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Are researchers following best storage practices for measuring soil biochemical properties?

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1. Survey Figure

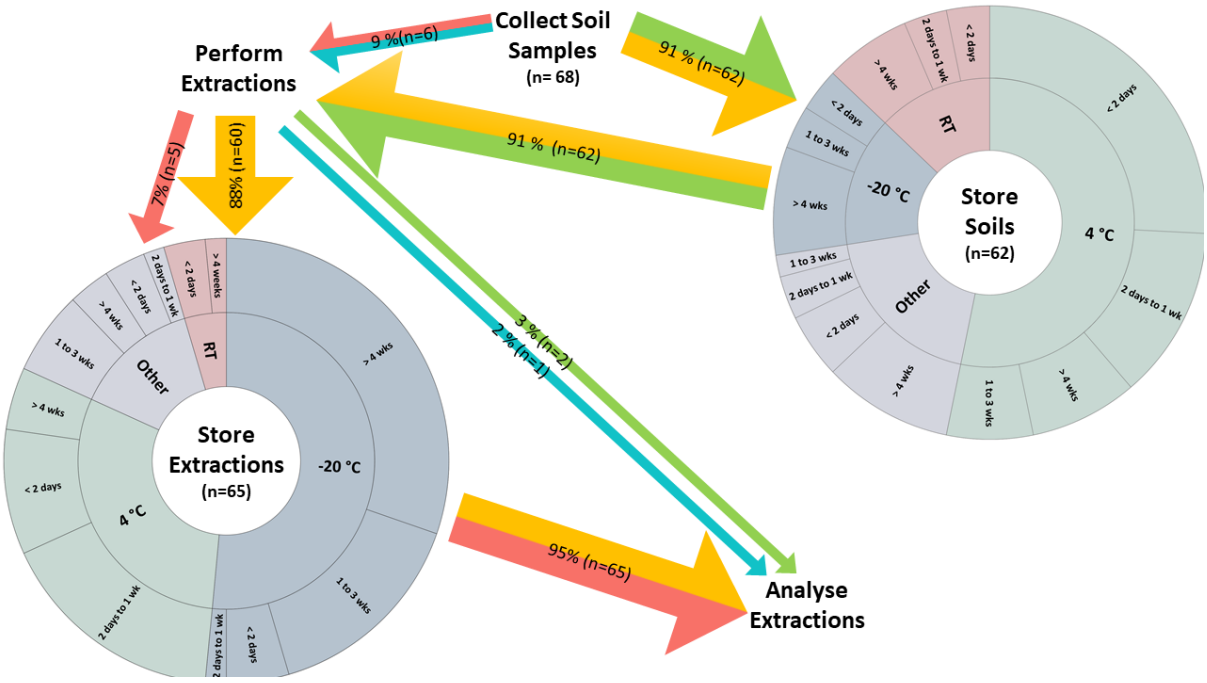


Figure S1– Flow chart indicating the results of an online survey to determine how and for how long people store soils and/or extracts prior to analysis. Room temperature is abbreviated as RT. Survey date: December 2018 – June 2019. The proportion of people that followed each methodological step is indicated as 'n' in each arrow, which are also scaled in size depending on 'n'. The arrow colours correspond to the differences in soil sample storage, processing and extract storage prior to analysis. Pink: soil samples are extracted immediately after collection and extracts are stored for future analysis. Blue: Neither soils nor extracts are stored. Green: soils are stored after collection, but extracts are analysed immediately after extraction. Yellow: both soils and extracts are stored.

2. Extended materials and methods

2.1. Experimental design

We designed a full factorial experiment with two different depths of soil (topsoil and subsoil), five field replicates, two different types of stored samples (soil or extract) and two different storage temperatures (4°C or -20 °C). We evaluated four different types of extracts: water, KCl, fumigated K_2SO_4 and unfumigated K_2SO_4; at 12 different time points: 1, 3, 7, 14, 21, 28, 57, 85, 113, 169, 281 and 430 days after sampling. Additionally, we measured and analysed the four different extracts immediately after soil collection (fresh sample), to use as the 'baseline' comparison value. This amounted to 1,952 extractions in total.

2.1.1. Soil collection and treatment preparation

Five replicated topsoil and subsoil samples were collected from a field located in Selside in the Yorkshire Dales National Park (54.17 N, 2.34 W), northern England. We sampled at two different depths in order to explore differences in key soil properties without the confounding factors of climate and parent material. This site was chosen as it has been widely characterised by

other experimental studies (eg. De Long et al., 2019; Leff et al., 2018) and is representative of typical permanent grasslands used for livestock production across the United Kingdom and parts of Europe (Rodwell, 1992). The soil in this area is described as a clayey brown earth over limestone bedrock from the Malham series of Eutric Endoleptic Cambisols (Leff et al., 2018; De Long et al., 2019), and the main physical and chemical characteristics of these soils are summarised in Table S1.

Table S1 Physical and chemical characteristics of topsoil (0-10 cm) and subsoil (20-30 cm) used in this experiment. OM: Organic matter, DOC: dissolved organic carbon, DON: dissolved organic nitrogen, MBC: microbial biomass carbon, MBN: microbial biomass nitrogen (means ± SD).

