Supplementary material

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

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Extended material and methods

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.

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 S 1 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). 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.

Stored extract treatments

Samples were extracted (methods described in section S1.2) from soils directly after homogenisation and then stored 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.

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.

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.

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 calculated as $mg^{-1}\,kg^{-1}$ of dry soil for each variable within each extract type.

Extractant	Soil weight (g)	Volume (ml)	Shaking time (min)	Time centrifuged (min)	Filter
Ultrapure water	5	35	10	30	0.45 µm syringe
KCl	5	25	60	5	Whatman 42 *
K_2SO_4	5	25	30	5	Whatman 42 *

Table S 2 Summary of extraction methods used in this experiment

* 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).

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

Statistical analyses

All statistical analyses were carried out in R (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:

$$Relative\ change\ = \frac{Measured\ variable\ for\ each\ treatment}{Measured\ variable\ from\ fresh\ sample}$$

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 (Knowles et al., 2016).

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 mathematically possible (DOC), a ratio of 1 signifies no change from fresh samples, and the similarity limits were set between 0.8 and 1.2.

We utilised WebPlotDigitizer (Rohatgi, 2019) a web-based tool to extract numerical data from plots, to determine when the upper or lower confidence intervals of our predicted fitted model values extend beyond the 20% similarity limits.

Results

Overall, we found significant impacts of storage method and duration of both topsoil and subsoil on several response variables. In topsoil, we found that refrigerating soils, freezing extracts up to 430 days, and refrigerating extracts up to 10 days successfully maintained similar DOC concentrations to those from fresh samples (Fig. S1a). Freezing soils always resulted in dissimilar DOC concentrations to fresh samples regardless of storage duration. DOC concentrations increased immediately after freezing and continued to increase over time. With regard to subsoil, freezing soils, refrigerating extracts up to 430 days, and refrigerating soils up to 8 days successfully maintained similar values to fresh samples, but freezing extracts led to significantly different DOC concentrations compared to fresh samples (Fig. S1a).

DON concentrations in water extracts from topsoil stored for up to 281 days in the refrigerator or freezer were similar to those of fresh samples (Fig. S1b). DON concentrations in stored topsoils were unaffected by refrigeration soils for up to 60 days, while freezing topsoils changed DON concentrations relative to fresh samples throughout the experiment. DON concentrations increased immediately after freezing and continued to increase with storage duration, as observed for DOC. For subsoils, refrigerating soil samples up to 3 days was deemed to be the only storage method to yield similar results to the fresh samples, with all other storage treatments of any duration yielding dissimilar results (Fig. S1b).

