As a group of researchers from the Soil Science Department of the Research Institute of Organic Agriculture (FiBL), Switzerland, we discussed the manuscript entitled "Are researchers following best storage practices for measuring soil biochemical properties?" by Rhymes at al. 2020. Rhymes et al. 2020 raise the discussion on an important topic that concerns the whole soil science community. We would like to acknowledge the authors enormous work in a comprehensive and valuable case study on best practice storage conditions for soil samples and soil extracts for various commonly investigated biochemical parameters (with almost 2000 extractions performed). We highly appreciate their initiative in raising awareness on this vital, but often neglected topic and hope that their contribution will spark further work and exchange among soil scientists.

Thank you for the positive response. We appreciate the evaluation your group have made and we respond to the comments individually.

However, in our opinion some important aspects were not considered adequately and we have the following suggestions for improvement:

1) The data on which Rhymes et al. base their guidance should be provided in the main manuscript rather than the supplementary information (SI). While the authors themselves claim that "[. . .] optimal storage conditions will vary across different soils and ecosystems" (Line 75), but also between top- and subsoil, as shown by their own case study, they come to very generalized recommendations on best practice storage in Table 2, which we find contradictory. To us it is not quite clear how the authors come to their recommendations, or at least some differentiation is lacking. For instance, in Figures S4a and S5a, storing frozen extracts up to 430 days seems tolerable for both MBC (only topsoil) and MBN (both top- and subsoil), but in Table 2 freezing extracts for assessment of microbial biomass is indicated as completely inappropriate.

As recommended, we will expand Section 2 by moving the entire results section from the supplementary into the main manuscript, which will include line 107 to line 172 from the supplementary. The figures will remain in the supplementary and will be referred to in the main manuscript.

Summarising our findings into generalised recommendations was a particularly difficult task considering the differences we found between soil depths. We also want to highlight that these are not guidelines that should be followed if using different soil types or soils at different depths to those explored. The general rational for the guidelines summarised in Table 2 were that: 1) a storage method must have deemed appropriate for both subsoils and topsoils and 2) where the same extractant is used to measure different parameters they too must deem appropriate for both sets of parameters. In the example you give for MBC and MBN, we would like to highlight that storing frozen extracts was not deemed appropriate for measuring MBC in subsoils and therefore did not meet our first clause. Furthermore, microbial biomass is generally measured as microbial biomass nitrogen and carbon and would use the same extraction solution to measure both and consequently does not meet our second clause.

To clarify this issue, we will add this statement to line 230: "However, freezing samples did not significantly affect the concentration of N in fumigated or unfumigated samples, and thus frozen extracts was a suitable storage method to measure MBN."

We will also clarify our rational for making such guidelines in the table caption. This will be revised to read: "Table 2. Storage method recommendations for both temperate topsoil and subsoil. Dark grey denotes inappropriate storage methods for a specific analysis. Light grey denotes appropriate storage method, where storage length is annotated. Where storage length is annotated as 430

days we are unable to advise storage length beyond this due to the length of the experiment. Storage methods are deemed appropriate: 1) if the storage method does not compromise the sample integrity (defined as stored samples yielding soil parameter values within 20% similarity limits to fresh samples) for both topsoil and subsoils explored; and 2) where the same extractant type is used to measure different parameters, the storage method does not compromise the integrity of each parameter measured."

We do also provide some guidance on this issue in Table 3 bottom row "deciding on the best storage practice" but hope that with the added information in the caption this will be much clearer.

2) The discussion of changes upon storage should be further elaborated and put in context with existing literature (e.g. the literature reviewed in Table 1). For example, Stenberg et al. (1998) suggest that soils can be stored frozen for up to 13 months for assessing microbial biomass, while Rhymes et al. recommend not to freeze soil at all for any kind of biochemical analysis they considered in their manuscript. How would the authors explain these differences?

We will expand the discussion and compare our results with those published in the literature (see below for more details). For example, the key difference between our study and Stenberg et al. is that they only measured MBC, whilst we measured both MBC and MBN. In line with the findings of Stenberg et al. we also found that freezing soils to measure MBC was acceptable (up to 75 days for topsoil and 430 for subsoil), but not for MBN (although acceptable for topsoil up to 430 days). As discussed above, due to subsoil sample integrity being jeopardised by freezing soils to quantify MBN we deemed this storage method inappropriate.