Soil	TOPSOIL	SUBSOIL
Clay (%) *	60.0 ± 2.1	62.8 ± 2.4
Silt (%) *	0.6 ± 0.2	0.7 ± 0.3
Sand (%) *	39.3 ± 1.9	36.4 ± 2.1
Bulk density (g cm^{-3}) *	0.63 ± 0.04	0.71 ± 0.04
OM (%) *	14.0 ± 2.5	7.1 ± 1.6
C (%)	3.9 ± 0.1	1.5 ± 0.2
N (%)	0.45 ± 0.01	0.17 ± 0.02
C: N Ratio	10.5 ± 0.2	10.5 ± 0.2
pH	5.9 ± 0.1	5.8 ± 0.1
Soil moisture (%)	47.9 ± 4.9	43.6 ± 0.5
NH_4^+ (mg kg^{-1} dry soil)	2.02 ± 0.97	0.94 ± 0.75
NO_3^- (mg kg^{-1} dry soil)	0.32 ± 0.13	0.23 ± 0.04
DON (mg kg^{-1} dry soil)	2.3 ± 0.7	7.5 ± 2.1
DOC (mg kg^{-1} dry soil)	19.6 ± 5.5	82.6 ± 13.6
MBC (mg kg^{-1} dry soil)	1772 ± 340	246 ± 200
MBN (mg kg^{-1} dry soil)	137.3 ± 27.3	35.0 ± 12.5
DOC: DON	8.8 ± 1.9	11.4 ± 1.8

* Unpublished data provided by and collected for published work by De Long et al. (2019)

Five 0.5 x 0.5 m plots were allocated 10 m apart along a transect in the field in June 2018. At each location, the first 20 cm of soil were removed and subsoil samples were collected down a further 10 cm (i.e. at a depth of 20-30 cm). This approach of sampling and keeping separate true field replicates was chosen to avoid pseudo replication, and to properly represent the high variability associated with typical ecological field experiments. High data variability was accounted for by calculating relative change for each individual replicate compared to corresponding fresh samples, and by increasing the acceptable similarity limit to 20% (see statistical analyses for details). Three weeks later, new 0.5 x 0.5 m plots were allocated approximately 10 cm apart to the previous ones. At each location, the first 2-3 cm of turf was removed and topsoil samples were collected with a spade from the top 3-10 cm. Staggering the sampling in this way enabled all necessary laboratory work to be completed at the same relative timepoints for both top- and subsoils. Both topsoil and subsoil samples were transported to the laboratory on the day of collection and placed at 4 °C overnight. On the next day, roots were removed by hand and soils passed through a 4 mm

sieve, as standard practice (Jones and Willett, 2006). Soil moisture was measured and extractions were carried out (see below for details) immediately after homogenisation on fresh samples.

2.1.2. Stored extract treatments

Samples were extracted (methods described in section S1.2) from soils directly after homogenisation and then stored vertically at either 4 °C or -20 °C in falcon tubes. Additionally, water and KCl blanks were stored in the same way (n = 3 for each time point and extract type). Stored 4 °C extract samples were removed from the refrigerator immediately prior to analytical analysis whilst those at -20 °C were removed the night before and allowed to thaw overnight (≈16 h) at 4 °C before analysis.

2.1.3. Stored soil treatments

Approximately 50 g of soil was stored in sealed plastic bags immediately after homogenisation at either 4 °C or -20 °C. For stored soil treatments at 4 °C, samples were taken out of the refrigerator immediately prior to extraction whilst those at -20 °C were allowed to thaw before extraction (< 1 h). Analysis was carried out immediately after extractions were completed.

2.1.4. Soil moisture

Soil moisture was measured on fresh samples and all soil treatment samples immediately after having been removed from the refrigerator or thawed from the freezer. Percentage soil moisture was measured by calculating the loss of mass from samples after drying at 105 °C for 48 h.

2.2. Extraction procedures

Different chemical forms of C and N were measured by means of soil extractions with different extractant solutions. All extractions were carried out on 5 g ± 0.1 of soil (exact weight annotated) weighed into falcon tubes where extractant was added, shaken horizontally at 200 rpm, centrifuged at 2900 x*g* and then filtered. Extractant volumes, shaking times, centrifugation times and filter type varied for each extractant type, and are summarised in Table S2. Extraction blanks (water and KCl) were included, i.e: water or KCl samples that were shaken, centrifuged and filtered following the same procedures as the soil samples. K_2SO_4 blanks were not performed as fumigated values were subtracted from unfumigated values, rendering subtraction of blanks from both unnecessary. Total and inorganic C were measured by a combustion catalytic oxidation method with a NDIR with 5000A TOC-L analyser (Shimadzu, Japan). Total N, NO_3^- and NH_4^+ were measured by a colorimetric segmented flow analyser AA3 (Seal Analytical, UK). All measured concentrations were corrected using blanks and corrected by soil moisture as $mg^{-1}\,kg^{-1}$ of dry soil for each variable within each extract type.