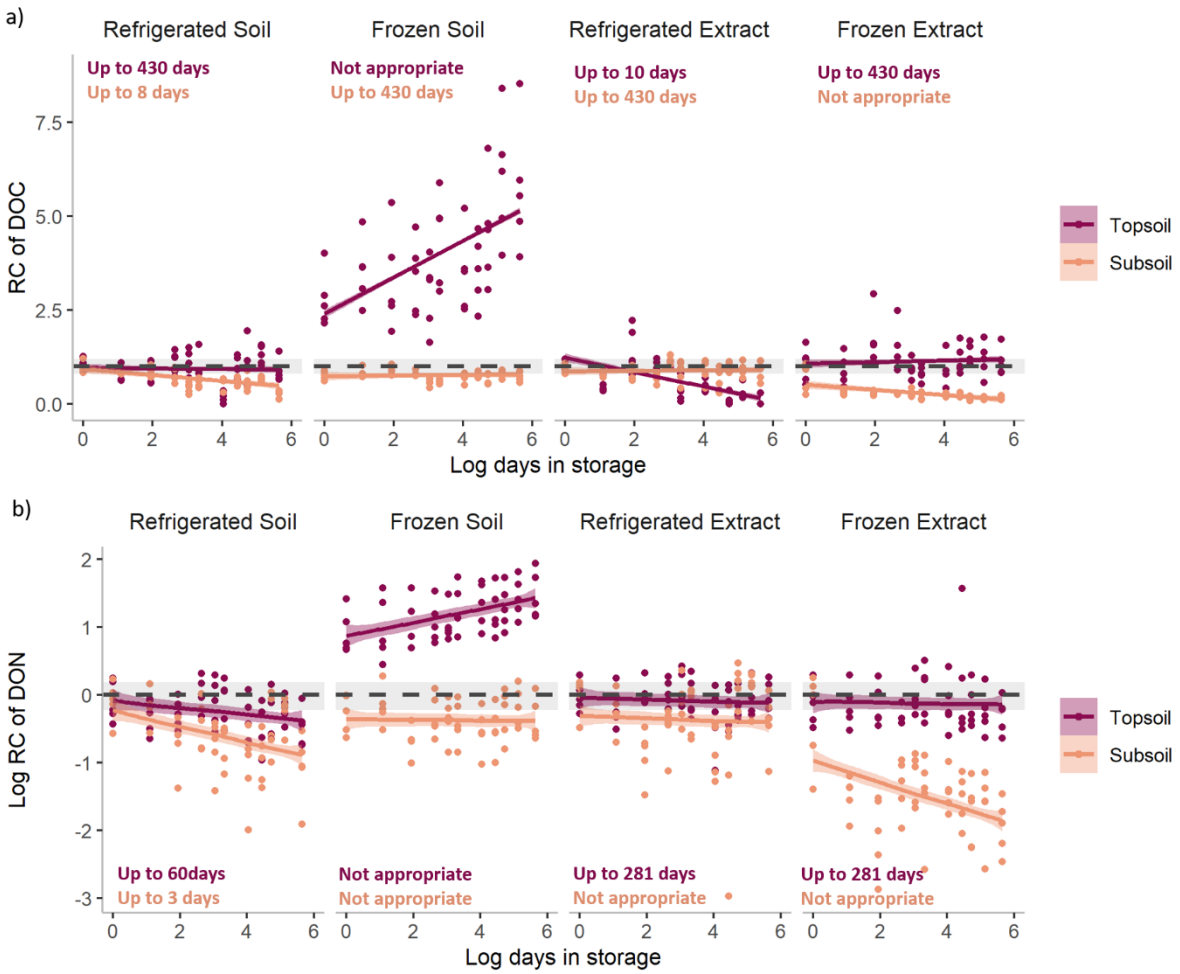

Figure S 1 The relative change (RC) of a) DOC and b) DON (log transform) along storage time (days, log transform) for both soil depths for each storage treatment. Points represent calculated relative change ratios (compared to fresh sample) for individual replicates. The trend lines represent the predictive 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).

DOC extracts from blank (ultrapure water) samples used for blank corrections only differed with storage length when stored in the refrigerator, where DOC concentrations increased with increased storage length doubling its concentration after 430 days (Fig. S2).

