis.

3. Results

INSERT TABLE 3 HERE

3.1. Influence of treatments on CO_2-C efflux from soils

Cumulative CO_2-C efflux from HN soils was significantly greater than from LN soils across the incubations ($p<0.001$). Further, digestate amendment exerted significant control on cumulative CO_2-C efflux ($p< 0.0001$), with higher cumulative CO_2-C efflux observed after the application of digestate to soils compared to control treatments, in the order Ctr<WD≈SD. However, an interaction between soil type and digestate amendment was observed ($p<0.0001$), with significant increases in cumulative CO_2-C efflux after WD and SD application only occurring within LN soils and not within HN soils.

A significant three-way interaction between time, soil type and digestate amendment was also observed for cumulative CO_2-C efflux as shown in Figure 1($p < 0.0001$). Within the LN soil, both WD and SD amendments increased cumulative CO_2-C efflux rapidly and significantly through time when compared to the control treatment, reaching +563% (SD) and +377% (WD) at 21 d compared to fluxes in the control treatment. Further, SD and WD diverged significantly from each other from 14 d

289 onwards. Within the HN soil, only the SD amendment generated significantly higher cumulative CO_2-
290 C efflux and only from 14 d of the incubation onwards (+20% at 21 d when compared with Ctr),
291 whilst WD and Ctr did not differ significantly.
292
293 **INSERT FIGURES 1 AND 2 HERE**
294
295 Figure 2 reports the percentage of TC present in the combination of soil and digestate amendment
296 that was respired as CO_2-C during the incubations. In contrast to cumulative CO_2-C efflux, no
297 significant difference in %TC respired was observed between HN and LN soils. However, both WD
298 and SD amendments resulted in significant increases in %TC respired compared to the Ctr ($p < 0.001$),
299 in the order Ctr<WD≈SD. Further, a significant interaction between soil and digestate amendment
300 ($p<0.001$) indicated that significant increases in %TC respired following SD or WD application only
301 occurred in the LN soil, consistent with observations related to cumulative CO_2-C efflux.
302
303 A highly significant three-way interaction between time, soil type and digestate amendment was
304 observed ($p<0.0001$), indicating that the temporal pattern in %TC respired after the addition of
305 digestate depended on the nature of the soil at the start of the incubation. In the HN soil, digestate
306 amendments followed the same temporal trend as the Ctr treatment. However, in the LN soil the
307 %TC respired increased significantly through time following both WD (+372% at 21d) and SD (+369%
308 at 21d) applications compared to the control treatment, an effect that was observed from 1 d
309 onwards in the incubations.
310
311
312

3.2. Influence of digestate amendments on the soil microbial community

Microbial biomass C was significantly higher in HN compared to LN soil ($p < 0.001$). Further, C_{micro} increased significantly after the application of SD compared to either Ctr or WD treatments ($p < 0.0001$), by +29% at 21 d in the HN soil and by +36% at 21d in the LN soil compared to the Ctr treatment (Figure 3). No significant interactions between soil type, digestate amendment or time were observed for C_{micro}, confirming that the significant increase following the application of SD was observed in both HN and LN soils and throughout the duration of the incubations.

Similarly to C_{micro}, the fungal to bacterial ratio increased significantly under the SD treatment compared to either the Ctr or WD treatments ($p<0.01$), an effect that was also consistent across both HN and LN soils. Further, time significantly affected the fungal:bacterial (Figure 4), with a marginally significant three-way interaction observed between time, soil type and digestate amendment ($p < 0.049$). The fungal to bacterial ratio increased significantly between 0 and 21 d following application of SD in both soils (+58% HN and +18% LN compared to Ctr), whilst the ratio decreased slightly (-8%) in the LN soil following the application of WD compared to the control ($p = 0.05$).

INSERT FIGURES 3 AND 4 HERE

3.3. Influence of digestate amendments on Dissolved Organic Carbon concentration

The concentration of water-extractable DOC was significantly higher in HN compared to LN soils ($p<0.0001$). Further, the application of SD to soils resulted in a significant increase in the concentration of water-extractable DOC, compared to either WD or Ctr treatments ($p<0.0001$). However, the impact of SD application differed between soil types, with a significant increase in DOC

concentration following SD application only observed in the LN soil (Figure 5). No interaction between time, soil type and digestate amendment was observed with respect to DOC concentration.

INSERT FIGURE 5 HERE

3.4. Estimation of CUE after digestate amendment

Table 4 reports the CUE for each combination of soil type and digestate amendment used within the incubation reported here. No significant difference in CUE was observed between the two soil types. However, digestate amendment exerted significant control on CUE ($p < 0.05$), with positive values of CUE observed following the application of SD and negative values after application of WD to soils; these effects were consistent across the two soil types used in the incubations.

ADD TABLE 4 HERE

4. Discussion

The application of digestate strongly influenced the C cycle within the soils examined during this research. This was evidenced by significant changes in the loss of C via gaseous pathways, the production of water-soluble DOC, and the biomass and composition of the soil microbial community. However, for many parameters the impact of digestate application depended on the initial soil nutrient status, on the physical fraction of digestate that was applied, and on time across the 21 d incubation. It should be noted that the history of soil management within the HN and LN soils likely drove different responses between these soils to the treatments applied in the experiments reported here. For example, past digestate application to the HN soil may have been responsible for differences in microbial community composition and functional traits, compared to the LN soil.

