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

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Abstract

It is widely accepted that the measurement of organic and inorganic forms of carbon (C) and nitrogen (N) in soils should be performed on fresh extracts taken from fresh soil samples. However, this is often not possible, and it is common practice to store samples (soils and/or extracts), despite a lack of guidance on best practice. Here, we demonstrate how differences in soil and/or soil extract storage can compromise sample integrity for the quantification of soil dissolved organic C and N, extractable inorganic nitrogen (NH_4^+ andNO_3^-), and microbial biomass C and N. We discuss and provide the appropriate tools that will ensure researchers consider best storage practice methods when designing and organising ecological research involving assessments of soil properties related to C and N cycling. We encourage researchers to use standardised methods where possible and to report their storage treatment (i.e. temperature, duration) when publishing findings on aspects of soil and ecosystem functioning. In the absence of published storage recommendations for a given soil type, we encourage researchers to conduct a pilot study and publish their findings.

Keywords: Soil, Sample Storage, Microbial Biomass C and N, Analytical Biogeochemistry

1 Introduction

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Biogeochemical cycles involve the turnover of essential nutrients between different organic and inorganic forms. For carbon (C) and nitrogen (N), many of these steps occur in the soil environment and hence the evaluation of different chemical forms of nutrients in soil is crucial to understand the recycling of nutrients and ecosystem functioning (Barrios, 2007; Datta, 2020; Robinson et al., 2014). While most soil biogeochemical analyses should ideally be carried out on fresh samples immediately after sampling (ISO18400-102:2017, 2017), this is not always possible due to the number of samples taken and the analytical procedures exceeding human and/or instrumental capabilities. In these cases, it is common practice to store samples for future analysis.

Soil extraction procedures are commonly used to quantify different biochemical parameters in soils. Typically, such procedures shake soils with a high soil weight-to solution volume ratio and separate the solution phase from the solid phase by centrifugation and/or filtration (Kachurina et al., 2000). This process poses further storage opportunities for future analysis, irrespective of how soils were initially stored. However, recommendations for both soil and/or soil extract storage vary substantially, and little is known about the impact storage methods may have on sample integrity.

Dissolved organic C and N are commonly extracted from soils with water (Forster, 1995). However, in cases where inorganic N is also being quantified, concentrated salt extractions, such as KCl, are used to evaluate 'plant available' N (Forster, 1995; Jones and Willett, 2006; Keeney and Nelson., 1982). Methodological factors for both extraction types differ substantially (Jones and Willett, 2006; Ros et al., 2009). In many comparative studies exploring the impacts from methodological factors overlook soil and/or extract storage temperatures and duration, and those that have considered these have considered few variables (Table 1) (e.g. Jones and Willett, 2006; Lee et al., 2007).

Table 1 –Summary of different recommendations for storage of soil or extract samples to measure soil nutrients found in the literature. This summary is non-exhaustive.

Variable evaluated	Extractant used	Soil type	Study	Recommendation based on	Storage methods explored	Storage recommendations	Limitations
Water extractable organic carbon	H_2O	Not applicable	Gregorich and Carter, 2007	No evidence provided		Minimal time, refrigerated maybe ideal	
		Loam, sandy loam, sandy clay	Rees and Parker, 2005	Comparative study	Extracts only at 4 °C, -18 °C and room temperature	Store extracts at 4 °C for 1 week. Store extracts frozen at -18 °C for 3 months Do not store extracts at room temperature	
		Yolo loam, which is a member	Rolston and Liss, 1989	Comparative study	Soils only stored as air dried and frozen	Store soils at -18 °C if storage is required	No recommendation for length of storage