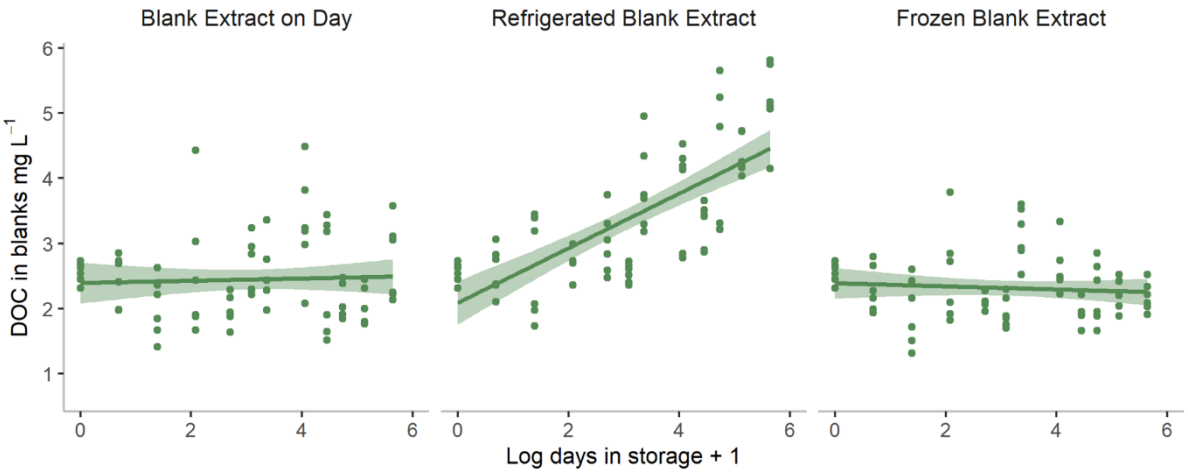

Figure S 2 Concentrations of DOC in blank samples either extracted on the day of analysis, refrigerated, or frozen and then analysed over the storage time. Points represent concentrations for individual replicates. Mixed effects models are represented by trend lines, where shaded areas represent 95% upper and lower confidence intervals.

All storage types were inappropriate for analysis of extractable NO_3^- in both soils, apart from refrigerating extracts up to 5 days and 42 days for topsoil and subsoil, respectively (Fig. S3a). There were no storage methods that were deemed appropriate for measuring extractable NH_4^+ in subsoils (Fig. S3b). However, refrigerating soils and extracts, and freezing extracts up to 135, 141 and 430 days from topsoil yielded NO_3^- concentrations similar to those in fresh samples. By contrast, freezing soils was not appropriate for any storage length in topsoil (Fig. S3b).