359 Further, our experimental system did not include the input of labile C to soil from root exudates that
360 may alter microbial requirements for digestate-derived C. Future research will be required in order
361 to examine the interactions within plant-microbial-soil systems including the net impacts of these
362 interactions for the fate of C derived from inputs of digestate to agricultural soil, and the impacts of
363 a wider range of soil management histories. .
364
365 ***4.1. The influence of digestate application on CO_2-C efflux***
366 The efflux of CO_2-C from soil, whether expressed as an absolute flux or as a proportion of the TC
367 within the combination of soil and digestate, increased significantly following the application of
368 digestate. This observation is consistent with both previous laboratory and field research (e.g.
369 Pezzolla et al., 2012; WRAP, 2016; Johansen et al., 2013), spanning grassland and arable soils. For
370 example, field experiments have reported an increase in cumulative CO_2 efflux occurring across a 12-
371 month period following four whole digestate application, (WRAP, 2016) and across a 5-month period
372 following three applications of whole digestate (Pezzolla et al., 2012). Further, a 9-day laboratory
373 experiment on arable soil revealed a two-fold increase in cumulative CO_2-C efflux after whole
374 digestate addition when compared with untreated soil (Johansen et al., 2013). Whilst the research
375 we report above used digestate from a single feedstock, it should also be noted that some past
376 research has demonstrated significant effects on CO_2 efflux associated with variation in digestate
377 feedstock and post-digestion processing (i.e. separation) techniques (e.g. Askri et al., 2016). These
378 variables were not incorporated within the experimental system used in the research reported here.
379
380 The data reported above confirm that CO_2-C efflux was influenced by a significant interaction
381 between soil type and digestate, in which increases in this gaseous flux of C following either WD or
382 SD application only occurred in the LN soil. Increases in CO_2-C efflux following digestate application
383 are partly consistent with de la Fuente et al. (2013) and Grigatti et al. (2011), who report
384 mineralization rates after the application of different fractions of digestate and their effects on CO_2-

C efflux. However, de la Fuente et al. (2013) and Grigatti et al. (2011) report higher CO_2-C efflux following the application of SD compared to WD, whilst in the research reported here CO_2-C efflux did not differ significantly between the two fractions of digestate. It should be noted that the research of de la Fuente et al. (2013) involved a calcareous soil with nutrient content similar to the HN soils used in our research, whilst Grigatti et al. (2011) also used a soil more similar in nutrient content to the HN compared to LN soil used in the current research. Differences in soil type may help to explain why no significant difference in CO_2-C efflux was observed between SD and WD within the LN soil in the research reported above. However, further work would be required in order to understand why similar variation in CO_2-C fluxes after application of different fractions of digestate were not observed in the HN soils.

The efflux of CO_2-C increased rapidly from the early stages of the incubations following the application of either SD or WD to the LN soil, whether expressed as cumulative CO_2-C or as a percentage of TC present in the soil-digestate system. The effects of digestate application in the LN soil likely reflect the activation of dormant bacteria and stimulation of maintenance respiration after the application of either fraction of digestate (Mondini et al., 2006). In the LN soil, rapid increases in bacterial catabolism likely followed the application of WD due to the input of readily available DOC, suggesting that this C source may have been utilised quickly for enzyme production and maintenance respiration within a few days after application and consistent with other research (e.g. Wang et al. 2013; Wang and Post, 2012). After exhaustion of readily available C in WD, bacteria may have started to mine SOM present in the soil to meet continued demand for nutrients (Fontaine et al., 2004, 2011), or alternatively turnover of the bacterial community may have occurred through the course of the incubation (Blagodatskaya et al., 2007), consistent with negative CUEs following the application of WD. However, the increase in CO_2-C efflux was higher and more persistent following the application of SD to the LN soil, possibly because fungal degradation of recalcitrant C compounds in SD produced C by-products which were subsequently consumed by bacterial catabolism. Alternatively, bacteria may have invested directly in enzymatic degradation of

411 recalcitrant C such as lignin within SD, as reported by Sierra (2012). In turn, this likely resulted in
412 prolonged increases in respiration and CO_2-C efflux, consistent with Fontaine et al. (2003),
413 Sinsabaugh et al. (2013) and Winogradzky (1924).
414 In contrast, within the HN soil, only during the later stages of the experiment and only after SD
415 application were increases in CO_2-C efflux observed, and only when CO_2-C was expressed as a
416 cumulative flux rather than as a percentage of TC present in the system. Following exhaustion of
417 readily-available C during the earlier stages of the incubation, by-products from fungal or bacterial
418 degradation of recalcitrant C within SD likely supported the higher efflux of CO_2-C from bacterial
419 respiration towards the end of the incubation (Six et al., 2006). In contrast, rapid exhaustion of
420 readily available C, combined with the absence of an input of more recalcitrant C in WD, meant that
421 CO_2-C efflux under this treatment did not differ significantly compared to the control within the HN
422 soil.
423
424 Varying effects of digestate application on CO_2-C efflux between HN and LN soils also likely reflects
425 differences in physico-chemical conditions between the two soil types that influenced microbial
426 metabolic responses to the input of resources within digestate (e.g. Larsson et al., 1995; Manzoni et
427 al., 2012; Russell & Cook, 1995). Within the HN soil, existing neutral soil pH, higher C_{micro}, higher DOC
428 and lower C:N meant that the changes in microbial respiration following digestate input were
429 relatively small compared to the control soil treatment. In contrast, the adverse soil conditions in the
430 LN soil (low pH, C_{micro}, DOC and nutrient concentration) created an environment in which respiration
431 of CO_2 from control soils was relatively low, and in which activation of dormant bacteria and
432 subsequent increases in respiration followed the application of resources within both WD and SD
433 (Mondini et al., 2006).
434
435 ***4.2. Changes in the soil microbial community following digestate application***