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		of the fine, silty, mixed, nonacid, thermic, typic, Xerorthents family					
Plant available N	KCl	Not applicable	Hefferna n, 1985	No evidence provided		Store extracts at -18 ºC indefinitely	
		Not provided	Li et al., 2012	Comparative study	Extracts only at 4 °C, -18 °C and room temperature	Analyse as soon as possible Store extracts at -18 °C and analyse as soon as possible	Storage length explored up to 6 weeks only
		Unclear as to which soil type used from 8 types. All located in temperate, oceanic locations.	Jones and Willett, 2006	Comparative study	Extracts only at 4 °C and 18 °C	Carry out soil extractions within 24 hours of collection Store extracts for days in the refrigerator Store extracts at -18 ºC for moths	Broad recommendations for storage length given
		Not applicable	Gregoric h and Carter, 2007	No evidence provided		Minimal time, refrigerated maybe ideal	
Microbial biomass	K_2SO_4	Arable sandy loam soil, grassland orchard soil and mixed forest soil with high organic carbon content	Černohlá vková et al., 2009	Comparative study	Soils only stored at 4 °C, -20 °C and air dried	Store sieved soil at 4 °C for up to 8 weeks	
		Not applicable	Vance, Brookes and Jenkinso n, 1987; Beck et	No evidence provided		Store extracts indefinitely at -18 ºC	

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			al., 1997; Coleman, Callaham and Crossley Jr, 2017				
		Agricultural mineral	Stenberg et al., 1998	Comparative study	Soils only at 2 °C and -18 °C	Store soils at -18 °C for up to 13 months	Extracts were also frozen at -20 ºC until analysed with no account for storage length
		Not applicable	Gregorich and Carter, 2007	No evidence provided		Minimal time, refrigerated but not frozen	

For example, a meta-analysis exploring methodological factors that impact soil extractable organic N did not account for soil or extract storage length, despite showing impacts from soil storage temperatures and soil extract temperatures (Ros et al., 2009). Nevertheless, while recommendations for storage of soil, as well as water and KCl extracts are reported, they are in many cases vague with no indication as to when samples deteriorate beyond usability, highlighting the need for comparative studies.

Microbial biomass C and N are commonly quantified using fumigation-extraction methods (Brookes et al., 1985; Vance et al., 1987). In their classic paper, Vance et al. (1987) recommended that K_2SO_4 extracts should be analysed immediately, and where this is not possible, stored for up to 2 weeks at 1-2 °C. However, these authors did not give any recommendations for storing soil samples prior to extraction, which is also commonly practiced. Nonetheless, many studies have since modified the Vance et al. (1987) and Brookes et al. (1985) methods, which has led to substantial variation in practice and storage of soil and extracts (Table 1). To the best of our knowledge, only the recommendations of Stenberg et al. (1998) and Černohlávková et al. (2009) were based on comparative studies of different storage methods, whereby sample integrity was best preserved when fresh soils were stored at -20 ºC for up to 13 months or when sieved soils were stored at 4ºC for up to 8 weeks, respectively. Despite these findings, Stenberg et al. (1998) still stored the extracts of both soil storage treatments at -20 ºC until analysis and made no account for storage length.

We highlight that recommendations for storage methods are vague and that there is a lack of comparative studies to determine best storage practices for the quantification of soil DOC, DON, inorganic nitrogen and microbial biomass, which are all commonly measured in ecological studies considering aspects of soil and ecosystem functioning. We also explored common practices across different laboratories with an online survey, which suggests that storage of both soils and extracts is common practice (Fig. S6). Generally, the storage of soil was done at 4 °C for a short period of time (<1 week), while extracts were stored at -20 °C and for longer (> 4 weeks, Fig. S6). Nonetheless, storage methods varied significantly, highlighting the need for common protocols to standardize methods across laboratories.

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In this commentary, we report a study that aimed to identify the best practice methods for storage of soil or soil extracts for the analysis of soluble pools of C and N and microbial biomass in soil. The study, which was based on both topsoil and subsoil of a well-characterised experimental grassland site that has been used in recent ecological studies (Leff et al., 2018; De Long et al., 2019), served to demonstrate how different, widely used storage methods can affect sample integrity. It also provides the tools required by researchers to determine best storage practice for their own studies, given that optimal storage methods will vary across different soils and ecosystem types. We encourage researchers to carry out their own pilot studies, for which our study provides an example and guidelines for.