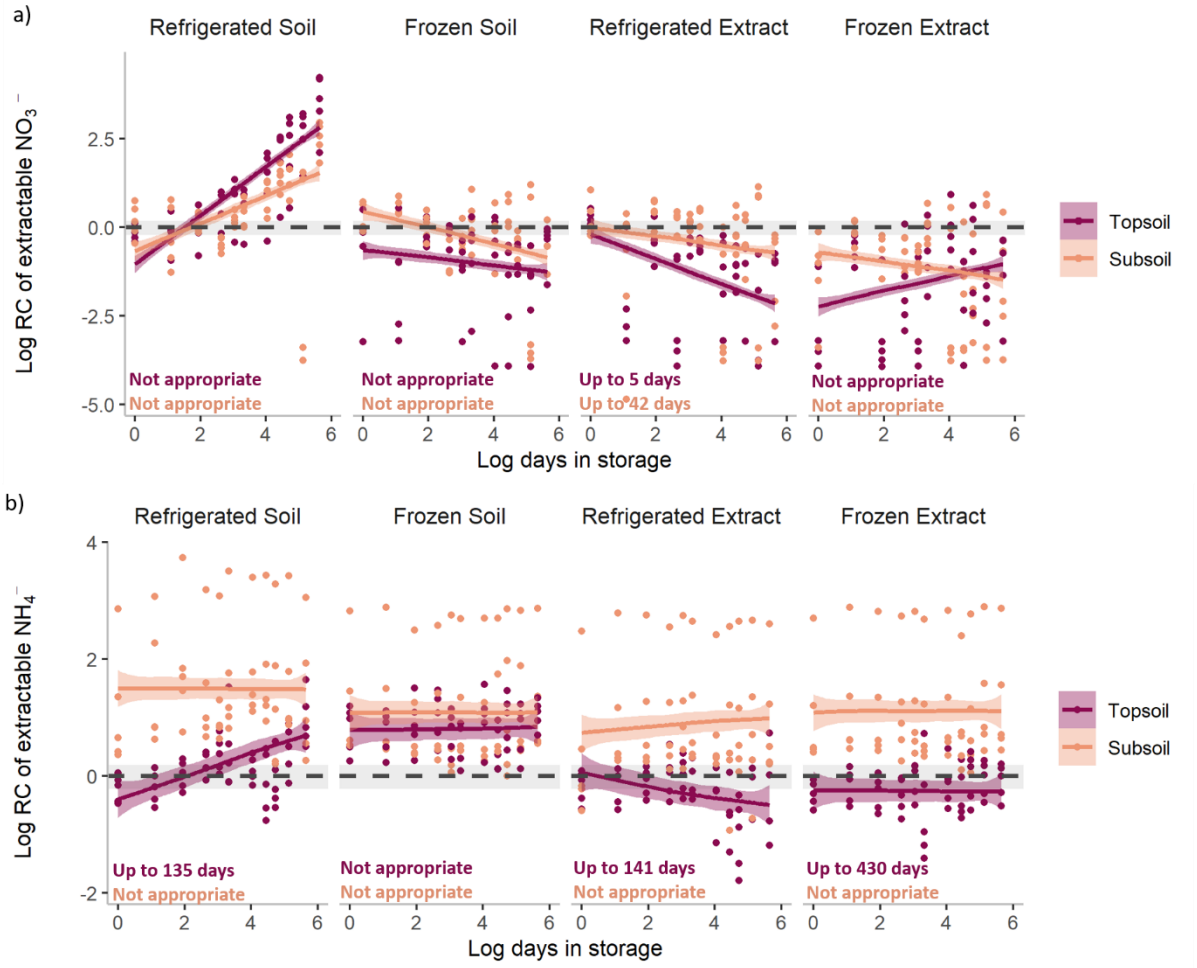

Figure S 3 The relative change (RC, log transform) of extractable a) NO_3^- and b) NH_4^+ extracted with KCl along storage time (days, log transform) in storage for each soil depth within each storage treatment. Points represent calculated relative change for individual replicates. The trend lines represent 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.

Subsoil MBC did not differ from fresh soil when soils were frozen for up to 430 days (Fig. S4a), while every other storage treatment did within just one day of storage. By contrast, MBC in topsoil was similar to fresh samples in refrigerated soils, refrigerated extracts and frozen extracts up to 430 days, and in frozen soils up to 75 days. However, separate evaluation of the fumigated and unfumigated samples revealed differences (Fig. S4b, c). Fumigated extracts were comparable to fresh samples in all storage methods for topsoil, but only when soil was stored (either in the refrigerator or frozen) for subsoils (Fig. S4b). For both soils, TC generally decreased in the fumigated refrigerated extracts with long storage times (starting after 3 months

of storage), at least for most replicates. Unfumigated extracts were only comparable to the fresh samples in topsoil if the soil was refrigerated, while all storage methods were comparable to the fresh samples up to 430 days in subsoil (Fig. S4c).