Both C_{micro} and the fungal to bacterial ratio increased significantly following the application of SD, a pattern that was consistent across both HN and LN soils. Increases in C_{micro} following the application of SD were likely driven by higher inputs of TC compared to the WD treatment, in order to achieve a consistent N application rate across both fractions of digestate. The additional input of C resources allowed greater opportunity for biosynthesis and the accumulation of C within new soil microbial biomass under the SD treatment. These observations related to C_{micro} are supported by other research that has examined the impact of digestate application on the soil microbial community. For example, de la Fuente et al. (2013) report increases in C_{micro} only 7 days after the application of SD, driven by the high TC applied to soil with this fraction of digestate. Further, Chen et al. (2012) carried out a 21d incubation and report an increase in C_{micro} which was related to a shift from r-strategists to K-strategists in soil that received biogas residues.

The fungal to bacteria ratio of control HN and LN soils indicated a microbial community that was dominated by bacteria, consistent with other research focussed on agricultural grasslands (Bardgett et al., 1996, 1995, 1993). However, this ratio increased significantly following the application of SD to both soils used in the incubations reported here, driven by an increase in fungal PLFA rather than a decrease in bacterial PLFA. This observation likely reflects the significant input of more recalcitrant C compounds, such as lignin, associated with SD compared to WD (Noka, 2014). Hydrolysis of these C compounds has been shown to rely predominantly on the action of fungi rather than bacteria (Hammel, 1997), consistent with the increase in total fungal PLFA through the incubations reported here following the application of SD and in agreement with other research (e.g. Rousk and Bååth 2011; Walsh et al., 2012). Fungal-produced C by-products following degradation of recalcitrant C within SD may also have sustained bacterial production (e.g. Dashtban et al., 2010; Bugg et al., 2011; Rüttimann et al., 1991), including through generating a flush of DOC which is available for the microbial community (Möller et al., 1998). In contrast, the limited input of recalcitrant C following WD application produced no significant change in fungal:bacterial within the HN soil, alongside a

462 relatively small and marginally significant decrease in this ratio within the LN soil, reflecting a
463 decrease in total fungal PLFA within the microbial community under this treatment.
464
465 Whilst the concentration of DOC was significantly greater in soil following the application of SD
466 compared to either Ctr or WD treatments, this effect was only observed within LN and not within HN
467 soils. Within the HN soil, DOC generated following the application of SD appeared to be efficiently
468 metabolised by the microbial community, evidenced by an increase in C_{micro} but no increase in CO_2-C
469 efflux compared to control soils. In contrast, the application of SD to the LN soil increased DOC
470 concentrations by the end of the incubation. This likely reflects unfavourable conditions for the
471 microbial community within the LN soil, including low pH and nutrient availability, which can limit
472 microbial metabolism of DOC as noted in previous research (David et al., 1989; Jardine et al., 1989;
473 Vance and David, 1989; Guggenberger et al., 1994).
474
475 ***4.3. Changes in CUE following digestate application***
476 Carbon use efficiency varied significantly between the digestate treatments used in the experiments
477 reported here, with consistent patterns observed across both soil types. The application of WD
478 resulted in negative values of CUE, driven by greater decreases in C_{micro} and by increased CO_2-C fluxes
479 compared to control treatments during the incubations. Decreases in C_{micro} may reflect grazing by
480 protozoa and/or microbial turnover (Frey et al., 2001). The input of readily degradable C substrates
481 within WD likely promoted the catabolic pathway and maintenance respiration of bacteria to a
482 greater extent compared to the anabolic pathway, resulting in enhanced CO_2-C effluxes and
483 decreased biosynthesis of C within microbial cells (Manzoni et al., 2012; Geyer et al., 2016). The
484 magnitude of the effect of WD on CUE was more pronounced in HN compared to LN soils. This
485 observation reflects the smaller cumulative CO_2-C efflux in HN soils compared to the respective
486 controls, generating a more negative value of CUE following the application of WD. Whilst C_{micro} also
487 decreased following the application of WD to LN soils, the relatively large increase in CO_2-C efflux

compared to control soils resulted in a smaller value of CUE for LN soils compared to the HN soils. These observations emphasise the potential for application of WD to result in net decreases in C_{micro}, rather than net accumulation of C within soil microbial biomass, due to the stimulation of maintenance respiration and associated utilisation of C from both native soil and substrate pools (e.g. Blagodatskaya et al., 2014; Moorhead & Sinsabaugh, 2006).

In contrast to WD, positive values of CUE were observed following the application of SD to both soil types, with CUE in the range 0 – 0.55 as reported for soil microbial communities by Sinsabaugh et al. (2013) who accounted for substrate C:N, the assimilation efficiency of N, bacterial C:N and a CUE_{max} in their research. However, it is notable that a higher CUE was observed after application of SD to HN compared to LN soils, reflecting substantial increases in C_{micro} and relatively small increases in cumulative CO_2-C efflux in HN soils following SD application, compared to control soils. Whilst C_{micro} also increased in LN soils after the application of SD compared to control soils, the increases in CO_2-C efflux was far more pronounced, resulting in lower values of CUE compared to HN soils. Increase in C_{micro} following SD application to soils indicate the potential for net accumulation of C within soil microbial biomass, in particular associated with increases in soil fungal community anabolism and biomass (Keiblinger et al., 2010). However, it should also be recognised that cumulative CO_2-C fluxes following the application of SD exceeded those under all other treatments used in our experiments. Therefore, application of SD to soils can potentially generate adverse effects on absolute fluxes of CO_2 to the atmosphere, whilst at the same time contributing positively to the accumulation of C within soils.