Another potential explanation for these differences could be differences in soil microbial communities. They worked with soils from Upsala, Sweden that experience marginally lower average winter temperatures than the soils we collected. In turn, the microbial communities in their soils could be more adapted to colder temperatures causing them to respond differently to freezing storage methods.

In Line 130, Rhymes et al. speculate about microbial processes as the main driver of changes in stored soils or extracts and they suggest storing samples under conditions which suppress microbial activity completely. Given the major changes still happening in frozen extracts over time for NO3 (Figure S3a), do the authors suggest that freezing is not suppressing microbial activity sufficiently? Could there be other mechanisms responsible for this trend?

We do not explore the mechanisms responsible for the changes to samples under different storage conditions and therefore can only discuss potential mechanisms. Indeed, freezing does suppress activity, but our results demonstrate that this is not enough to maintain sample integrity. As a result, (on line 130) we recommend other potential storage options that completely halt microbial activity such as the acidification of extractants. To avoid confusion, we will change "completely suppress microbial activity" to "completely halt microbial activity".

In response to freezing not supressing microbial activity, there is evidence for this; freezing has been shown to decrease microbial biomass as a result of damaged cell structures (Černohlávková et al., 2009). As a result microbes that are not damaged by the freezing process profit from the organic molecules made available from the proportion of microbes that have died (Stenberg et al., 1998). This could be a potential mechanism to explain the shift over time whilst in the freezer but we are unable to conclude this as we did not investigate the mechanisms.

The discussion currently provided in the SI should be moved to the main manuscript in order to increase its visibility to the scientific community.

We agree with the reviewer's comments and would like to include some of the potential mechanisms for the sample deterioration we observed under different storage conditions. We will move paragraphs starting from line 182 to 230 from the supplementary material into a new section with two subsections to discuss mechanisms involved in storing soils and extracts (Section 2.3 – Results and discussion).

3) The importance of the underlying research questions is neglected: The authors only look into relative changes in the measured parameters in comparison to freshly extracted and immediately analyzed samples. However, many studies aim at investigating relative differences between treatments rather than obtaining absolute data on fresh samples. In fact, appropriate storage conditions are not only part of the method, but also depend strongly on the research question. In many cases, standardized pretreatments (for example pre-incubation of soil after refrigerated storage for microbial N and C), freezing of all samples before extraction etc. might produce smaller errors than immediate extractions, where differences upon sample collection, transport, outside temperature upon sampling etc. would arguably cause bigger effects than the storage treatment. With this regard, especially the change in the measured parameters upon prolonged duration of storage is relevant. For instance, Rhymes et al. consider freezing of soil or extract for analysis of NH4 inappropriate (Table 2 or Figure S3b), however, changes here seem to appear immediately upon freezing, with marginal changes thereafter (Figure S3b). For studies only interested in relative differences between treatments or sites, freezing thus would be a tolerable storage method. Again, we think that the recommendations should be more differentiated and take potential research questions into account.

We agree that other procedures for sample collection and preparation will also affect sample integrity. We are happy to refer to these other procedures in the introduction to ensure readers also consider other aspects that will impact sample integrity. This will include sample collection, transport, processing and analytical practices that can also influence results. We will add these sentences to the introduction from Line 30 to address this issue: "It is therefore integral that researchers consider each factor that can impact accurate and reliable analytical measurements, which can include sampling procedures (e.g. strip removal of turf), transport (e.g. transport length and temperature), storage (e.g. temperature), preparation for analysis (e.g. sieving mesh size and when samples are sieved) and analytical methods (e.g. temperature, shaking times and filter types). Here we focus solely on sample storage. 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."