Table S2 Summary of extraction methods used in this experiment

Extractant	Soil weight (g)	Volume (ml)	Shaking time (min)	Time centrifuged (min)	Filter

Ultrapure water	5	35	10	30	0.45 µm syringe
KCl (1 M)	5	25	60	5	Whatman 42 *
K_2SO_4 (0.5 M)	5	25	30	5	Whatman 42 *

* Whatman 42 filter paper has a 2.5 µm pore size

Water extractions were utilised for the quantification of DOC and DON as:

$$DOC = Total\ Carbon - Inorganic\ Carbon - DOC\ in\ blanks$$

$$DON = Total\ Nitrogen - (NO_3 + NH_4) - DON\ in\ blanks$$

KCl extractions allowed for the quantification of NO_3^- and NH_4^+. K_2SO_4 fumigation–extraction techniques described by Brookes et al. (1985) and Vance et al. (1987) were used to calculate microbial biomass C (MBC) and N (MBN). Fumigated K_2SO_4 extractions were extracted with soils that had been fumigated with excess $CHCl_3$ under vacuum for 48 h. Unfumigated extractions were performed on soils that had not been pre-treated with $CHCl_3$. Both extractions were then measured for total C and N. MBC and MBN were calculated with the below calculation, using the k_{EC} correction factor 0.35 for MBC (Sparling et al., 1990) and k_{EN} correction factor 0.54 for MBN (Brookes et al., 1985).

$$MB = (\text{Fumigated concentration} - \text{Unfumigated concentration}) \text{ x } 1/\text{correction factor}$$

2.3. Statistical analyses

All statistical analyses were carried out in R Version 3.6.1 (R Core Team, 2019). In order to standardize the relative change of each variable for each soil depth, storage type and storage length to the measurements made immediately on the fresh samples, we calculated a ratio for each corresponding replicate with the below equation:

$$relative\ change = \frac{measured\ variable\ for\ each\ treatment}{measured\ variable\ from\ fresh\ sample}$$

Normality and homoscedasticity of the data were first checked using Anderson Darling and Levene's tests, respectively. All variables were subjected to natural log transformation except for DOC, which was not transformed. Mixed-effects models were used for each measured variable with the *lme4* package (Bates et al., 2018) to test the effects of fixed factors (soil depth, storage type and storage length) and random factor (replicate) and their interactions on the calculated relative change ratio from fresh samples. Predicted fitted values from the multi-level model were calculated with *predictInterval* with the *merTools* package (Frederick, 2019).

Similarity between fresh samples and soil storage treatments was determined when the upper or lower limit of the predicted fitted value confidence intervals fit within 20% positive and negative variance from fresh samples; we refer to these as similarity limits (Rita and Ekholm, 2007; Wallenius et al., 2010). In the instance where relative change is log transformed, a ratio of 0 signifies no change from fresh samples. Where log transformed relative change fits within our lower and upper similarity limits, which are -0.2231 and 0.1823, we accept that similarity with fresh samples is met. Where log transformation was not carried out (DOC), a ratio of 1 signifies no change from fresh samples, and the similarity limits were set between 0.8

and 1.2. The storage method was deemed no longer appropriate when the upper or lower confidence intervals of our predicted fitted model values extended beyond the 20% similarity limits.

2.4. Survey

A survey was conducted to identify trends in soil storage methods and the proportion of researchers that carry out each. The survey was conducted anonymously on Google surveys and promoted through Twitter social media platform. A total of 68 participants provided information on how they typically store their soil and/or extract samples. The survey questions asked are provided below.

1) Realistically, how do you process your samples prior to carbon and nitrogen analysis (KCl, K_2SO_4, H_2O)?
    i) Extract and run all my samples immediately
    ii) Extract immediately and store the extract until analysis
    iii) Store the soil and carry out extraction for immediate analysis
    iv) Store the soil and store the extract
2) At what temperature do you generally store your extracts?
    i) 4°C
    ii) -20°C
    iii) -80°C
    iv) Room temperature
    v) I do not store extracts prior to analysis
    vi) Other:
3) At what temperature do you generally store your soil?
    i) 4°C
    ii) -20°C
    iii) -80°C
    iv) Room temperature
    v) I do not store soil prior to analysis
    vi) Other:
4) How long do you typically store your soils for prior to extraction?
    i) 2 days or less
    ii) 2 days - 1 week
    iii) 1 - 3 weeks
    iv) 3 weeks - 2 months
    v) Longer than 2 months
    vi) I do not store soil prior to analysis

5) How long do you typically store your extracts for prior to analysis?

i) 2 days or less

ii) 2 days - 1 week

iii) 1 - 3 weeks

iv) 3 weeks - 2 months

v) Longer than 2 months

vi) I do not store extracts prior to analysis

6) Any additional comments?