5. Conclusions

The research reported here provides important new insights into how changes in the soil C cycle may follow the application of digestate to agricultural grasslands. The precise nature of these impacts is

contingent on the physical fraction of digestate applied to land and on the nutrient status of the soils that receive digestate. The solid fraction of digestate drove substantial increases in CO_2-C efflux, an effect that appears to be inversely related to soil nutrient status. Microbial biomass C and the fungal to bacterial ratio in soil also increased following the application of the solid fraction of digestate, regardless of initial soil nutrient status. The effects of applying whole digestate to soil were more variable. Whilst CO_2-C efflux increased following the application of whole digestate to soil at low initial nutrient status, no significant changes in microbial biomass C or in fungal to bacterial ratio followed the application of whole digestate. Carbon use efficiency in soils receiving solid digestate was positive, indicating the potential for C accumulation within soil microbial biomass. However, the accumulation of C within soil was exceeded by the additional C lost from soils via CO_2-C efflux. Further, CUE was negative in both soil types following treatment with whole digestate, driven by decreases in C stored within microbial biomass and loss of C as CO_2-C.

These findings emphasise the need to carefully plan the management of digestate in agricultural production systems, in order to minimise negative impacts on C storage within soils whilst maximising the agronomic value derived from digestate. Future research should seek to examine the impacts of a broader range of digestate fractions (whole, liquid, solid) on the soil C cycle in long term field experiments, including the effects of plant-soil interactions and longer-term changes in CUE and SOM. In addition, research should seek to quantify the impacts of digestate application on other environmental parameters of concern, including the emission of greenhouse gases beyond CO_2 and the potential leaching of pollutants into the subsurface.

Acknowledgements

536 We are indebted to the contribution from Chris Parry who provided access to the digestate fractions
537 and soils used during this experiment. We are also grateful to Dr. Annette Ryan for the technical
538 support and training provided related to analysis of the samples reported in this paper. We also
539 thank two anonymous reviewers for their comments that helped to substantially improve this paper.

540

541 **Conflict of interest**

542 The authors declare no relevant conflict of interest with respect to the content of this paper.

543

544 **Funding**

545 This project was funded by the Natural Environment Research Council (NERC) and Biotechnology and
546 Biological Sciences Research Council (BBSRC) through the STARS Centre for Doctoral Training (CDT).

547

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757 *Table 1 Initial physio-chemical characteristics of soils used in the microcosm incubations (mean*
758 *values reported, ±1 SE in parentheses, n=3)*

Soil characteristics	High nutrient soil	Low nutrient soil
Bulk density (g cm^{-3})	1.54 (0.14)	1.48 (0.014)
pH water (1:5 w/v)	7.31 (0.035)	5.06 (0.018)
NO_3^- (mg kg^{-1} DW soil)	71.05 (0.51)	66.66 (0.32)
NH_4^+ (mg kg^{-1} DW soil)	0.47 (0.044)	1.94 (0.10)
Olsen P (mg kg^{-1} DW soil)	40.66 (1.18)	10.42 (1.10)
P index UK (Agriculture and Horticulture	4	1

Development Board, 2017)		
Water extractable Total Organic C (mg kg^{-1} DW soil)	228.61 (14.23)	61.43 (0.76)
Soil Tot C (mg C kg^{-1} DW soil)	50298.14 (68.49)	31817.73 (39.3)
Soil Tot N (mg N kg^{-1} DW soil)	4396.73 (160.30)	
2363.93 (199.82)		
TC:TN	11.46 (0.07)	13.68 (0.50)
DM (%)	73.06 (0.10)	75.49 (0.02)

DM (Dry Matter); Tot C (Total Carbon, non-acidified analysis); Tot N (Total Nitrogen); Water Tot C (Water Extractable Total Organic Carbon, acidified analysis); NH_4^+ (Ammonium); NO_3^- (Nitrate); P (Phosphorus); P index (mg L^{-1} P Olsen)

Table 2 Physio-chemical characteristics of whole and solid digestate used in the microcosm incubations (n=1)

Parameter in fresh weight (FW)	Whole digestate (WD)	Solid digestate (SD)
DM (%)	11.6	24.3
Organic Matter (%)	8.36	84.3
pH (1:6 w/v)	8.18	8.20
TN (mg kg^{-1} FW)	8500	4836
NH_4^+-N (mg kg^{-1} FW)	4921	752.81
TP (mg kg^{-1} FW)	2869	4209
TC (mg kg^{-1} FW)	37000	109107
TC:TN	4.35	22.56

DM (Dry Matter); TP (Total Phosphorus); TC (Total Carbon); TN (Total Nitrogen); NH_4^+-N (Ammonium Nitrogen)

Table 3 Summary of one-way and two-way ANOVA results from microcosm incubations. Columns from left to right describe effects of initial soil nutrient status (high (HN) vs low (LN)); effects of digestate amendment (Control (Ctr), whole digestate (WD), solid digestate (SD)); and interactions between soil nutrient status and digestate amendment. "n.s" represents effects that were not

statistically significant (p>0.05). Tukey tests were employed to determine differences between individual levels of soil type and digestate amendment, with significant differences between levels denoted using superscript letters. For interactions between soil type and digestate amendment, first superscript letter represents differences between digestate amendments within each soil type, second superscript letter represents differences between soil type within each digestate. amendment.