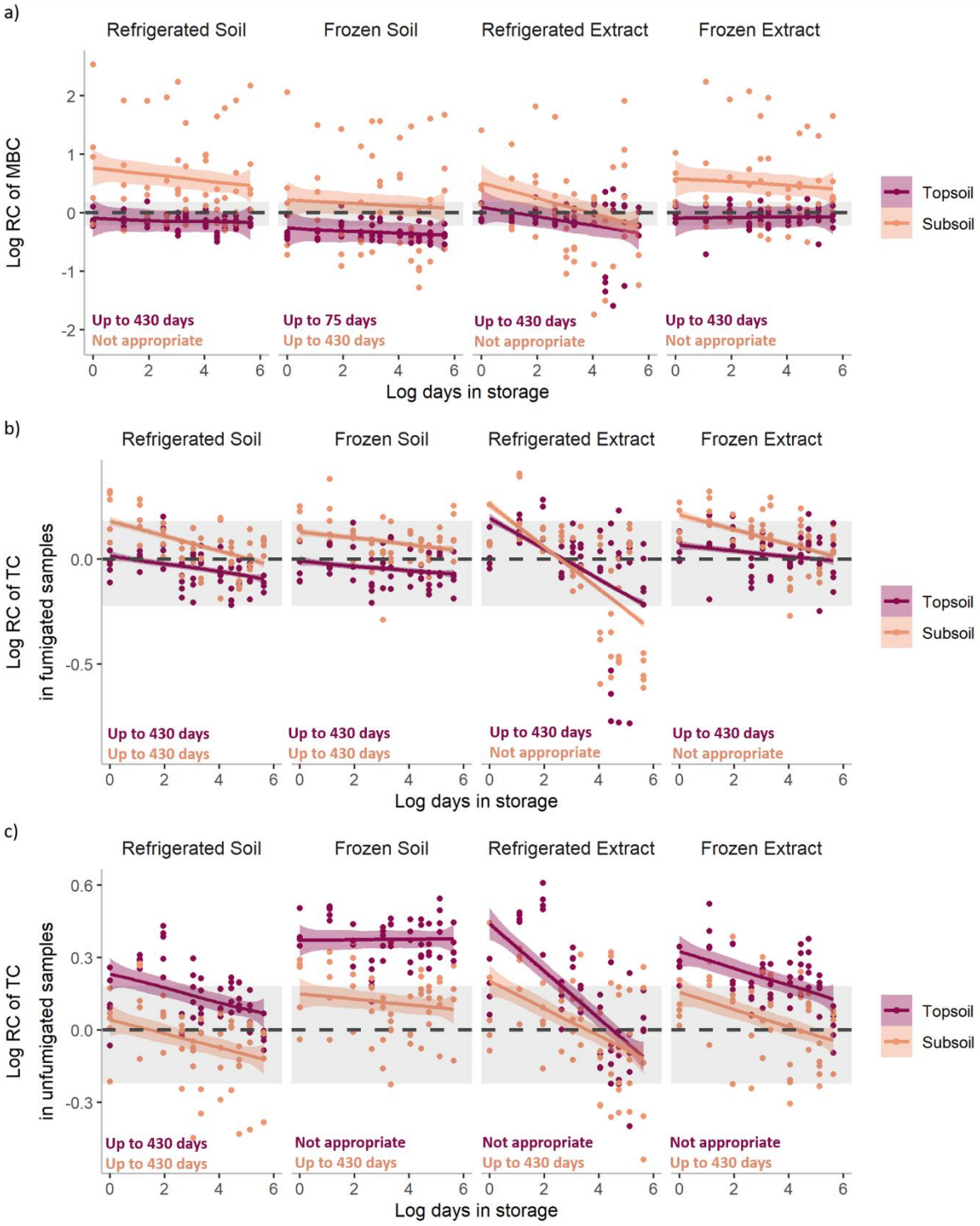

Figure S 4 The relative change (RC, log transform) of a) microbial biomass carbon b) total carbon in fumigated and c) unfumigated K_2SO_4 extracts along storage time (days, log transform) for each soil depth within each storage treatment. Colour distinguishes

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.

MBN data were comparable to the fresh measurements for both soils and all storage types, except for the frozen soil from subsoils (Fig. S5a). As for MBC, fumigated and unfumigated extracts did not follow the same trend. TN in fumigated extracts was comparable to the fresh for both soils and for all storage times (Fig. S5b). However, TN in unfumigated extracts showed more variability (Fig. S5c). Storing extracts was an appropriate storage method for both soils, but storing subsoil only deemed appropriate when stored in the refrigerator. Freezing soil led to an immediate increase of TN in both soil depths.

