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Short communication

Response of C-cycling enzymes in soil aggregate-size fractions to slurry application over a one year period in two contrasting soil types

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ABSTRACT

The distribution of enzymatic activity in soil aggregates is a key determinant in the turnover of organic matter. This study aimed to address how the application of a carbon-rich slurry influences extracellular enzymatic activity within aggregate-size fractions over a one-year period. Twenty-four pots of either a loam or sand soil, sown with *Lolium perenne*, were kept slurry-free (control), or were treated with a high dry matter slurry. Pots were sampled after 31, 137 and 361 days ($n = 4$). Soils were physically fractionated to obtain four aggregate-size fractions: ≥2 mm, ≥250 μm, ≤250 μm and ≤53 μm with enzyme assays of seven C-cycling enzymes conducted on each, and the potential extracellular enzymatic activity (pEEA) calculated. The strongest response in individual pEEA was seen after 137 days, where the pEEA of at least one C-cycling enzyme was significantly higher in the slurry treatment in each of the four aggregate-size fractions in both soil types (all at least $P \leq 0.05$). Additionally, nearly all seven C-cycling enzymes were significantly higher in the slurry treatment compared to the control in the ≤53 μm fraction (all at least $P \leq 0.05$) in both. No significant increase in pEEA was seen in the slurry treatment in any aggregate-size fraction after 361 days in the loam soil (all $P > 0.05$), and this was also largely the case for the sand soil. The results of this study clearly indicate that pEEA within aggregate-size fractions was strongly influenced by slurry application in the initial months, though this was not sustained.

1. Introduction

Soil aggregate-size fractions represent distinct micro-habitats which govern both microbial community structure and diversity (Bach et al., 2018; Fox et al., 2018; Wang et al., 2020) and activity (and thus biogeochemical cycling) primarily through substrate availability as well as O_2 and water permeability (Bach and Hofmockel, 2014). This varied distribution of soil organic carbon (SOC) and microbes caused by soil aggregation is a key determinant in the regulation of organic matter turnover in grassland soils (Torres-Sallan et al., 2017). Globally, grasslands contain approximately 30 % of soil organic carbon, with their management being a key determinant of their sequestration potential (Hewins et al., 2015). This is a particularly important consideration in temperate agricultural grasslands, as the over-wintering of livestock produces a large quantity of carbon (C) rich slurry, which is then applied back onto the land the following spring to recycle nutrients (Abbruzzese et al., 2021; Bourdin et al., 2014; Harris et al., 2011). The theory of aggregate formation postulates a sequential incorporation of C into soil aggregate structures, being first occluded within the macroaggregates. After a period of months, this C can become incorporated into the microaggregates, as these are formed within the macroaggregates (Oades, 1984). While studies have examined potential extracellular enzymatic activity (pEEA) within aggregate-size fractions in cropland and semi-arid grasslands (Bach and Hofmockel, 2016; Fansler et al., 2005; Sharma et al., 2022; Wang et al., 2015), more research is required in agriculturally managed temperate grassland systems. This is a key research gap, as a comprehensive understanding of the interactions of soil structure, agricultural management and temporal progression on pEEA is key to elucidate the mechanisms underpinning C sequestration and mineralization (Bach and Hofmockel, 2016; Luu et al., 2022). This study was established to determine how the application of slurry-derived C can influence the pEEA within aggregate-size fractions in two highly texturally contrasting soils. Specifically, we hypothesized that:

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(i) Slurry amendment will increase pEEA in the larger aggregate-size fractions (macroaggregates), which contain younger, more labile carbon (Jastrow, 1996) in the weeks following its application.
(ii) As slurry-derived C becomes increasingly incorporated into the soil matrix (after months), pEEA will be higher in smaller aggregate-size fractions (microaggregates and silt and clay) compared to the slurry-free control.