	Soil	Mean	Std error	p-value	**Digestate**	Mean	Std error	p-value	**Soil × digestate interaction**	Mean	Std error	p-value
CUE	HN LN	-0.087 -0.025	0.038 0.013	n.s.	WD SD	-0.22[a] 0.11[b]	0.17 0.05	0.015				n.s.
Cumulative CO_2-C (mg C kg DW soil^{-1})	HN LN	1382.18[a] 942.62[b]	133.9 125.3	0.0009	Ctr WD SD	738.02[a] 1250.56[b] 1417.88[b]	132.20 156.50 192.50	0.00002	HN	Ctr: 1247.67[a,a]	228.53	0.00007
										WD: 1323.85[a,a]	239.73	
										SD: 1413.04[a,a]	275.16	
									LN	Ctr: 228.88[a,b]	45.75	
										WD: 1178.26[b,a]	204.43	
										SD: 1420.72[b,a]	275.34	
% TC respired	HN LN	1.44 1.60	0.25 0.34	n.s.	Ctr WD SD	1.43[a] 2.66[b] 2.38[b]	0.39 0.38 0.40	0.0002	HN	Ctr: 2.22[a,a]	0.47	0.0001
										WD: 2.06[a,a]	0.43	
										SD: 1.25[a,a]	0.41	
									LN	Ctr: 0.63[a,b]	0.14	
										WD: 3.21[b,a]	0.63	
										SD: 3.18[b,a]	0.71	
C_{micro} (mg kg DW soil^{-1})	HN LN	796.31[a] 698.01[b]	24.38 21.63	0.0007	Ctr WD SD	684.60[a] 663.18[a] 891.38[b]	20.14 22.78 30.93	4.3*10^{-10}				n.s.
Fungal:bacterial	HN LN	0.11 0.11	0.0052 0.0035	n.s.	Ctr WD SD	0.11[a] 0.11[a] 0.13[b]	0.0030 0.0031 0.0067	0.005				n.s.
DOC (mg kg DW soil^{-1})	HN LN	166.11[a] 117.03[b]	9.66 10.15	0.000002	Ctr WD SD	110.45[a] 120.15[a] 194.12[b]	10.68 10.87 12.72	4*10^{-9}	HN	Ctr: 157.12[a,a]	15.68	0.000002
										WD: 161.93[a,a]	15.66	
										SD: 179.30[a,a]	19.10	
									LN	Ctr: 63.79[a,b]	5.69	
										WD: 78.36[a,b]	9.36	
										SD: 208.94[b,a]	16.75	

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Table 4 Carbon Use Efficiency (CUE) following whole (WD) and solid fraction (SD) digestate amendments in high nutrient (HN) or low nutrient (LN) soils (mean values reported, ±1 SE in parentheses, n=3).

Amendment	Estimation of CUE
HN × WD	-0.37 (0.33)
HN × SD	0.20 (0.050)
LN × WD	-0.07 (0.035)
LN × SD	0.02 (0.042)