However, we disagree with the reviewers' view that freezing is a tolerable method for storage when studies are only looking at relative differences between treatments and/or site. Our findings demonstrate that storage can impact soil from different depths differently, which implies that soils of different types are likely to respond differently to storage methods. We did not explore the mechanisms for the differences observed and therefore cannot assume that this is correlated to soil depth alone. It might be as a result of other factors such as differences in nutrient status or microbial populations for example. If this were to be true, two samples of the same soil type with

different nutrient statuses would respond differently to a soil storage method. Or two soils under different experimental treatments that had affected their nutrient status or microbial communities would also respond differently. We therefore do not recommend that researchers assume that samples from the same site will respond the same to a storage method and that the relative differences between treatments will not change. Additionally, not all variables respond in the same way to storage, and if researchers are to study C/N ratios for example, storage effect will have a strong impact on them. Finally, it is true that there are some changes that occur immediately and some that occur over storage time. However, this immediate change was not equal in the two soil depths studied (for example frozen extracts for NH_4 determination, or freezing soil for DON), and hence, the same logic applies.

We will now include this in our discussion to address this issue: "It is commonly assumed that any changes to soil biochemistry from storage methods will occur equally for all samples. Here, we provide evidence to show that changes do not occur equally which could have major implications for the findings of ecological studies. We did not investigate the mechanism behind different responses of two soils to the storage treatments, but it could be related to many factors such as differences in nutrient status or microbial communities. As a result, any treatment that affects soil properties have the potential to also affect the response of soils to storage. Even if sample biochemistry changes immediately as a result of storage but subsequently remains stable over storage time, in our study this effect varied between the two soil depths. Therefore, even if the research question is to compare between treatments applied to the same soil type, strict storage limits should be followed. We suggest that all samples should be stored under the same conditions that allow the preservation of samples from the soil type, site and/or treatment with the highest sensitivity to storage."

4) From our own experience, but also highlighted by the results of the survey which Rhymes et al. conducted amongst different laboratories (note that the documentation on how the survey was conducted could be expanded), storage of both soil and soil extracts are common practice. This is owed to the mere impossibility to collect, extract and analyze samples in one day, especially with high sample numbers or when sample collection has to be conducted at large spatial distance to the lab. In this context, we find their conclusion on "appropriate" or "inappropriate" storage too general. How about defining an acceptable relative error, e.g. by handling the samples in one way or the other? Furthermore, as indicated above, relative errors occurring immediately (e.g. upon freezing or un-freezing) should be distinguished from continued changes upon prolonged storage.

We agree that we need to provide more information about our survey approach. The survey was conducted on Google surveys and promoted through twitter a social media platform. We will now include the survey questions in supplementary materials (see details in response to referee 1). We will now include the rational for the survey and the survey questions.

Regarding the "appropriate" or "not appropriate" recommendations, they are already taking into account and accepting a 20% error for each storage treatment explored. But if researchers consider that a higher % error is acceptable for a particular study due to strong logistical limitations, a higher error to determine appropriateness could be used. Regarding changes happening immediately upon storage, please, refer to our response above.

5) With their study, Rhymes et al. made an important point on the effect of storage conditions, but we miss the broader picture. The discussion should expand also on other aspects potentially compromising the integrity of soil samples, such as sampling procedure, transport, pre-treatments or handling of the samples in the laboratory. We believe that the whole soil science community

should put more effort into defining common standards and evaluating potential errors during the whole procedure from sample collection, transportation and storage until analysis. Comparing the effect of storage conditions with the effects of these other aspects would help to identify sources of major errors and design experiments accordingly.

We agree that the manuscript would be strengthened if a comment were to be added to the introduction to address other aspects of sample collection and processing beyond storage methods can also impact sample integrity. We have addressed this in your 3rd comment (please see 3 above) and will also include an amended sentence in section 5: Conclusions.

"We stress that researchers must also consider other practices beyond just storage (e.g. sieving samples, transport, extraction procedures…) as each methodological step between sample collection and analysis can introduce errors to measurements that are intended to be field representative. We 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."***

6) If each group has to carry out their own pilot studies and resulting storage conditions will vary substantially, then meta-analyses will become even more difficult than they are now. Besides, the recommendations for such pilot studies would need to be really concise, e.g. how many time points would need to be analyzed? It would be important to learn as much as possible from the experiment conducted by Rhymes et al. As an alternative to pilot studies, why not put an effort into identifying suitable reference materials that can be included in each study?