3. Results figures

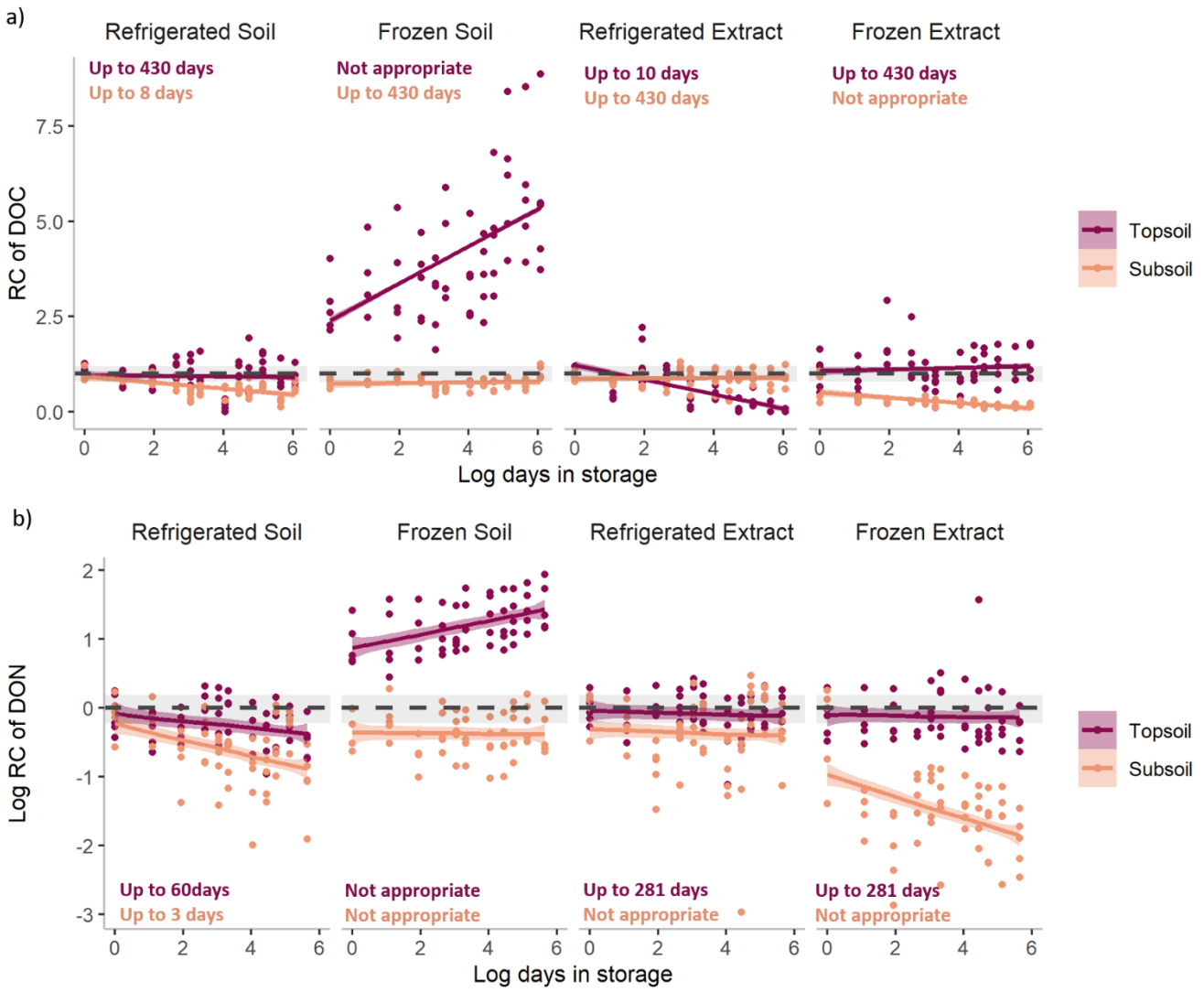


Figure S2 The relative change (RC) of a) DOC and b) DON (log transform) along storage time (days, log transform) for both soil depths for each storage treatment. Points represent calculated relative change ratios (compared to fresh sample) for individual replicates. The trend lines represent the predictive fitted ratio change values based on the mixed effects models, where coloured shaded areas represent 95% upper and lower confidence intervals of the mean. The grey shading represents the previously established similarity limits. Similarity is no longer met when both the upper and lower limit of the fitted values (coloured shading) extend beyond the grey shading. The appropriate number of storage days for each soil depths and storage method is annotated on the graph. DON data are presented only to day 281 due to technical issues on the last measuring date (day 430).