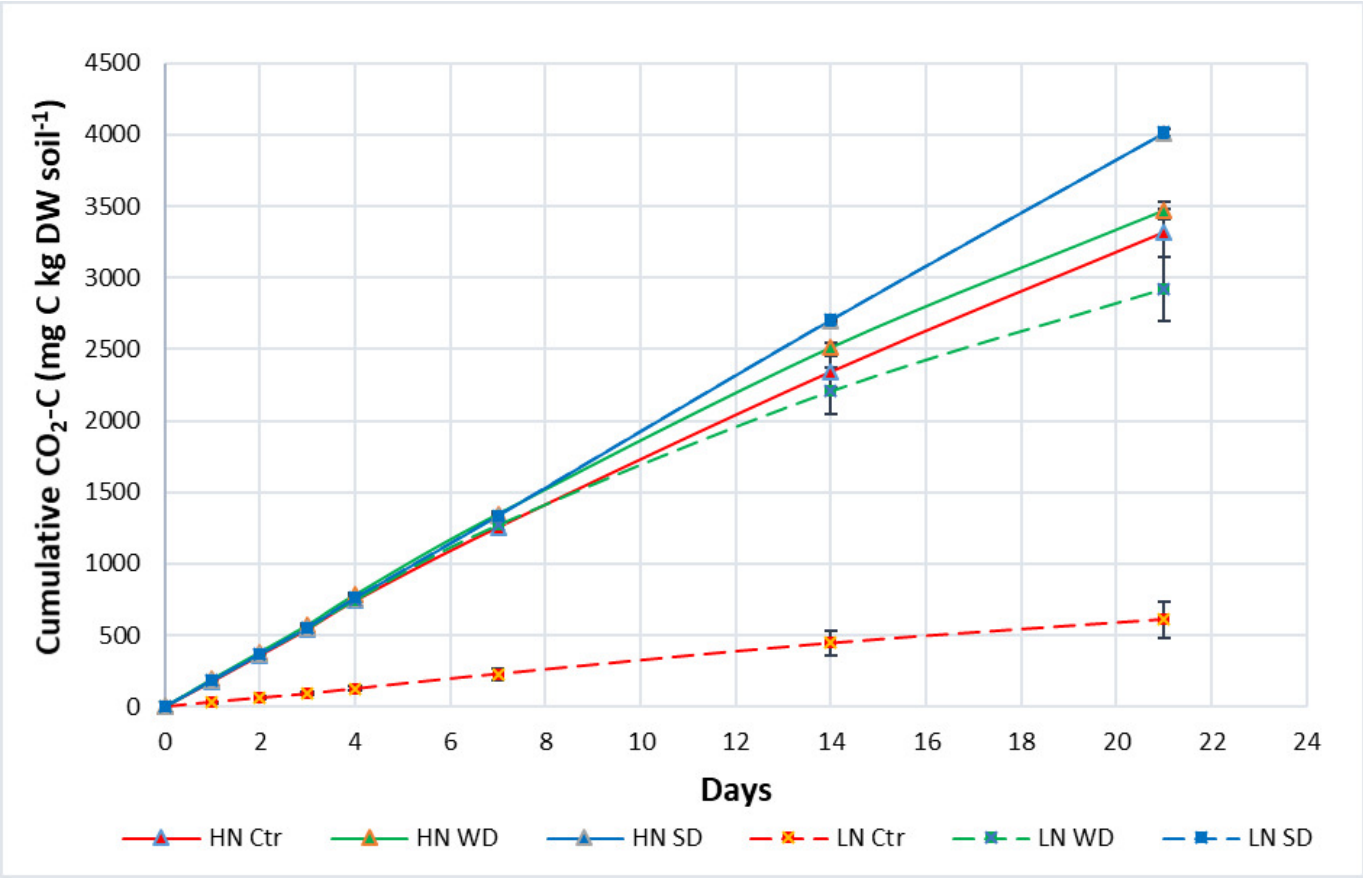


Figure 1 Cumulative CO_2-C produced from control (Ctr) soils or after addition of whole (WD) or solid (SD) fractions of digestate in soils at high (HN) or low (LN) initial nutrient status. HN × SD and LN × SD overlapping in the figure. Error bars ± 1SE

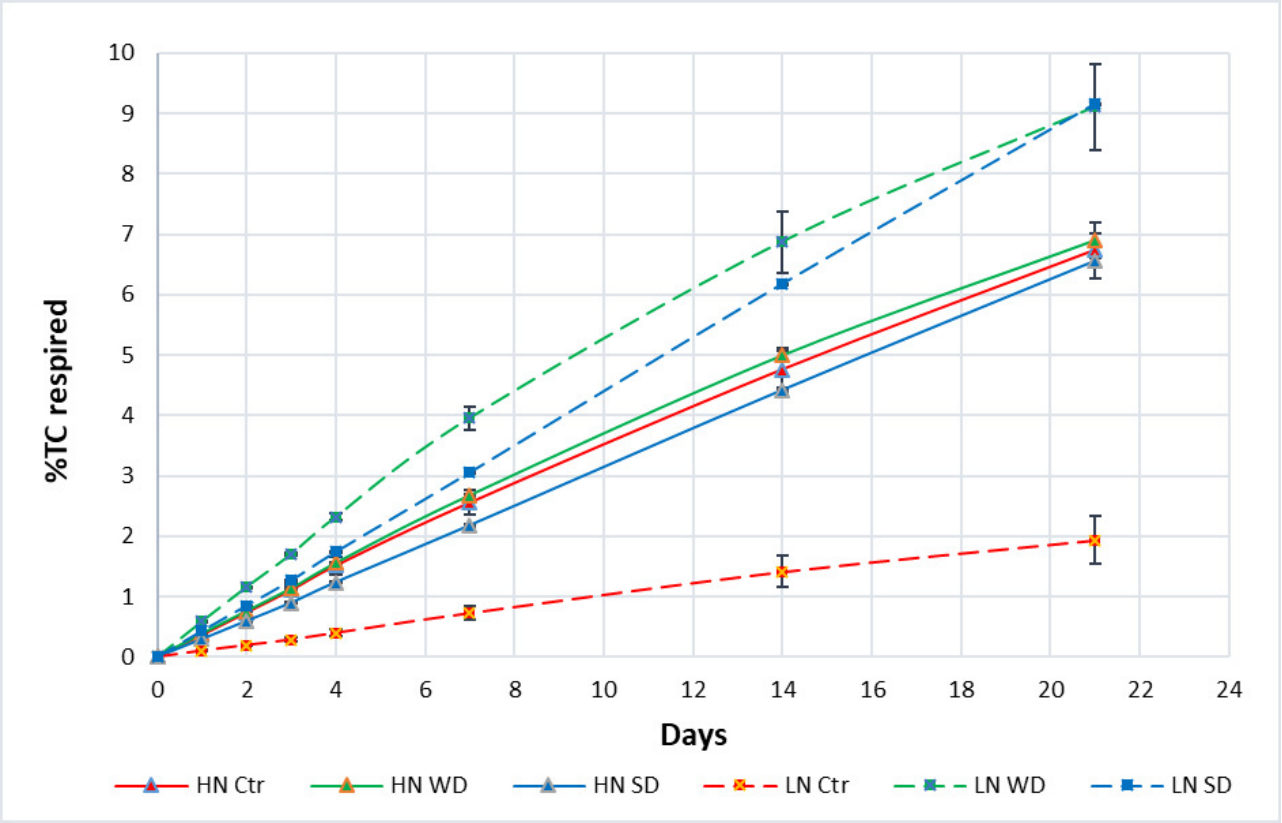
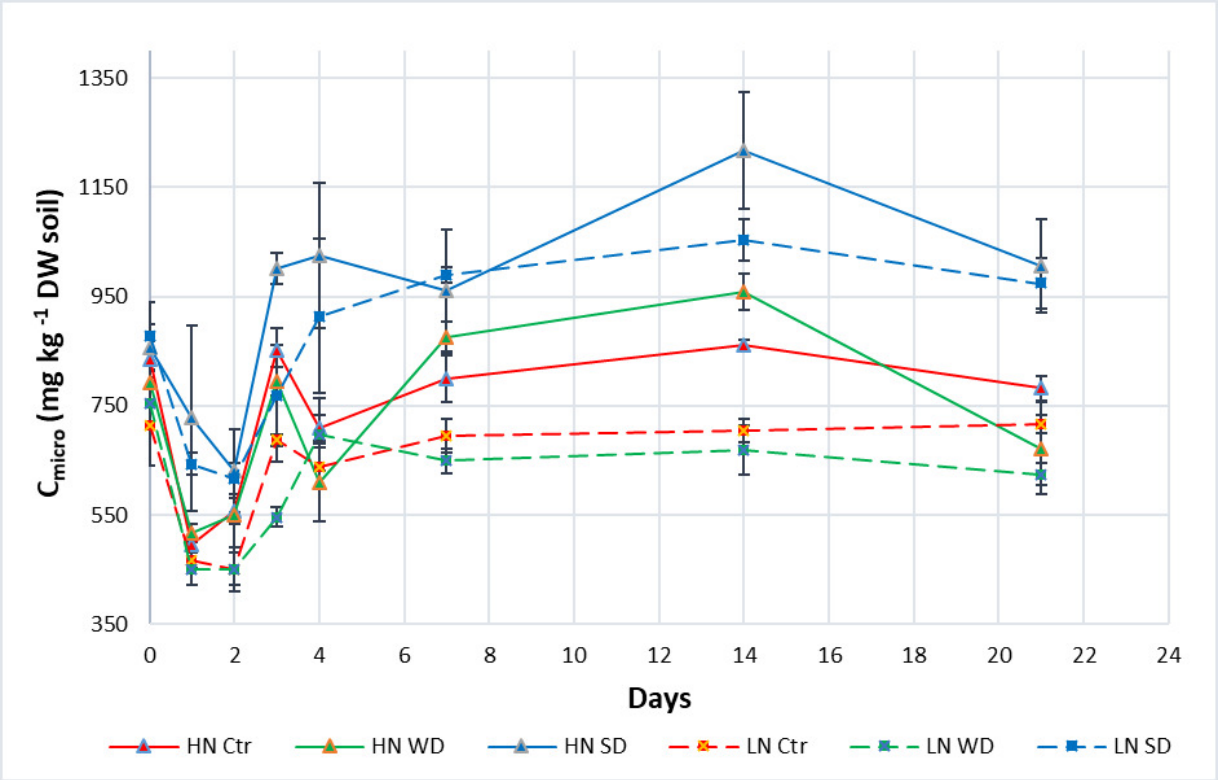