2. Materials and methods

Forty-eight pots were established, twenty-four pots of 900 g of sieved (8 mm) loam soil (49 % sand, 32 % silt and 19 % clay, pH 5.2, 2.38 % % total C) or twenty-four pots of a sand soil (89 % sand, 4 % silt and 7 % clay, pH 5.5, 1.43 % % total C). Each pot was sown with 0.8 g of *Lolium perenne* L seed. The *Lolium* sward then established itself over 45 days. Pots were either kept slurry-free as a control or treated with 60 g of a high dry matter slurry (9.13 % DM, 7.56 pH, 39.80 % C, 2.30 % N), and placed in a glasshouse from May 2015 to June 2016 and were subject to ambient Irish temperatures. Pots were destructively sampled 31, 137 and 361 days after slurry application. There were four replicate pots per treatment at each sampling time. The 'wet-sieving' technique (Six et al., 1998) was used to obtain the large macroaggregate (LM, >2 mm), macroaggregate (MAC, >250 μm), microaggregate (MIC, <250 μm) and silt and clay fraction (SC, <53 μm) as previously described (Fox et al., 2018). However, aggregate-size fractions isolated for later enzymatic analysis were not subjected to the final drying step. In that instance, aggregates were placed into sterilized centrifuge containers and centrifuged at 750 rpm for 10 min at 4 °C. Seven different fluorogenic model substrates; 4-MUF-*N*-acetyl-β-D-glucopyranoside, 4-MUF-α-D-glucopyranoside, 4-MUF-β-D-glucopyranoside, 4-MUF-α-L-arabinopyranoside, 4-MUF-β-D-galactopyranoside, 4-MUF-β-D-Cellobioside and 4-MUF-β-D-xylopyranoside (all Sigma Aldrich, St. Louis, MO), were used to determine pEEA (Marx et al., 2001). 3 g of fresh soil was placed into 30 ml of sterile dH_2O and extracellular enzymes were extracted by shaking on a Gyratory shaker (New Brunswick Scientific) at 150 rpm for 10 min, followed by centrifugation at 750 rpm for 10 min at 4 °C. 200 μl of this supernatant was pipetted into individual wells of black microtiter plates (VWR, Radnor, PA). Plates and assays were prepared and conducted as previously described (Fox et al., 2017; Hendriksen et al., 2016). The pEEA was calculated as follows:

$$\text{pEEA} = \text{EEA}\left(\mu\text{mol h}^{-1}\ \text{g}^{-1}\ \text{dry soil}\right)*\text{Prop}\left(\text{aggregate} - \text{size fraction}\right)$$

where EEA is the potential extracellular activity per gram of dry aggregate soil and Prop (aggregate-size fraction) is the proportion of the whole soil mass comprised within the aggregate of interest (Bach and Hofmockel, 2014). The % total C within each aggregate-size fraction was measured using a LECO Truspec CN analyser (LECO Corporation, Saint Joseph, MI).

Analysis of variance was conducted to determine the effect of slurry application on the % proportion and % total C of aggregate-size fractions, as well as the pairwise differences between the control and slurry treatment in individual pEEA of each measured enzyme at each sampling event. A Kruskal-Wallis test was performed on non-normally distributed data. Linear regression analysis was conducted to determine the relationship between the pEEA of individual enzymes within an aggregate-size fraction at a given sampling event and the % total C of that aggregate-size fraction. All statistical tests were done in the R statistical software (R Development Core Team, 2019).

3. Results

In the loam soil, there was a significant increase in the % proportion of LM between the slurry treatment (18.71 %) and the control (10.67 %) after 137 days ($P < 0.05$, Table 1a). The % total C within LM was significantly increased in the slurry treatment after both 31 and 137 days (36.24 % and 58.92 % increase respectively, $P < 0.05$, Table 2a). Both MAC and MIC had significantly higher % total C in the slurry treatment after 137 days (16.39 % and 10.61 % increase, respectively, $P < 0.05$, Table 2a), with the effect still seen in MAC after 361 days (10.47 % increase, $P < 0.005$). No such effect was seen in SC at any time point ($P > 0.05$, Table 2a). In the sand soil, slurry application did not significantly increase the % proportion of any aggregate-size at any time point (Table 1b). Rather, the % proportion of MIC was lower in the slurry treatment compared to the control ($P = 0.40$) after 137 days, as it was for SC after 361 days ($P = 0.001$, Table 1b). The % total C significantly increased in the slurry treatment (4.63 %) over the control (2.80 %), in LM in the sand soil after 31 days ($P = 0.019$, Table 2b). This was also seen in SC after 137 days, with the slurry treatment (6.71 %) having more % total C than the control (5.87 %, $P < 0.001$, Table 2b).