2 Case Study

2.1 Brief description of methods and experimental design

Our study aimed to determine best practice methods for storage of soil or soil extracts for the analysis of dissolved organic C (DOC), dissolved organic N (DON), inorganic N (NO_3^-, NH_4^+), and soil microbial biomass (MBC and MBN). This was tested on both topsoil (0-20 cm) and subsoil (20-30 cm) of a brown earth (Cambisol) taken from a well-studied experimental grassland site (De Long et al., 2019; Leff et al., 2018; Table S1), which is representative of typical permanent grasslands used for livestock production across the UK and parts of Europe (Rodwell, 1992). We designed a full factorial experiment with both topsoil and subsoil, two different types of stored samples (soil or extract), and two different storage temperatures (4°C or -20 °C), replicated five times. 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 a 'baseline' comparison value (amounted to 1,952 extractions in total).

In order to standardize the relative change of each variable measured for each soil type, 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 to test the effects of fixed factors (soil type, storage type and storage length) and random factor (replicate) and their interactions on the calculated relative change ratio from fresh samples (baseline). Predicted fitted values from the multi-level model were calculated with *predictInterval* with the *merTools* package (Knowles et al., 2016).

Similarity between fresh samples (baseline) 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 (baseline); we refer to these as similarity limits (Rita and Ekholm, 2007; Wallenius et al., 2010). For further detail on sample collection and

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preparation, storage treatments, extraction procedures and statistical analysis please read our full study description in the supplementary material provided.

2.2 Key findings

Our study provides strong evidence that storing soils and extracts can have significant consequences for the quantification of soluble C and N pools of relevance to key ecosystem processes. These findings are important given increasing emphasis on the need to understand soil processes as regulators of ecosystem services (Coe and Downing, 2018; Dangi, 2014), and calls for standardised and robust indicators of soil health made in recent policy interventions (DEFRA and EA, 2018), where consistency in protocols across studies and measurements is essential.

Overall, we found significant impacts of storage method and duration demonstrating that it is generally not advisable to store soils or soil extracts. Nonetheless, through appropriate experimental design we were able to determine a limited range of storage type and storage length recommendations for both topsoil and subsoil (Table 2).

Table 2. Storage method recommendations for both topsoil and subsoil. Red squares denote inappropriate storage methods for specific analysis. Green denotes appropriate storage method with additional recommendations for storage length. Where we do not specify, stored samples did not differ from fresh samples through the entire experiment, 430 days.

Measured Variable			**Topsoils**			**Subsoils**		
			Extractable Dissolved Organic N and C	Inorganic N	Microbial Biomass	Extractable Dissolved Organic N and C	Inorganic N	Microbial Biomass
Extractant			Water	KCl	K_2SO_4	Water	KCl	K_2SO_4
Storage Type	Soil		<1 month		<430 days	<1 week		<430 days
								
	Extract		10 days	<1 week				
			<430 days					
Dark grey denotes inappropriate storage method and light grey appropriate.								

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We found that storing soil and extracts by freezing at -20 ºC was generally least effective at maintaining measured values of fresh material. Appropriate storage recommendations include refrigerating (4 °C) brown earth soils for less than a week for DOC/DON, up to a year for MBC/MBN, and refrigerating extracts for less than a week for NH_4^+/NO_3^-.

It is commonly assumed that shifts in concentrations of nutrients as a result of sample storage will occur more or less equally for all soil types. While we only considered soil from one experimental grassland, the appropriateness of different storage treatments varied between topsoil and subsoil, which highlights the need to consider appropriate storage methods based on soil type and soil properties through rapid review methods and/or pilot studies which we discuss in section 3. Such effects were likely due to differences in physical and chemical properties of soils at different depths, and lower substrate availability with increasing depth (Bardgett et al., 1997) resulting in smaller microbial biomass (Lavahun et al., 1996), reduced microbial activity (Schnecker et al., 2015), and a decreased capacity for substrate utilisation (Kennedy et al., 2005). We discuss the potential mechanisms that compromise sample integrity of soil in supplementary material (S.4)

Our study highlights the need to explore storage effects on other soil types, and ideally to determine the mechanisms leading to changes in nutrient concentrations with storage. Although we found it to be unadvisable to store soil extracts, this procedure may be appropriate if samples are sterilised or stored in conditions that completely supress microbial activity, which is likely to be one of the main mechanisms leading to changes in nutrient concentrations. For example, adding acid prior to storage (Zagal, 1993) or microbial inhibitors (Rousk and Jones, 2010) has been suggested, but this may not be compatible with instrumentation and the quantification of inorganic nutrient pools, and requires further investigation.