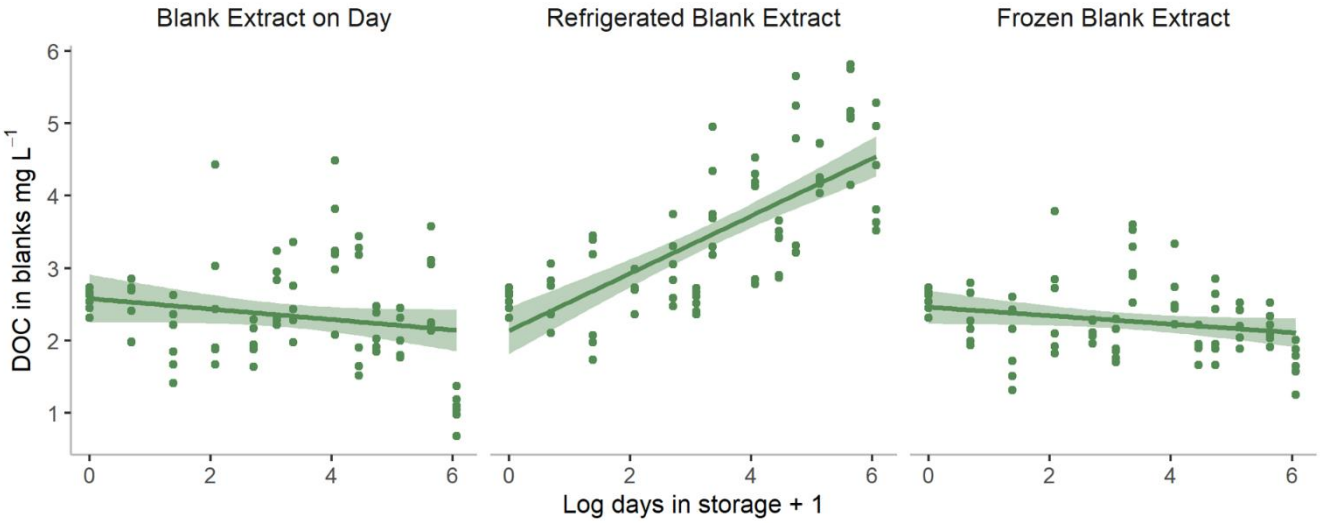


Figure S3 Concentrations of DOC in blank samples either extracted on the day of analysis, refrigerated, or frozen and then analysed over the storage time. Points represent concentrations for individual replicates. Mixed effects models are represented by trend lines, where shaded areas represent 95% upper and lower confidence intervals.