Looking at the individual enzyme responses in the loam soil (Fig. 1a-l), there were significant increases in pEEA in individual enzymes in the slurry treatment over the control in LM (β-*N*-acetyl-glucosaminidase, β-galactosidase and α-arabinosidase, Fig. 1a), MIC (α-glucosidase and α-arabinosidase) and SC (β-glucosidase, β-*N*-acetyl-glucosaminidase, α-glucosidase, β-galactosidase, all at least <0.05), but not in MAC. There was also a significant linear relationship between the pEEA of the three responsive enzymes in LM with the % total C in that fraction (Table S1). The strongest response in pEEA of individual enzymes in the loam soil was seen after 137 days, with enzymes from each of the four aggregate-size fractions increasing in activity in the slurry treatment compared to the control. The pEEA of α-glucosidase, cellobiosidase and β-xylosidase were significantly higher in the slurry treatment in each of the four aggregate-size fractions (Fig. 1e-h, all at least $P < 0.05$). In SC, each of the seven measured enzymes increased in the slurry treatment, five in LM, and four in the MAC and MIC fractions (Fig. 1e-h). Three of these

Table 1
Averages and standard errors (±) of the % proportion of; large macroaggregate (LM), macroaggregate (MAC), small microaggregates (MIC) and silt and clay (SC) after 31, 137 and 361 days after slurry application in the slurry-free control and the slurry treatment in both the loam (a) and sand (b) soils. Significant differences and trends in the proportion of individual aggregate-size fractions between Cont and HS within each sampling time are also shown. Significance codes: '**' $P \leq 0.01$ '*' $P \leq 0.05$.

Time	31 days		137 days		361 days	
Fraction	Control	Slurry	Control	HS	Control	Slurry
(a) Loam						
LM	29.49 % (±2.574 %)	37.53 % (±2.099 %)	10.67 % (±1.083 %)	18.71 % (±4.166 %)*	23.90 % (±5.319 %)	38.88 % (±3.235 %)
MAC	39.27 % (±1.999 %)	33.00 % (±1.930 %)	31.39 % (±1.225 %)	28.26 % (±3.737 %)	38.20 % (±1.664 %)	32.62 % (±3.290 %)
MIC	22.78 % (±0.555 %)	20.57 % (±1.823 %)	34.40 % (±1.076 %)	30.57 % (±1.405 %)	25.62 % (±2.790 %)	19.59 % (±0.353 %)
SC	8.46 % (±0.139 %)	8.90 % (±1.015 %)	23.54 % (±0.437 %)	22.46 % (±2.146 %)	12.28 % (±1.671 %)	8.92 % (±0.406 %)
(b) Sand						
LM	5.18 % (±0.618 %)	7.08 % (±1.784 %)	27.29 % (±5.108 %)	41.17 % (±4.229 %)	31.33 % (±4.806 %)	38.80 % (±2.584 %)
MAC	52.19 % (±3.649 %)	49.42 % (±1.430 %)	31.34 % (±2.049 %)	27.56 % (±1.868 %)	31.73 % (±2.830 %)	29.01 % (±3.070 %)
MIC	38.93 % (±2.963 %)	39.94 % (±2.514 %)	35.12 % (±2.788 %)	26.05 % (±2.085 %)*	32.03 % (±2.107 %)	28.57 % (±3.130 %)
SC	3.70 % (±0.333 %)	3.56 % (±0.135 %)	6.25 % (±0.657 %)	5.21 % (±0.703 %)	4.91 % (±0.173 %)	3.62 % (±0.144 %)**

Table 2
Averages and standard errors (±) of the % total carbon in; large macroaggregate (LM), macroaggregate (MAC), small microaggregates (MIC) and silt and clay (SC) after 31, 137 and 361 days after slurry application in the slurry-free control and slurry treatment in both the loam (a) and sand (b) soils. Significant differences and trends in the proportion of individual aggregate-size fractions between the control and slurry treatment within each sampling time are also shown. Significance codes: '**' $P \leq 0.01$ '*' $P \leq 0.05$.