3 How to determine best storage practice for your experiment

We provide a step by step systematic flow chart to determine best storage methods for soil and soil extracts (Figure 1).

Figure 1 Schematic flowchart depicting necessary steps to determine best storage practices for soil and soil extracts in ecological studies.

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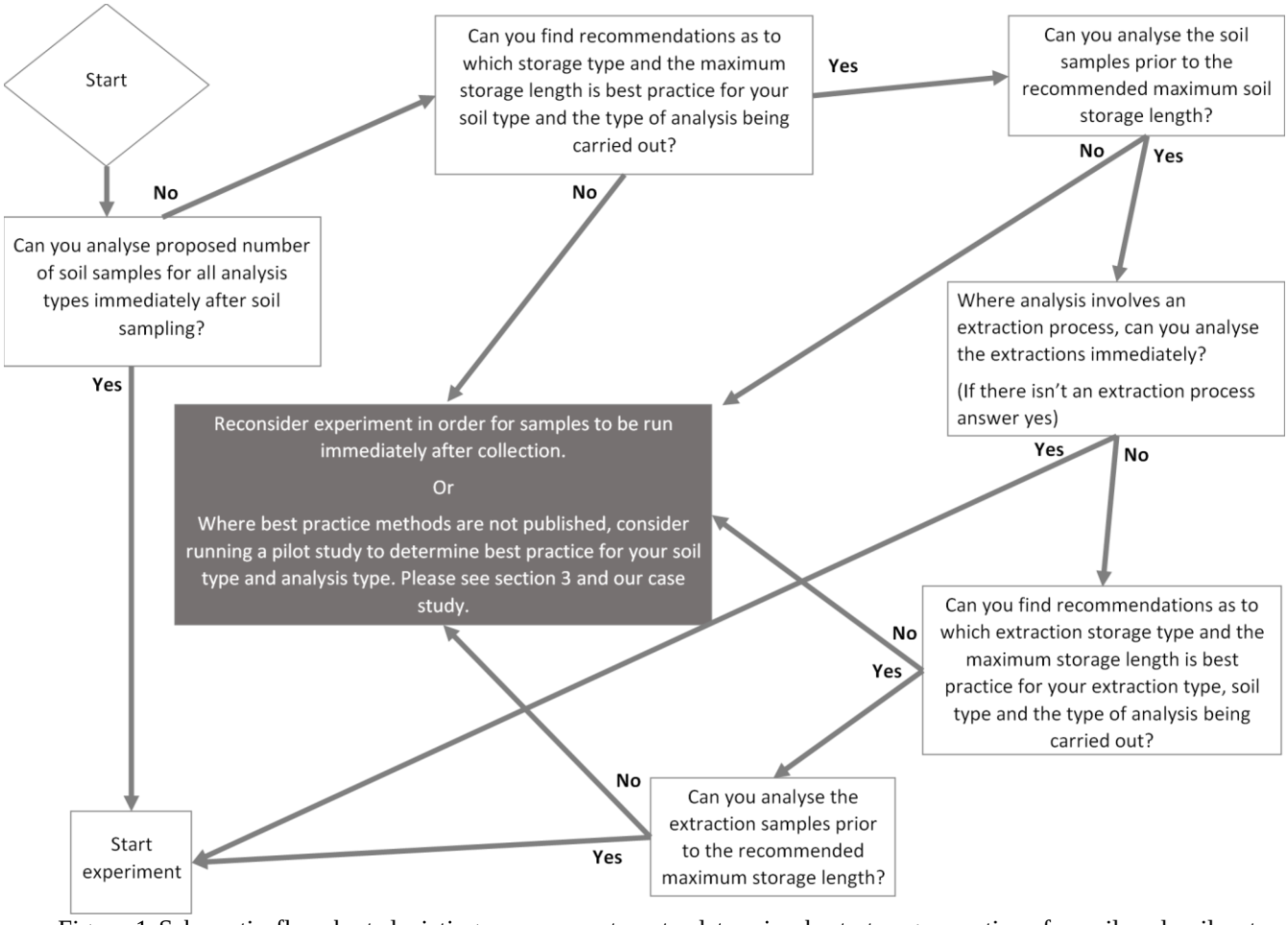

Figure 1 Schematic flowchart depicting necessary steps to determine best storage practices for soil and soil extracts in ecological studies.