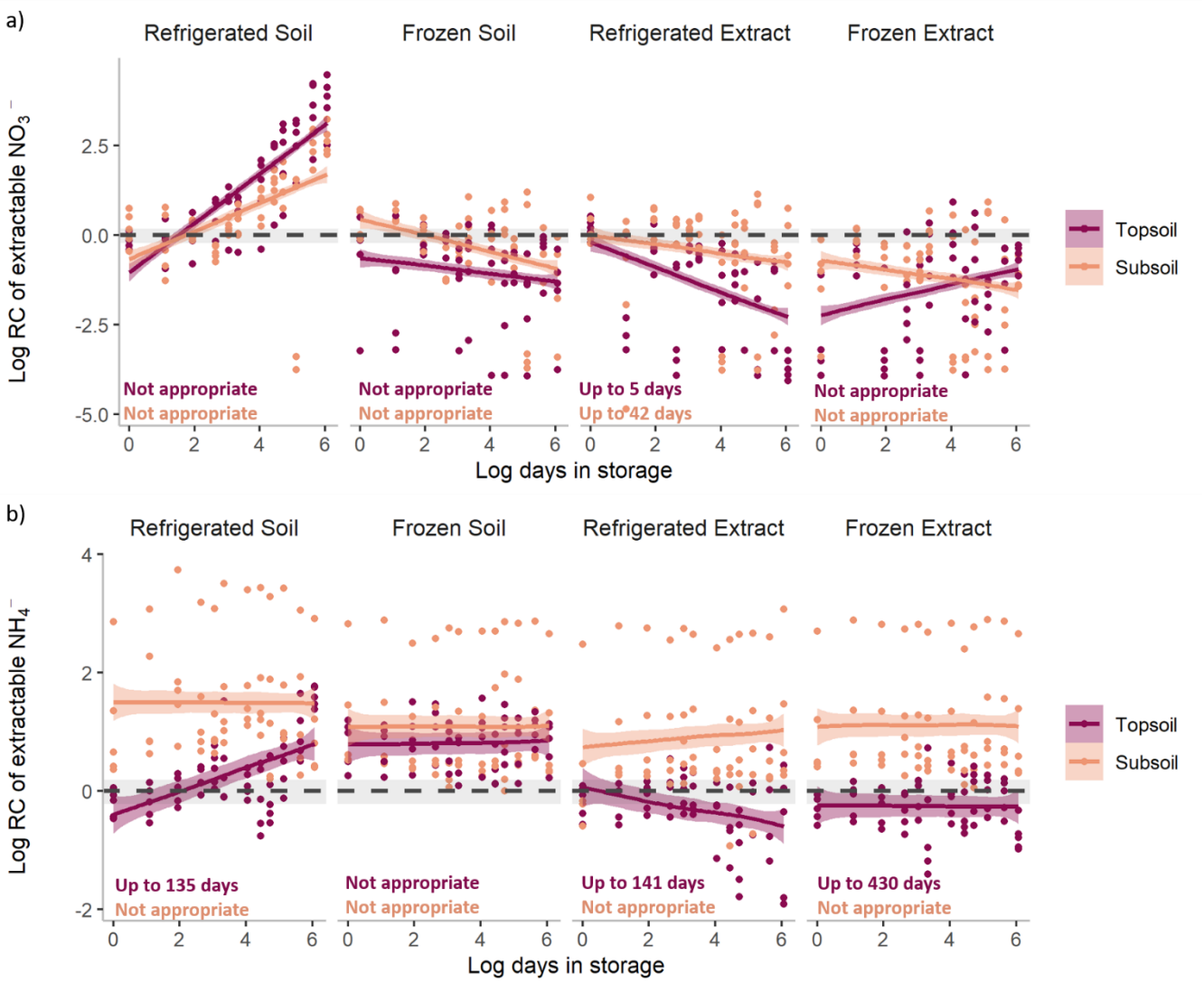
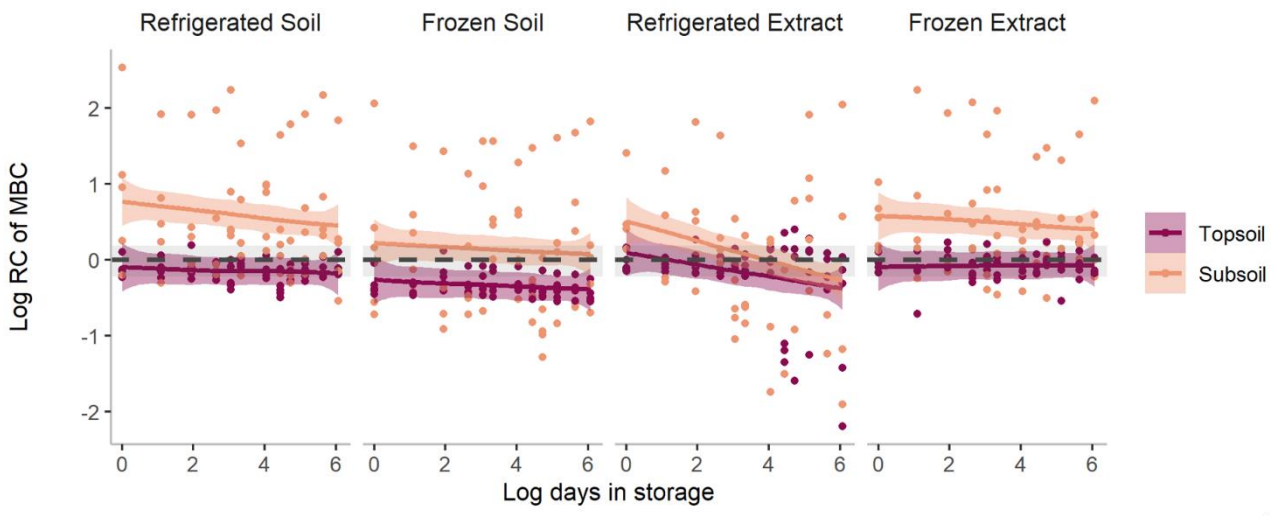