Time	31 days		137 days		361 days	
Fraction	Control	Slurry	Control	Slurry	Control	Slurry
(a) Loam						
LM	2.65 (±0.049)	3.61 (±0.096)*	3.14 (±0.111)	4.99 (±0.692)*	2.57 (±0.024)	2.66 (±0.074)
MAC	3.15 (±0.264)	3.40 (±0.083)	2.99 (±0.121)	3.48 (±0.101)*	3.11 (±0.050)	3.44 (±0.057)**
MIC	2.67 (±0.173)	2.63 (±0.120)	2.71 (±0.078)	3.17 (±0.067)*	2.48 (±0.115)	2.60 (±0.012)
SC	2.23 (±0.180)	2.34 (±0.101)	2.91 (±0.074)	2.94 (±0.079)	2.32 (±0.386)	2.82 (±0.087)
(b) Sand						
LM	2.80 (±0.337)	4.63 (±0.463)*	2.17 (±0.260)	2.23 (±0.242)	1.85 (±0.104)	3.35 (±0.882)
MAC	3.10 (±0.230)	2.88 (±0.255)	2.63 (±0.103)	3.14 (±0.617)	2.66 (±0.142)	3.09 (±0.459)
MIC	2.55 (±0.138)	2.59 (±0.281)	2.72 (±0.053)	2.55 (±0.110)	2.47 (±0.077)	2.90 (±0.189)
SC	5.67 (±0.164)	5.74 (±0.301)	5.87 (±0.069)	6.71 (±0.061)***	6.46 (±0.352)	5.91 (±0.197)

enzymes in MIC had a significant linear relationship to the % total C in that fraction, β-glucosidase ($r = 0.59$), cellobiosidase ($r = 0.77$) and β-xylosidase ($r = 0.81$, all at least $P < 0.05$, Table S1). There were no differences in the pEEA of individual enzymes seen in the loam soil after 361 days in any aggregate-size fraction (Fig. 1i-l).

In the sand soil, there were no significant increases in pEEA in individual enzymes in the slurry treatment over the control in LM (Fig. 1m all $P > 0.05$), in contrast to that seen in the loam soil. There were, however, increases in pEEA in individual enzymes in the MAC, MIC and SC aggregate-size fractions. β-*N*-acetyl-glucosaminidase was significantly higher in the slurry treatment compared to the control in all three, α-glucosidase was significantly higher in both MAC and MIC, while β-glucosidase and β-xylosidase were significantly higher in MIC (all at least $P < 0.05$). After 137 days, six of the measured C-cycling enzymes had significantly higher pEEA in the slurry treatment compared to the control in SC (all at least $P < 0.05$), with β-*N*-acetyl-glucosaminidase being the exception ($P > 0.05$). This was the most of any of the aggregate-size fractions at this sampling point. Unlike with the loam soil, however, the pEEA of five of these enzymes had a significant linear relationship with the % total C in SC ranging from $r = 0.54$ for β-glucosidase to $r = 0.72$ for β-xylosidase (all at least $P < 0.05$, Table S2). Three of the four enzymes with higher pEEA in the slurry treatment in MAC also had a significant linear relationship with the % total C in that fraction, β-glucosidase ($r = 0.54$), cellobiosidase ($r = 0.75$) and β-galactosidase ($r = 0.52$, all at least $P < 0.05$, Table S2). In contrast to that seen after 137 days, after 361 days six of the seven measured enzymes (with the exception of cellobiosidase) had significantly lower pEEA in the slurry treatment compared to the control (Fig. 1x, all $P < 0.05$).

4. Discussion

These results clearly indicate that (i) pEEA was significantly increased by slurry application in the initial months, though no evidence of a sequential response through the larger to smaller aggregate-size fractions was seen in either soil type, (ii) the significant increase in pEEA was largely not sustained after 361 days in either soil type. The data would not support either of the studies stated hypotheses. In the loam soil, while the pEEA of individual enzymes in LM was significantly increased with slurry application after 31 days, this was also the case in MIC and SC with no significant increases seen in MAC. In the sand soil, there was no increase in the pEEA of any enzyme to slurry application after 31 days in LM, but there were in MAC, MIC and SC. After 137 days, the pEEA of at least one individual enzyme in all aggregate-size fractions were significantly higher in the slurry treatment in both soil types. Rather than seeing a significant increase in pEEA in the smaller aggregate fractions (i.e., MIC and SC) in the slurry treatment after 361 days, no such effect was observed in either soil type.