Where there are publications outlining best soil storage practices, ensure recommendations are based on comparative studies carried out on the correct soil type. Where published recommendations are not found, we advise researchers to carry out a targeted pilot study using less extensive yet similar approaches to that outlined in our case study. We identified key considerations that need to be made in Table 3 to ensure that comparisons between storage methods tested for are appropriate for determining best storage practices.

Table 3. Considerations to be made and their associated issues and recommendations when designing a pilot study.

Consideration	**Issues**	**Recommendations**
Soil type	Responses to storage methods vary between soil types.	When working with different soil types we recommend making comparisons between storage methods for each soil type.

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Storage Methods	Not applicable	Storage methods are not limited to those explored in our case study.
Time points	Limited by resources.	Choose a reasonable set of time points within resource limitations. Include best- and worst-case scenario for the timeframe that you typically need to analyse samples after collection.
Extraction matrix	Each extraction matrix will respond differently to each storage method.	Storage methods for each extraction matrices should be considered separately.
Extraction methods	Extractant volumes, shaking times, centrifugation times and filter types can influence measurements.	Use the same extraction methods throughout all storage treatments and for baseline measurements. Where possible utilise standardised methods (e.g. Halbritter et al., 2020).
Baseline[1]	Without reliable baseline measurements conclusions on best storage practice cannot be made.	Double the number of replicates for this time point (day 0) and ensure analysis is carried out immediately after soil collection.
Replicates	Heterogeneity.	We recommend using a minimum of 5 replicates for each storage treatment and time point. Consider including more replicates when not sieving as samples are not homogenised.
	Pseudo replication.	Do not take replicates from same sampling location, ensure replicates capture the range of soil variability.
		Do not store soils or extracts in bulk. The same weight or volume of soil or extract must be stored separately for each storage treatment and time point.
Blanks	Some storage vessels can leach DOC.	Ensure you have a minimum of three replicate blanks for each extract type, storage method and time point.
Setting your upper and lower similarity limit[2]	Heterogeneity.	Replicate baseline measurements of the same soil sample will indicate the level of variation in measurements due to subsampling, handling (e.g. filtering) and instrument (e.g. calibration and accuracy) effects. This variation can inform the decision on the similarity limits or you can choose to accept a 10% or 20% upper and lower limit.

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Deciding on the best storage practice	When working with more than one soil type.	Samples should be subjected to the same storage method and length that is deemed appropriate for all soil types. For example, we found that it is appropriate to store brown earth subsoils at 4 °C for less than a month to quantify DOC/DON by water extractions (Table 2). However, for brown earth topsoils we found that this storage method was only appropriate for soils stored for less than a week, thus limiting the storage length to one week for both soil types.

[1]Soil measurements immediately after soil collection, not subjected to any storage method

[2]The negative and positive percentage variance from baseline measurements accepted between baseline and storage method measurements to deem storage method appropriate

Where possible, we strongly advise researchers to publish pilot studies (as a minimum within supplementary materials) to ensure approved methods are adopted by the wider ecological community and for the future synthesis in development of a standardised practice handbook for all soil types.

4 Improving method reporting

Comprehensive reporting of storage practices based on pilot studies and published recommendations in the literature is important for improving storage practices amongst the ecological community. We provide an example in Table 4 for best reporting practice.

Table 4. Examples of poor method reporting, reporting requirements and examples of good reporting of methods.