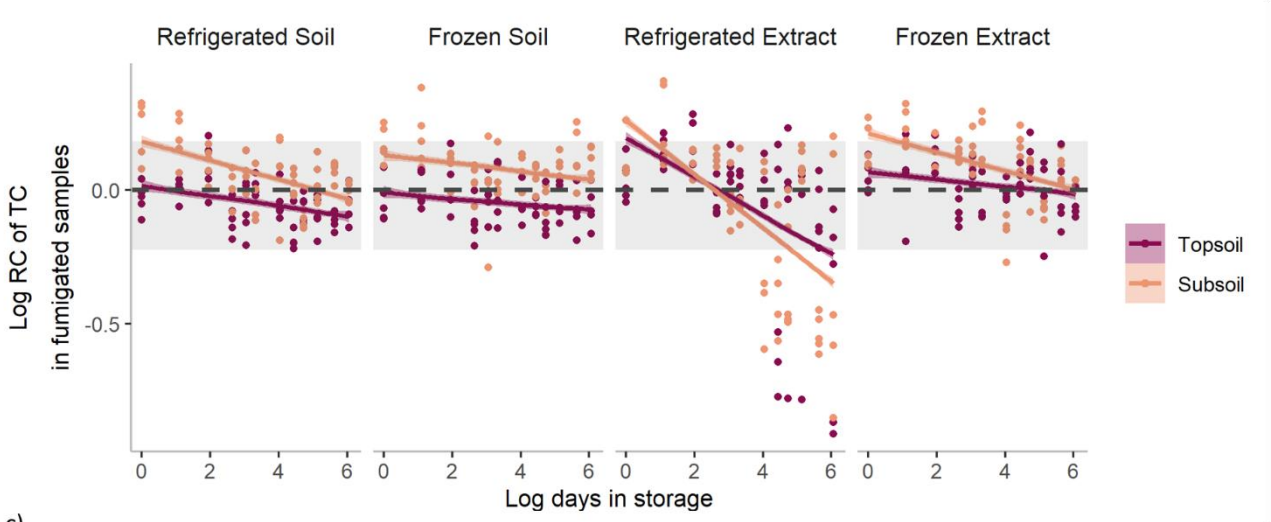
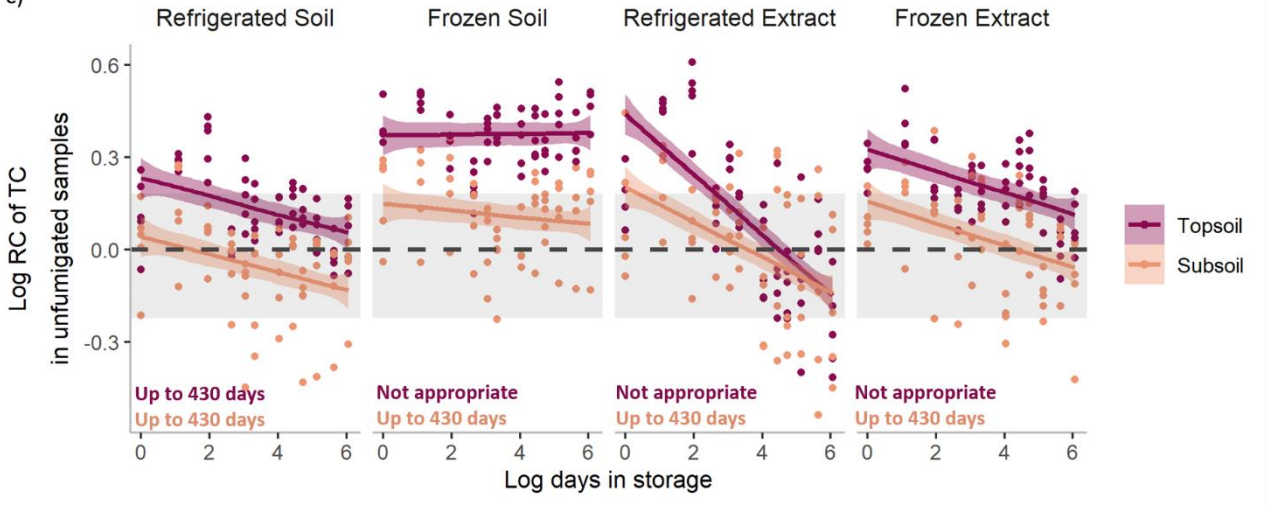