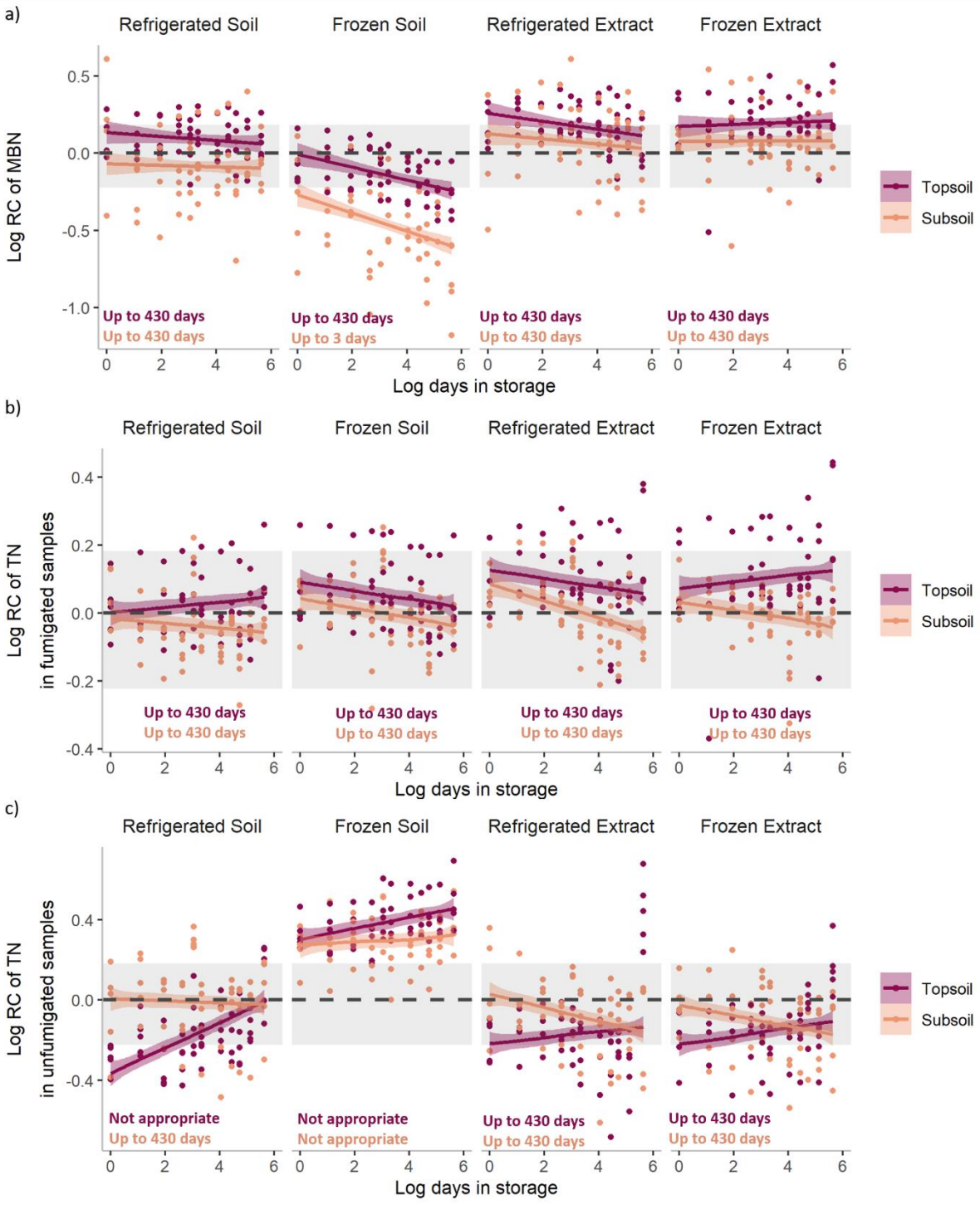

Figure S 5 The relative change (RC, log transform) of of a) microbial biomass nitrogen b) total nitrogen in fumigated and c) unfumigated K_2SO_4 extracts along storage time (days, log transform) for each soil depth within each storage treatment. Colour distinguishes between soil depths where red is representative 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.