Figure 2 %TC respired in control (Ctr) soils or after addition of whole (WD) or solid (SD) fractions of digestate in soils at high (HN) or low (LN) initial nutrient status. Error bars ± 1SE



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858 *Figure 3 C_{micro} trends over time in control (Ctr) soils or after addition of whole (WD) or solid (SD)*
859 *fractions of digestate in soils at high (HN) or low (LN) initial nutrient status. Error bars ± 1SE*

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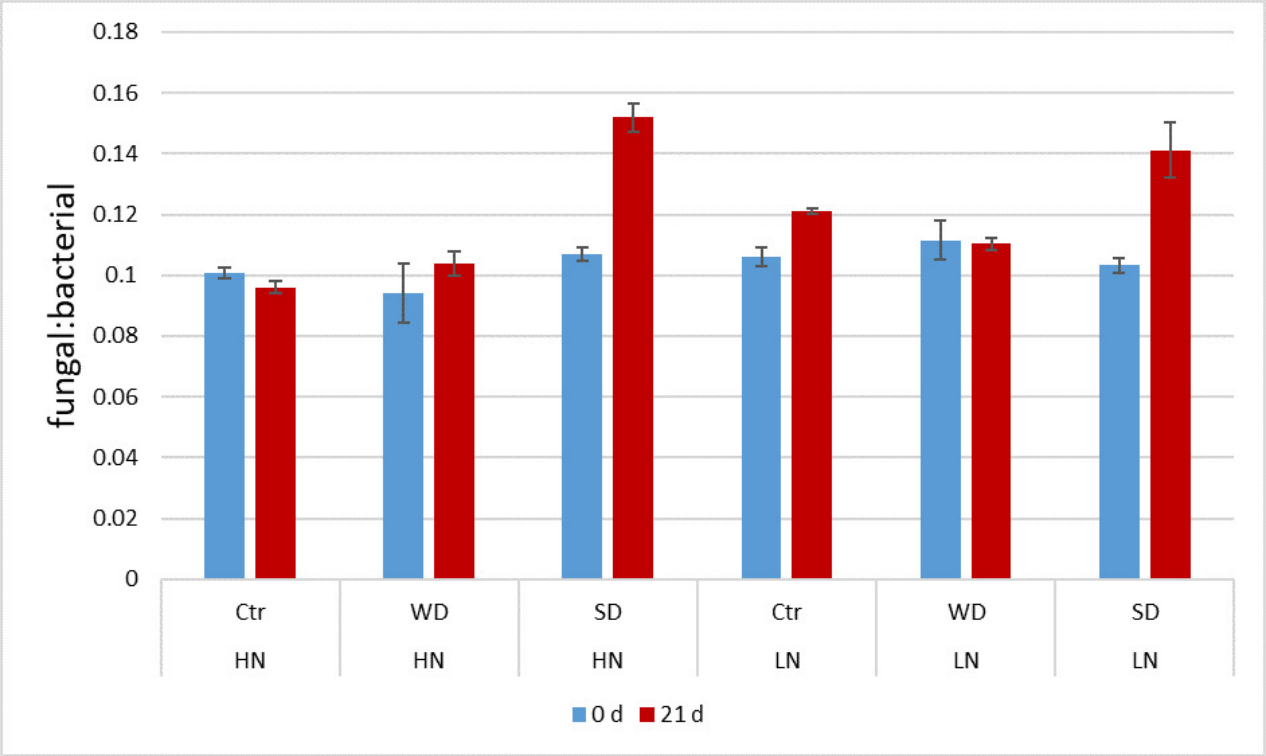