We believe that if more people that publish and report their pilot studies, this will generate the data required to conduct a large meta-analysis that paints a much clearer picture as to how storage impacts our results. This could potentially result in a standardised storage protocol being produced. But to date we know very little about the impacts of storage methods. In the supplementary material "Extended material and methods", we provide all the details necessary to replicate our study. Furthermore, in Table 3, we consider the different aspects that researchers should take into account when designing their own pilot studies. We do not suggest or think that it should be necessary that all the pilot studies should be an exact replica of our study. It is more appropriate that they focus on the aspects (e.g. storage methods, length, similarity limit) that can better inform their own experiments. Indeed, storage conditions will vary amongst these pilot studies but high variability in ecological studies already exist too and are commonly used for meta-analysis. Efforts should still be made despite this hurdle. We believe this commentary will spark the necessary discussions amongst the soil community to give sample storage more consideration than it currently does.

Suitable reference materials may be an option for quality control purposes. However, this would need to be explored for future recommendation as little is known about the mechanisms behind the shifts associated with storage and most importantly whether reference materials would behave similarly to living soils.

In addition to these general thoughts, here are some more detailed comments:

Sampling procedure and soil sample preparation

- While we understand the reasoning behind their sampling approach (topsoil sampled three weeks after sampling the subsoil), in most of our experiments this is simply not an option, e.g. due to distant sampling locations and the importance of a uniform sampling time point.

This approach was due to logistical constraints, and we do not suggest that researchers stagger their sampling times for different soils routinely.

- Soil samples were taken in June. Would results be different if soils had been sampled in winter or at a different initial water content? Generally speaking, the effect of seasonality should be discussed.

We agree that seasonality will affect any soil biochemical results measured. It is unknown whether they would also respond differently to storage methods.

We appreciate we do not make note of this in the manuscript and agree that we should. We will add this sentence in the discussion to address this comment: "We would like to note that due to the high temporal variability that the temperate soils explored experience, there is the potential that storage methods could impact sample integrity differently depending on when the samples were collected. Understanding the mechanisms responsible for jeopardising sample integrity under different storage methods will help determine the best storage methods for the time in which samples are collected (e.g. season), soil type and depth."

- Apparently, Rhymes et al. use "field replicates" for their extractions (SI Line 43ff): Soil was sampled from five locations (transect over the field with 10m distance between plots) in 0.5 x 0.5 m pits. These replicates were later on used for the extraction/different storage treatments. This sampling approach explains the high data variability upon the individual time points and storage conditions and should have been discussed by the authors.

We agree that true field replicates always result in higher variability, but we used this approach to avoid pseudo replication, and to provide a more robust representation of variability within our field experiment. We calculated relative change to help account for high variability. Additionally, we included a 20% similarity limit, instead of a stricter 10% similarity limit, which also helps accounting for this high variability.

We will include a statement on this issue in the supplementary methods in line 44: "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)."

We consider that adding that information in the methods is sufficient and we do not feel it is necessary to include a statement in the discussion.

- The time of sieving/homogenization was not investigated, since Rhymes et al. sieved all soil samples on the day after sample collection and stored all the soils sieved. Would the results have been different if soils had been sieved only after storage, immediately before extraction? Extraction procedure and handling of extracts

Yes, this will certainly yield different results, we address this comment both in the introduction and conclusion (Please see our response to points 3 and 5). We do not feel that it is necessary to discuss in too much detail as the purpose of this study was to look at storage methods. We feel that with the inclusion of the sentences we propose in the introduction and the conclusion we have made it much clearer that researchers need to consider all of these aspects including sieving, from sieving mesh size to when the soils are sieved immediately after collection or just before analysis.