Extended discussion and recommendations based on findings

Storing soils

Refrigerating sieved soils for up to 3 days was deemed the most appropriate storage method for the quantification of DOC and DON in both topsoil and subsoil. Rolston and Liss (1989) recommended to freeze soils if storage is required; by contrast, for the quantification of DOC, we found freezing sieved soils to result in the largest shifts in DOC and DON concentrations. Topsoil DOC and DON concentrations increased beyond comparison with fresh samples within just one day of freezing. A combination of factors associated with increasing labile C and N availability from a freeze-thaw cycle were likely to have contributed to these results, including the release of DOC and DON from microbial death (Černohlávková et al., 2009), a change in soil structure (van Bochove et al., 2000) and root decomposition (Tierney et al., 2001). However, shifts in DOC and DON concentrations also persisted with longer storage length implying that there are other factors contributing to these shifts beyond those related to the freeze-thaw process.

Storing refrigerated soils was the least appropriate method for the quantification of extractable N, as NO_3^- concentrations increased considerably and continued to increase with storage time in both topsoil and subsoil. This was likely due to a combination of: 1) the inability of refrigerated temperature to stop mineralisation (Tyler et al., 1959); 2) increased rates of N mineralisation after sieving (Hassink, 1992); and 3) reduced NO_3^- uptake by plants due to plant removal. This is supported by our observed decrease in soil DON concentration.

In general, refrigerating soils was an appropriate storage method to evaluate MBC and MBN, in line with the findings of Černohlávková et al. (2009). However, microbial biomass may be calculated inappropriately as an artefact of divergent changes in fumigated and unfumigated samples incurred from storage treatments and therefore requires both fumigated and unfumigated extraction samples to meet similarity limits. Contrary to recommendations to freeze soils as an appropriate storage method to quantify microbial biomass (Stenberg et al., 1998), we found that freezing soils generally increased extractable C and N concentrations in unfumigated extracts, but did not affect concentrations in fumigated samples. This suggests freezing caused some microbial death (Černohlávková et al., 2009) precluding reliable quantification of microbial biomass using fumigation. Refrigerating soil for the quantification of C in unfumigated soil was appropriate for up to 430 days, yet deemed inappropriate for N in topsoil. Generally, topsoils are susceptible to more storage-related changes than mineral soils (Lee et al., 2007), as a result of their greater microbial biomass. In this instance, topsoil had 720 % greater MBC and 390% greater MBN than subsoil making them more susceptible to nutrient turnover (Schnecker et al., 2015), where increased mineralisation from sieving may have contributed to this (Hassink, 1992).

Storing Extracts

Although refrigerating extracts for the quantification of DOC was appropriate for up to 10 days, we identified an underlying issue with longer periods of this storage method as blank extracts accumulated DOC over time when stored in the refrigerator. We were unable to determine what may have caused this, but it highlights the importance in considering the implications of every methodological step within a procedure and the necessity to include blanks for analysis. For example, the potential leaching of DOC from the polypropylene tubes where the extracts were stored could have contributed to this as it has been demonstrated that plastic can leach DOC into the water, even if kept in the dark and under sterile conditions (Romera-Castillo et al., 2018). Freezing the sample might have prevented this leaching. In support of and in line with recommendations made by Rees and Parker (2005), we found that freezing topsoil extracts was an appropriate storage method throughout the duration of the experiment; however, this was not the case for subsoil.