Figure 4 Fungal to bacterial ratio at 0 d and 21 d in control (Ctr) soils or after addition of whole (WD) or solid (SD) fractions of digestate in soils at high (HN) or low (LN) initial nutrient status. Error bars ± 1SE

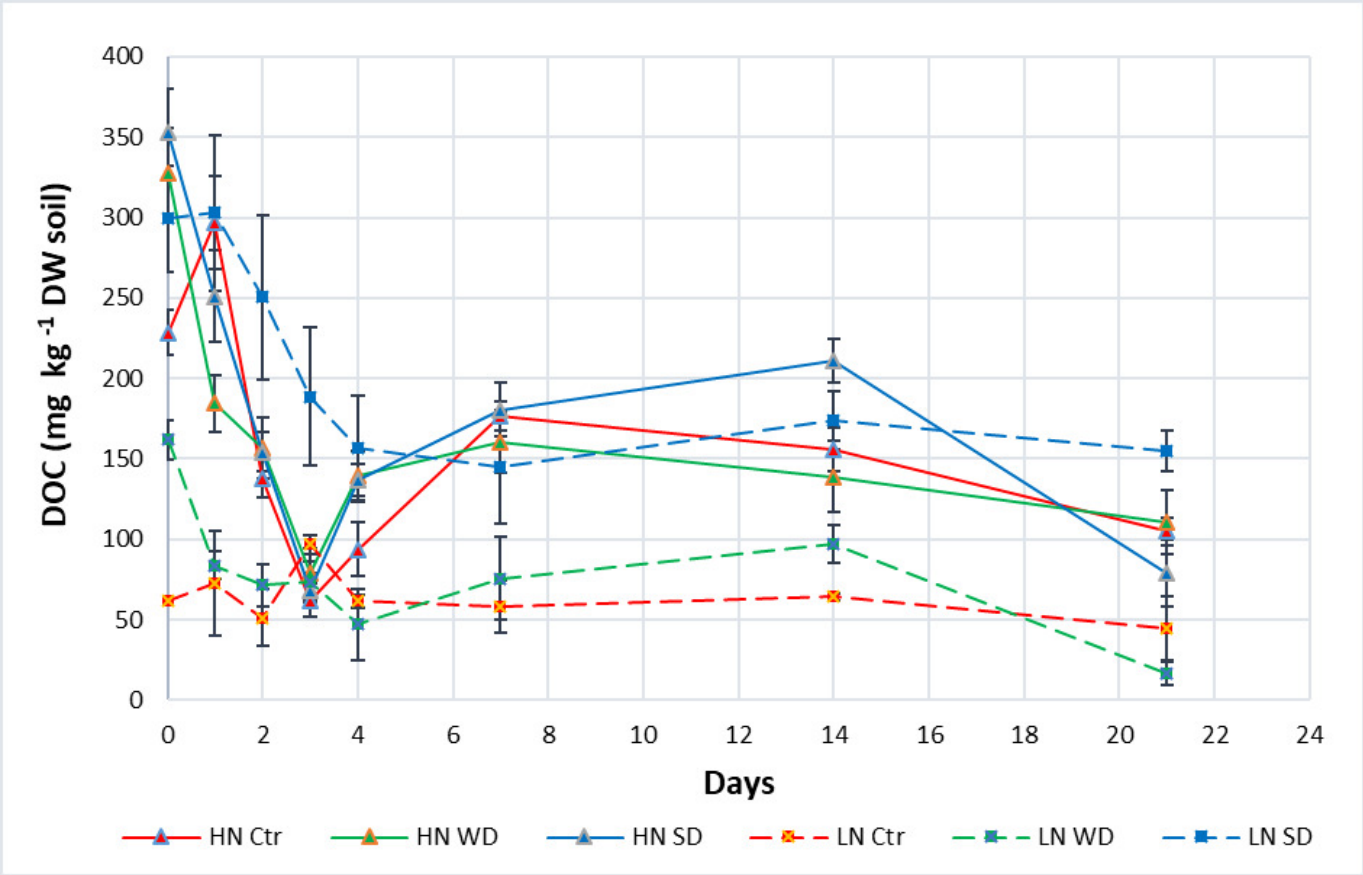

Figure 5 Dissolved organic carbon trends through time in control (Ctr) soils or after addition of whole (WD) or solid (SD) fractions of digestate in soils at high (HN) or low (LN) initial nutrient status. Error bars ± 1SE