- SI Line 71: For K2SO4 extraction, no blanks were performed. While this seems valid for the calculation of microbial C and microbial N as difference between fumigated and non-fumigated extracts, we find this problematic for reporting values on total C and total N in both fumigated and non-fumigated extracts, which were not corrected for blanks (compare Figures S4 b, c and Figure S5 b, c)

We agree that our results would have benefit from K_2SO_4 blanks. When the experiment was designed, we did not consider the possibility of presenting the raw unfumigated and fumigated data, and only the microbial C and N, deeming unnecessary to process and store K_2SO_4 blanks (which would have increase the number of extracts to an additional 180 samples). However, for the purpose of this study, we are only looking at relative differences between storage treatments and feel that presenting the raw unfumigated and fumigated data without blank correction is acceptable. In accordance, the recommendations we make are based on the microbial C and N values and not the unfumigated and fumigated values. The fumigated and unfumigated samples only helped in the discussion to explain some of the effects observed.

- The molarities of extractants (K2SO4, KCl) are not reported throughout the whole manuscript.

This will be added throughout. The molarity used was 0.5M for K_2SO_4 and 1M for KCl.

- Scaling of extractions procedure: Authors report that 5 g of moist soil were extracted. This is a very low amount considering any potential inhomogeneity in the soil. Due to the high number of replicates (n=5) this might be acceptable.

This is very common practice and is reflected in numerous publications, some even use less soil (e.g. 2.5g of soil to 25ml extractant in Jones and Willett).

Additionally, soil moisture content (e.g. between top- and subsoil) was ignored upon extraction, which might lead to differences in the soil-to-solution ratio. Equal amounts of dry soil equivalents should be used for a standardized extraction procedure.

Although we did not utilise equal amounts of dry soil equivalents, we did correct for differences in soil weights as a result of differences in soil moisture for final nutrient concentration calculations. It has come to our attention that this is not appropriately detailed in our methods and we will amend this. We would like to highlight that it is common practice to correct for the difference in dry weight when calculating concentrations, please see the standardised protocol in Halbritter et al. (2020). We would also like to draw your attention to Table S1 and note that we only saw a 2% variation in soil moisture and therefore feel that this will not have impacted our results.

- Scaling could also be added as another point to consider for a pilot study within Table 3 (extraction methods; recommendation: do not up-/down-scale the used amounts but use the same amounts as planned for the main experiment).

We already elude to this concept in table 3, under replicates, pseudoreplication "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." But we agree that it could include the idea of scaling down. We will modify this and create another row in the table for scaling. It will read:

"Consideration: Scaling Issues: pseudoreplication, reproducibility. Recommendations: Do not scale your soils or extracts for storage up (bulk storage) or down. The same weight or volume of soil or extract must be stored separately for each storage treatment and time point as the one planned for the main experiment."

- Freezing and un-freezing procedures were not investigated as further factors. From our experience, it makes a difference in which position extracts are frozen (e.g. vertical or horizontal placement of tubes) and under which conditions extracts or soils samples are thawed (e.g. thawing soils over night at 4◦C or extracting frozen soil immediately with the solution).

This is an interesting point and would make for a good exploratory study. We will add details on the specific conditions in which extracts were frozen in the methods (they were all frozen vertically). Details on how soils and extracts were thawed are already in the supplementary information, extended methods (please see line 56).

We will add the idea of the potential effects of freeing/thaw procedures on measurements in the discussion. We will add this sentence: "Due to the potential for freeze-thaw cycles to impact sample biogeochemistry (Černohlávková et al., 2009) it is important to consider and be consistent with the freeze/thaw procedure, such as the position in which extracts are frozen (vertical or horizontal placement of tubes) or under which conditions extracts or soils samples are thawed (e.g. thawing soils over night at 4°C or extracting frozen soil immediately with the solution)."