Filtering extracts before storage can also pose issues with sample preservation. When extracts are filtered through pore sizes larger than 0.22 μm the sample is not sterilised, resulting in biologically active extracts that are susceptible to microbial transformations of C and N (Ghuneim et al., 2018; Wang et al., 2007). This issue is likely to have also contributed to NO_3^- losses in refrigerated KCl extracts, as denitrification is accelerated in anaerobic conditions, and the decreasing C trend with longer storage in both refrigerated and frozen fumigated and unfumigated K_2SO_4 extracts. This is supported by observations of fungal growth in many K_2SO_4 extracts after three months of extract refrigeration. Consequently, refrigerating extracts for up to 5 days proved to be the only viable option for the quantification of extractable NO_3^- for both topsoil and subsoil, contradictory to reports that recommend freezing KCl extracts for months (Jones and Willett, 2006; Li et al., 2012), or in some instances indefinitely (Heffernan, 1985). Furthermore, storing fumigated extracts of subsoils either in the refrigerator or freezer were also not appropriate storage methods for the quantification of MBC, despite recommendations to refrigerate extracts for up to 1-2 weeks (Vance et al., 1987) or at -18 ºC for an indefinite period (Beck et al., 1997).

Recommendations

Our results demonstrate that it is generally not advisable to store soils or soil extracts. However, we recognise that this is not always possible, and therefore recommend storing refrigerated soils for less than a week for the quantification of DOC and DON, up to 430 days for quantifying microbial biomass C and N, and refrigerating extracts for less than a week for the analysis of extractable inorganic N.

Additional figures

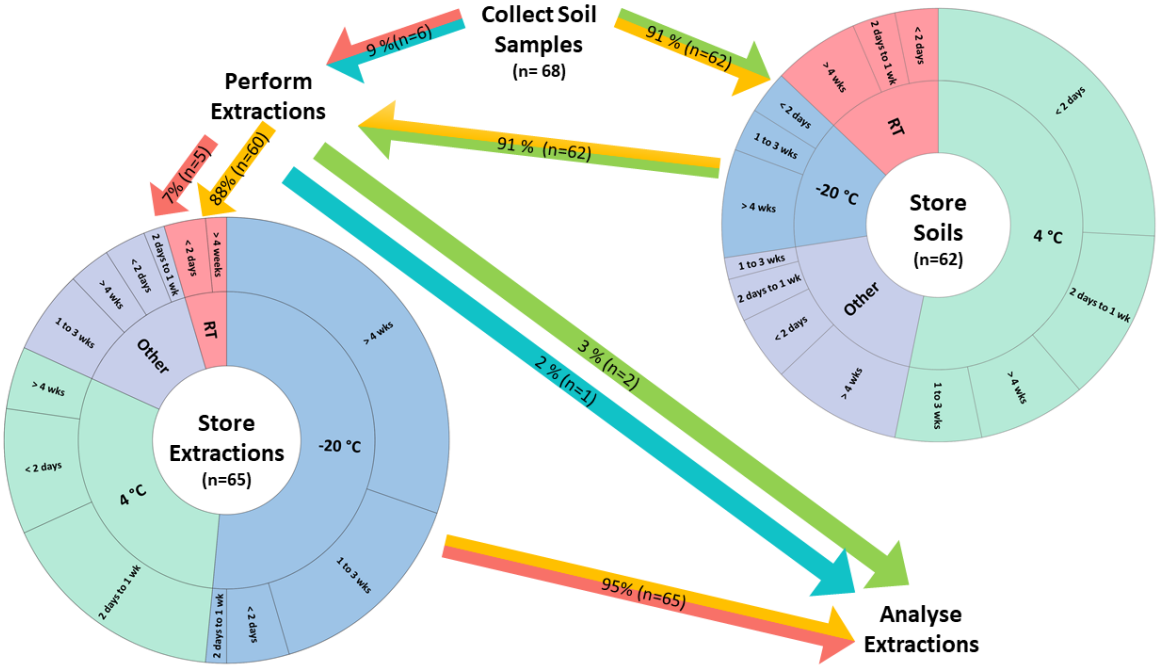

Figure S 6 – Flow chart indicating the results of an online survey to determine how and for how long people store soils and/or extracts prior to analysis. Survey date: December 2018 – June 2019. The proportion of people that followed each methodological step is indicated in each arrow.

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