Examples of poor methods reporting	**Reporting requirements**	**Examples of good reporting**
We tested the effects of drought on soil nutrients and microbial biomass across several plots, on a grassland field in the UK.	Report location for sampling as coordinates.	We tested the effects of drought on soil nutrients and microbial biomass across several grassland plots located in Selside in the Yorkshire Dales National Park (54.17 N, 2.34 W), England.
We collected organic soil from the top 10cm.	Report detailed information on soil, including World Reference Base for	The soil in this area is described as a clayey brown earth over limestone bedrock from the Malham series of

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<table>
<tr>
<td></td>
<td>Soil Resources WRB soil type and characteristics.</td>
<td>Eutric Endoleptic Cambisols (De Long et al., 2019), and the main physical, chemical and biological characteristics of these soils are summarised in Table 2. We collected samples from the top 10 cm of the organic layer.
Table 2- Soil physical, chemical and biological parameters
<table>
<tr><th>Soil</th><th>TOPSOIL</th></tr>
<tr><td>Clay (%)</td><td>60.0 ± 2.1</td></tr>
<tr><td>Silt (%)</td><td>0.6 ± 0.2</td></tr>
<tr><td>Sand (%)</td><td>39.3 ± 1.9</td></tr>
<tr><td>Bulk density (g cm^{-3})</td><td>0.63 ± 0.04</td></tr>
<tr><td>OM (%)</td><td>14.0 ± 2.5</td></tr>
<tr><td>C (%)</td><td>3.9 ± 0.1</td></tr>
<tr><td>N (%)</td><td>0.45 ± 0.01</td></tr>
<tr><td>C: N Ratio</td><td>10.5 ± 0.2</td></tr>
<tr><td>pH</td><td>5.9 ± 0.1</td></tr>
<tr><td>Soil Moisture (%)</td><td>47.9 ± 4.9</td></tr>
<tr><td>NH_4^+ (mg kg^{-1} dry soil)</td><td>2.02 ± 0.97</td></tr>
<tr><td>NO_3^- (mg kg^{-1} dry soil)</td><td>0.32 ± 0.13</td></tr>
<tr><td>DON (mg kg^{-1} dry soil)</td><td>2.3 ± 0.7</td></tr>
<tr><td>DOC (mg kg^{-1} dry soil)</td><td>19.6 ± 5.5</td></tr>
<tr><td>MBC (mg kg^{-1} dry soil)</td><td>1772 ± 340</td></tr>
<tr><td>MBN (mg kg^{-1} dry soil)</td><td>137.3 ± 27.3</td></tr>
<tr><td>DOC: DON</td><td>8.8 ± 1.9</td></tr>
</table>
</td>
</tr>
<tr>
<td>We modified the chloroform-fumigation methods described by (Vance et al., 1987)</td>
<td>Report detailed information for any modifications made to referenced methods.</td>
<td>We utilised modified chloroform-fumigation methods described by (Vance et al., 1987), where soils were fumigated with excess CHCl3 and modified under vacuum for 48 h.</td>
</tr>
<tr>
<td>Soil extracts were stored in the refrigerator until analysed.</td>
<td>Report the storage methods used along with length and the basis for using them</td>
<td>Extractions were carried out on soil samples immediately after soil collection. Soil extract samples were stored at 4°C for one week as recommended by our own pilot study reported in supplementary material.</td>
</tr>
</table>

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5 Conclusions

Our results demonstrate that it is generally not advisable to store soils or soil extracts when assessing soluble C and N pools and microbial biomass. We also show that the appropriateness of different storage treatments varied between topsoil and subsoil, suggesting that appropriate storage methods need to be tailored for different soils. However, we recognise that it is not always possible to avoid storing soils and therefore recommend using the tools provided to determine best practice.

We highlight that every step within an extraction procedure, including soil sample preparation, can generate biogeochemical differences in samples, and therefore encourage researchers to utilise standardised methods where possible (see e.g. Halbritter et al. (2020)) and to follow best storage practices for specific soil types to allow reliable comparison of data from different studies. Given the potential for storage treatment to affect results, we also urge researchers to report detailed information about their storage treatment (i.e. temperature, duration) and the basis for the chosen treatment when publishing findings. In the absence of published storage recommendations for a given soil type, we encourage researchers to conduct a pilot study and publish their findings. This will allow for future synthesis and development of a comprehensive handbook for standardised methods for soil and/or soil extract storage as many published standardised methods currently give unsubstantiated advice.

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