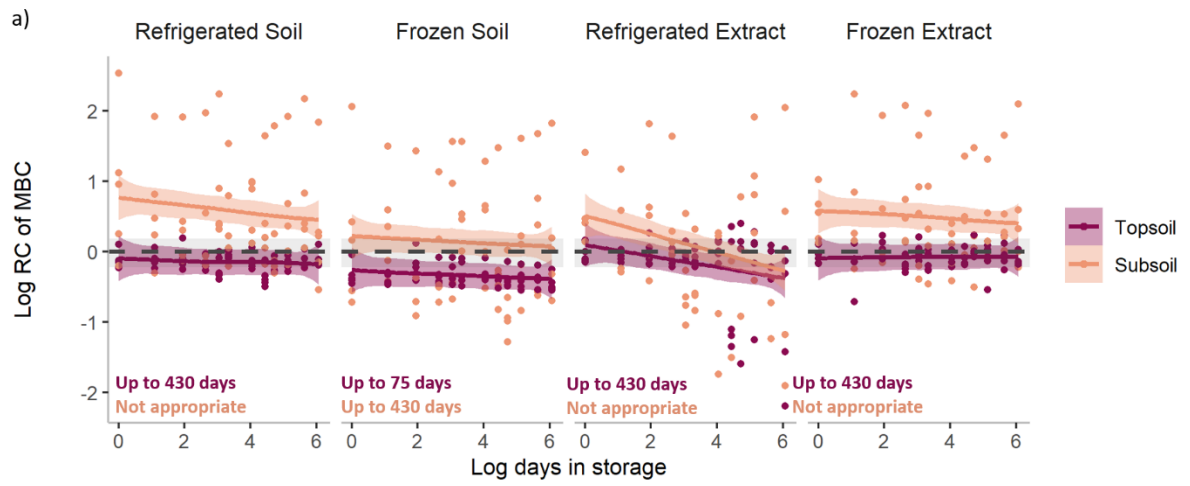
Statistics/Figures/Data presentation

SI Line 104: Why did the authors use a plot digitizer to extract numeric data from their own plots?

We define sample integrity as unusable when the confidence interval of the predicted model intercepts the outline of the similarity limit which is shaded in grey. We didn't consider it was important as to how we calculated this and chose what seemed to be the easiest way. This can of course be calculated through the predicted confidence intervals from our models, which has yielded the same results. We will amend this accordingly in the methods.

- Figure S1b: In the figure caption, authors indicate that there was a technical problem with the DON measurement on the last time point (Day 430) and thus, data should not have been included. However, in the figure there a data points also for this sampling time.

Graph S1b is correct. However, you have brought to our attention that the 430 day sampling point was not visible on all other graphs due to the x axis only going up to 6 rather than 6.06 (log of 430 days). This is a graphical plotting error rather than a statistical error, whereby no sampling points were excluded from out the linear mixed models. We will amend all the graphs. As an example, here is our corrected graph forS4a, which now includes the last time point.



- For some of the analyzed parameters, the replicates show a very high data variability. However, this seems not always represented in the confidence interval displayed (e.g. Figure S3 a: NO3 values for frozen extracts vary widely, while the confidence interval seems to be very small).

We would like to highlight that these are 95% upper and lower confidence intervals for the predictive fitted ratio change values based on the mixed effects models carried out. This is not an individual confidence interval at each time point, and that is the reason in this instance why data variability is high, but the represented confidence interval is small.

- In Table 3, authors recommend to use twice the number of replicates for the baseline (freshly extracted and analyzed samples). However, for their own case study they did not follow this recommendation or at least did not report it.

We did not use twice the number of replicates, but upon reflection for such a study we think this is valuable and have therefore added it into the recommendations.

Technical comments:

- Typo in Table 1: Plant available N, reference "Jones and Willett 2006", under storage methods explored it should probably be -18◦C

This shall be corrected.

- Line 60: Wording is misleading. Stenberg et al. 1998 also sieved the soil prior to storing it at different temperatures.

We have removed the word sieved so it is no longer misleading.

- Table 2: There seems to be a mistake in the table header. We do not see any red or green squares. We assume that the information given below the table ("Dark grey denotes inappropriate storage method and light grey appropriate.") gives the same information?

We will correct this and add grey colour information to the table caption. This will read:

"Table 2. Storage method recommendations for both temperate topsoil and subsoil. Dark grey denotes inappropriate storage methods for specific analysis. Light grey denotes appropriate storage method, where appropriate storage length is annotated. Where storage length is annotated as 430 days we are unable to advise storage length beyond this due to the length of the experiment."

- Line 135: Figure 1 should only have a figure caption below, but not additionally above.

This shall be corrected.

- Table S2: Table header is missing.

This shall be corrected. Table S2 header: "Table S2 Summary of extraction methods used in this experiment"

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