While the proposed study hypotheses could be expected in light of soil aggregate formation theory (Oades, 1984; Six et al., 2000), the results obtained here may be the result of the differential incorporation of differently labile slurry C. Slurry-derived C (labile and recalcitrant components) is incorporated into soil sequentially (Bourdin et al., 2014), with the labile C component entering the soil matrix in the days following application, and the recalcitrant component being incorporated in the succeeding weeks and months. In a field study investigating the influence of slurry application on C-cycling enzymes (the same reported in this study) in the bulk soil over a two-month period, a significant increase in extracellular enzymatic activity was seen 5 days after slurry application, with this increase assumed to be associated with the incorporation of the labile C component (Fox et al., 2017). In this study, the labile C likely percolated through the soil matrix after 31 days, and was accessible to microbes in the smaller aggregate-size fractions, in particular the small number of microbes which reside on the aggregate surface (Wilpiszeski et al., 2019). This C was not incorporated into either MIC or SC as evident by the lack of increase in the % total C in these fractions at this time point in either soil type.

Three possible explanations for the strong effect of slurry application after 137 days may be; 1. the incorporation of the recalcitrant (less labile) C slurry fraction (Bourdin et al., 2014), 2. microbial priming effects on native soil C (Bernard et al., 2022; Kuzyakov et al., 2000) and 3. the mineral-stabilization of extracellular enzymes (Allison and Jastrow, 2006; Olagoke et al., 2019). The % total C of LM, MAC and MIC aggregate-sizes in the loam, and SC in the sand, was significantly increased in the slurry treatment compared to the control after 137 days. This would indicate the incorporation of the recalcitrant slurry C into these aggregate-size fractions, as would the significant linear relationship between the % total C and the pEEA of individual enzymes within MIC in the loam and MAC and SC in the sand soil. It has been shown that the application of cattle slurry to an agricultural grassland increased C mineralization of native soil C (Bourdin et al., 2014). Such effects have been attributed to a microbially-induced soil 'priming effect', where the application of a C source induces the soil microbiome to breakdown more structurally complex native soil C (Bernard et al., 2022). Such a process may lie behind the observed increase in pEEA of individual enzymes with no significant linear relationship to the % total C within the associated aggregate (i.e., LM and MAC in the loam soil). All seven of the C-cycling enzymes were significantly higher in SC in the slurry treatment in the loam after 137 days. Unlike that seen with SC in the sand soil, in the loam there were no significant linear relationship between the % total C and the pEEA of individual enzymes. This may be due to a microbial-induced priming effect (as described above), or be linked to enzyme stabilization on the clay surfaces associated with SC (Allison and Jastrow, 2006; Olagoke et al., 2019).