Figure S4 The relative change (RC, log transform) of extractable a) NO_3^- and b) NH_4^+ extracted with KCl along storage time (days, log transform) in storage for each soil depth within each storage treatment. Points represent calculated relative change for individual replicates. The trend lines represent the predictive fitted ratio change values based on the mixed effects models, with coloured shaded areas represent 95% upper and lower confidence intervals for fitted values. The grey shading represents our similarity limits. Similarity is no longer met when both the upper and lower limit of the fitted values (coloured shading) extend outside of the grey shading. The appropriate number of storage days for each soil depths and storage method is annotated on the graph.


Refrigerated Soil
Frozen Soil
Refrigerated Extract
Frozen Extract
Log RC of MBC
Log days in storage
Topsoil
Subsoil

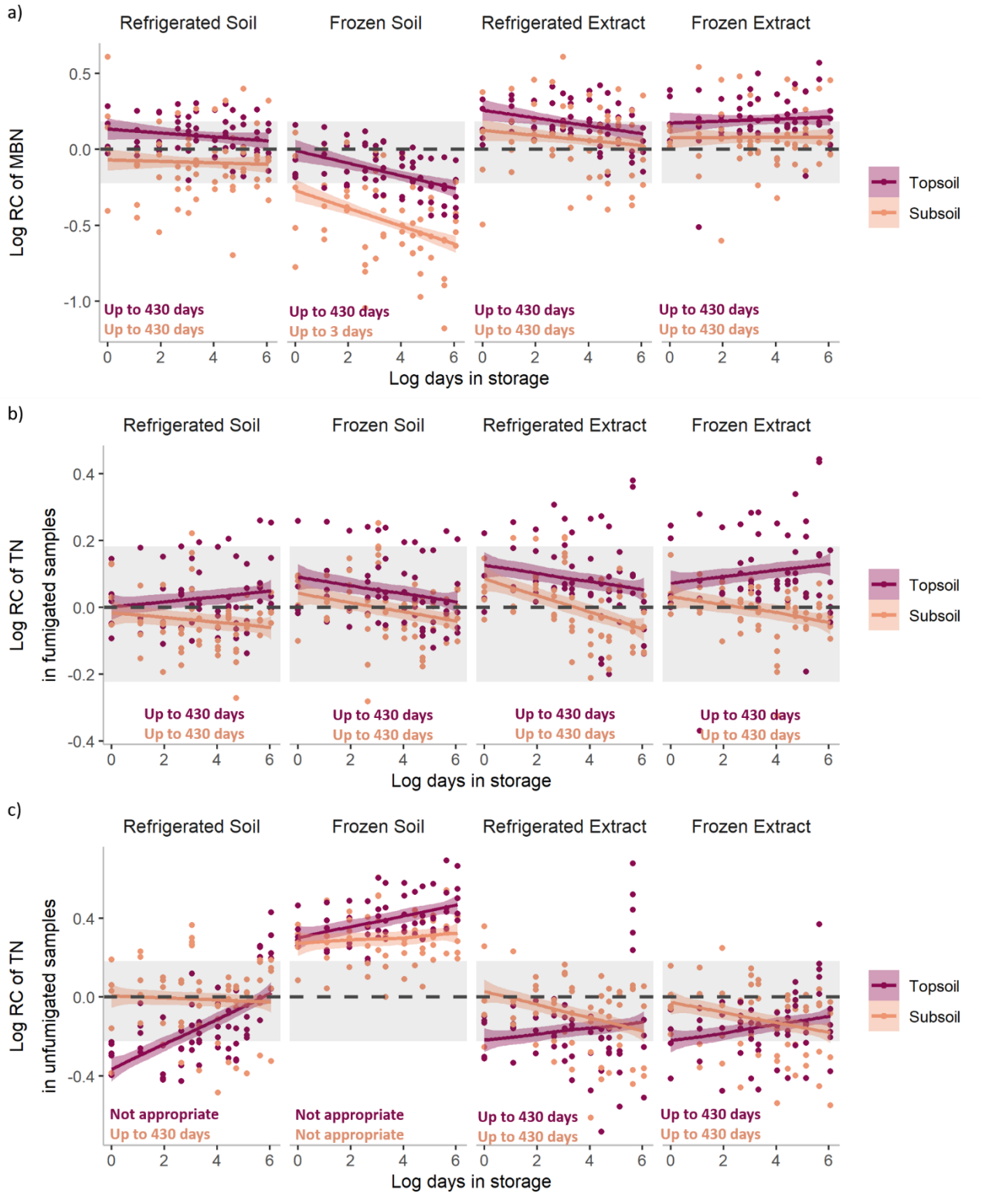

Refrigerated Soil
Frozen Soil
Refrigerated Extract
Frozen Extract
Log RC of TC
in fumigated samples
Log days in storage
Topsoil
Subsoil

c)


Refrigerated Soil
Frozen Soil
Refrigerated Extract
Frozen Extract
Log RC of TC
in unfumigated samples
Up to 430 days
Up to 430 days
Not appropriate
Up to 430 days
Not appropriate
Up to 430 days
Not appropriate
Up to 430 days
Log days in storage
Topsoil
Subsoil

175

Figure S5 The relative change (RC, log transform) of a) microbial biomass carbon b) total carbon in fumigated and c) unfumigated K_2SO_4 extracts along storage time (days, log transform) for each soil depth within each storage treatment. Colour distinguishes between soil depths where red is representative of topsoil and blue subsoil. Points represent calculated relative change ratios for individual replicates. The trend lines represent the predictive fitted ratio change values based on the mixed effects models, with coloured shaded areas represent 95% upper and lower confidence intervals for fitted values. The grey shading represents our similarity limits. Similarity is no longer met when both the upper and lower limit of the fitted values (coloured shading) extend outside of the grey shading. The appropriate number of storage days for each soil depths and storage method is annotated on the graph.


a)
Refrigerated Soil
Frozen Soil
Refrigerated Extract
Frozen Extract
Log RC of MBN
0.5
0.0
-0.5
-1.0
Up to 430 days
Up to 430 days
Up to 430 days
Up to 3 days
Up to 430 days
Up to 430 days
Up to 430 days
Up to 430 days
0
2
4
6
Log days in storage
Topsoil
Subsoil
b)
Refrigerated Soil
Frozen Soil
Refrigerated Extract
Frozen Extract
Log RC of TN
in fumigated samples
0.4
0.2
0.0
-0.2
-0.4
Up to 430 days
Up to 430 days
Up to 430 days
Up to 430 days
Up to 430 days
Up to 430 days
Up to 430 days
Up to 430 days
0
2
4
6
Log days in storage
Topsoil
Subsoil
c)
Refrigerated Soil
Frozen Soil
Refrigerated Extract
Frozen Extract
Log RC of TN
in unfumigated samples
0.4
0.0
-0.4
Not appropriate
Up to 430 days
Not appropriate
Not appropriate
Up to 430 days
Up to 430 days
Up to 430 days
Up to 430 days
0
2
4
6
Log days in storage
Topsoil
Subsoil

185

Figure S6 The relative change (RC, log transform) of a) microbial biomass nitrogen b) total nitrogen in fumigated and c) unfumigated K_2SO_4 extracts along storage time (days, log transform) for each soil depth within each storage treatment. Colour distinguishes between soil depths where red is representative of topsoil and blue subsoil. Points represent calculated relative change ratios for individual replicates. The trend lines represent the predictive fitted ratio change values based on the mixed effects models, with coloured shaded areas represent 95% upper and lower confidence intervals for fitted values. The grey shading represents our similarity limits. Similarity is no longer met when both the upper and lower limit of the fitted values (coloured shading) extend outside of the grey shading. The appropriate number of storage days for each soil depth and storage method is annotated on the graph.

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