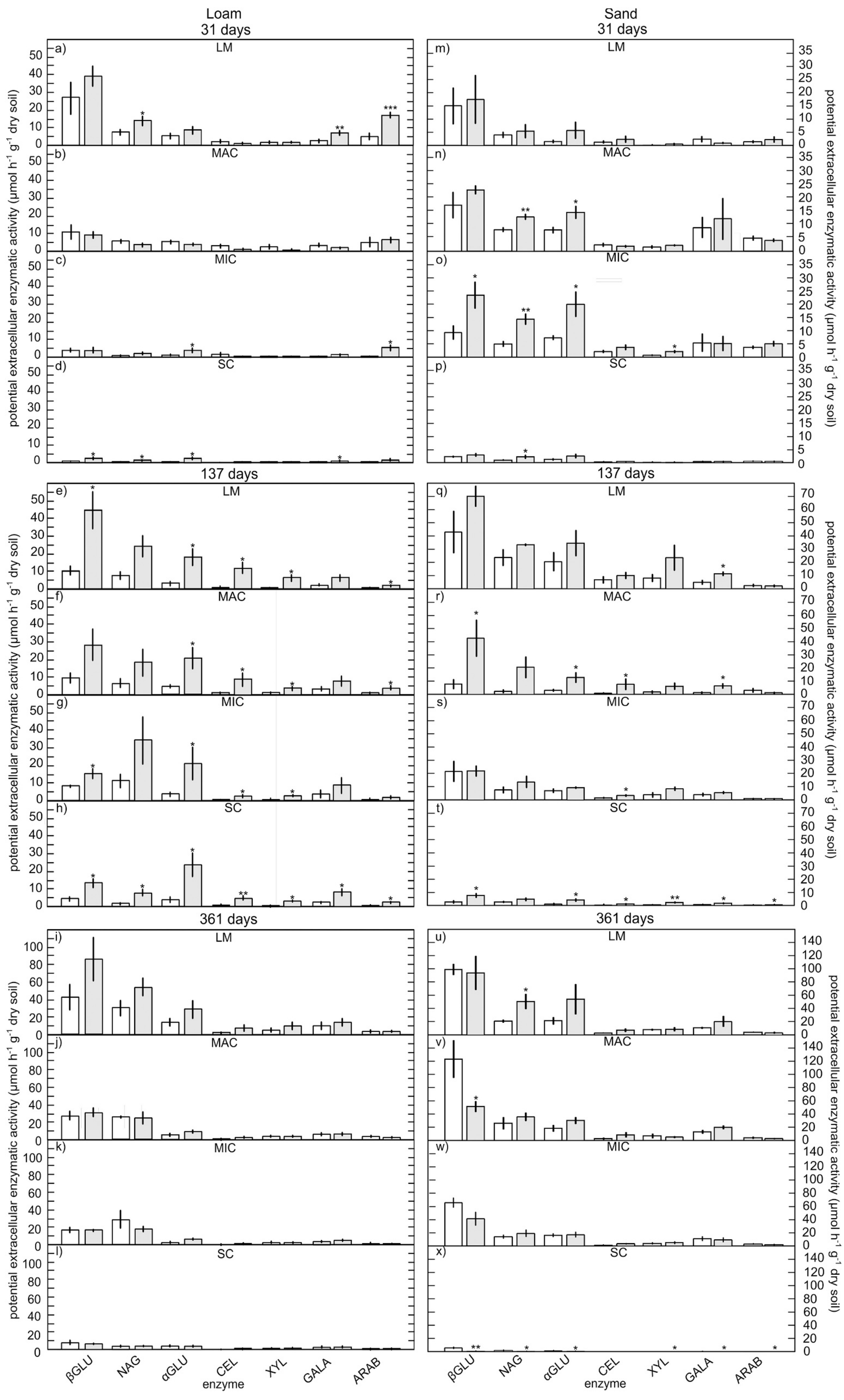

Fig. 1. Averages and standard errors (±) of 7 carbon cycling enzymes potential activities (μmol h^{-1} g $^{-1}$ dry soil) within each aggregate-size fraction. This included the large macroaggregate (LM, panels a, e, i, m, q & u), macroaggregate (MAC, panels b, f, j, n, r & v), microaggregate (MIC, panels c, g, k, o, s & v) and the silt and clay fraction (SC, panels d, h, l, p, t & x) from both the slurry-free control (white) and slurry treatment (light grey) 31, 137 and 361 days after slurry application, in both the loam and sand soil. Enzyme abbreviations are as follows; β-glucosidase (βGLU), β-*N*-acetylglucosaminidase (NAG), α-glucosidase (αGLU), cellobiosidase (CEL), β-xylosidase (XYL), βgalactosidase (GALA) and α-arabinosidase (ARAB). Pairwise differences between Cont and HS were determined by analysis of variance. Significance code: '**' $P \leq 0.01$ '*' $P \leq 0.05$.

After 361 days, the slurry-derived C appears to have been exhausted as an easy utilizable C source for the soil microbiome in the loam soil, as no significant differences were seen in the pEEA of any individual enzyme in any aggregate-size fraction at this sampling point. This is despite the fact that the % total C of MAC was still higher in the slurry treatment in this soil type, indicating that this C may be physically protected from microbial breakdown (O'Brien and Jastrow, 2013). Only one enzyme had a significantly higher pEEA in the slurry treatment after 361 days in the sand soil (α-glucosidase in LM), also suggesting that slurry-derived C had been exhausted as a microbial carbon source in this soil type as well. Unexpectedly, in the sand soil, six of the C-cycling enzymes had a higher pEEA in the control treatment in SC at this sampling point. This is most likely reflective of the higher % proportion of SC in the control, compared to the slurry treatment (Bach and Hofmockel, 2014).

In conclusion, this study did show that there is a response of pEEA to slurry application in the initial months following application, though it was not sustained after 1 year. While a progressive response in enzymatic activity to carbon application through the larger to smaller aggregate-size fractions over time could be expected, in light of aggregate formation theory, this was not observed in this study. Further work is required to study this response under field conditions in temperately managed grasslands, which can be subjected to intensive management throughout the growing season, and at different application rates of slurry.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2022.104728.

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