Discussion on referee comments: Spectroscopic assessment of three ecologically distinct organic carbon fractions of mineral soils by Walden et al.

We thank the referees for their reviews and comments. Below, we provide our responses in blue text and preceded by **Authors:**. We denote 'page' as 'P', 'line' as 'L', when referring to locations in the submitted manuscript.

Referee 2

Comment 1: In their manuscript, Spectroscopic assessment of three ecologically distinct organic carbon fractions of mineral soils the authors present how MIR spectra can be used to identify organic matter composition and mineral components in two particle size fractions. This is surely an interesting and important aspect of using MIR spectra qualitative analysis of soils and can help to better understand carbon stabilisation in soils.

Authors: Thank you for the comment and acknowledging the importance of our research.

However, the current manuscript does not clearly present a way forward to advance our understanding here. This is mainly caused by the more descriptive presentation of the results rather than an improvement and discussion of our mechanistic understanding. This is also related to the rather small set of soils and important missing information. Please see below my concerns and further comments.

Authors: We agree that our results can be more clearly presented and the discussion improved to tease out the understanding gained from the combined use of the physical (granulometric) fractionation and FT-IR spectroscopy—see responses to referee 1. Regarding the small sample set, to test our ideas, we used three different soils found in cropping regions of Australia. As also suggested by referee 1, we will add information on the soils and improve the description. This will include, soil type, climate zone, annual rainfall, total nitogren (%), and CEC.

Comment 2: I struggle with the title and the use of the term "ecologically distinct". While this sounds catchy, the authors do not discuss any ecological aspects of the two isolated fraction. To my opinion, the presented study does not include any data to discuss such ecological aspects.

Authors: We used 'ecologically distinct' because the different types of soil organic C in a particular ecosystem are ecologically meaningful. The organic C fractions have specific roles in C transformations and storage, microbial activity, mineralisation, nutrient cycling, energy transfer, and other ecological processes. Assessing their differences is important for understanding the dynamics and functioning of ecosystems and overall ecosystem health. However, we agree that using 'ecologically distinct' in this particular study is not entirely clear, and perhaps also somewhat overstates the research presented. Therefore, we propose to amend the title to: 'Spectroscopic assessment of three distinct organic carbon fractions of mineral soils'.

In consequence, the discussion section is rather a repetition of the results and the arguments that the presented findings are advancing our understanding are not well supported

Authors: As above, we propose to improve the discussion section by removing repetition and teasing out the understanding gained from our research.

Comment 3: The research aim to perform particle size fractionation of three different soils is rather weak and the novelty is not clear here. The second research aim is also more or less weak considering the small set of soils in this study. The general novelty is missing here

Authors: Thank you for the comment. The reason we added the fractionation to the aims is to emphasise that our analysis combines the granulometric fractionation with FT-IR spectroscopy to gain insights. However, we agree that perhaps we haven't articulated the aims clearly and we will do so in the revision. We will also highlight the novelty of our study.

Comment 4: One of my major issues is the presentation of the considered soils. It is

not clear from section 2.1 from where the soils are, if they are derived from different climate, what soil types the represent, under which land use they were or from which depth. With all this basic information missing, it is not clear how relevant the presented findings are. I agree that there is a strong range of TOC and clay content, which will certainly affect the organic composition in the fractions.

Authors: We will include more information on the selected soils. Whilst not all-encompassing, the three soils are representative of highly weathered soils that span large areas of Australia, with low organic C content. In the revision we will include more background information on the soils selected in this study. Additional to land use and other properties we reported, this will include, soil type, climate zone, annual rainfall, total nitrogen (%), and CEC.

I would argue that a larger range would be need to include different stabilisation mechanisms. This would include a larger range of pH especially in the sandy soil to include changes in stabilisation processes by pedogenic oxides. The rational of only considering samples with TOC <2% is also not fully clear to me.

Authors: A broader range of soils would potentially allow us to identify other forms of C stabilisation, however, this is outside the scope and aims of our study. In the discussion we will further clarify that future research needs to include a diverse range of soils to test the concepts we present here.

However, The argument that we can use NMR for higher TOC soils is not convincing for me.

Authors: We do not make the argument that NMR is not suitable for soils with high TOC content. We argue that to conduct assessments on the soils we study, with low C content and high levels of paramagnetic material the use of other methods to characterise the organic matter (i.e NMR) is not entirely suitable (Mathers et al., 2002; Baldock et al., 1989; Kinchesh et al., 1995). Here we present the FT-IR approach for low C soils, however further research could investigate using this technology for high C soils.

Comment 5: The authors present briefly why they selected a simple particle size fractionation. However, I would argue that for the intended purpose in this study, it is important to discuss the fact that the separation by size only can result in artifacts as a limitation. This is especially true when a large range of texture is considered. Thus, the POM of the sandy soils will be more diluted by sand than the POM in a clay rich soil, which might contain more stable aggregates.

Authors: First, the granulometric fractionation isn't a 'simple' method. It involves many critical steps that alone might appear 'simple' but require careful consideration and precise analyses. The method is also rather time consuming. We do not agree that the granulometric procedure that we used produces 'artefacts'. But of course, we agree that no analytical method is without error—other methods for organic C fractionation (density or chemical) will also produce errors in the allocations to each fraction and each method has advantages and limitations. Please note that the dispersion step is particularly important and a delicate operation for achieving the objectives of the fractionation. In our case, because we used soils with different textures with <2% organic C, we used ultrasonic dispersion. We tested different applied energies to achieve the best results for our soil types—see Supplement. Recovery was >98% for all three soils tested, which suggests that our fractionation procedure is robust. The organic C content of the whole soil and the POC fractions of the two sandier soils is <0.05%, we would argue that the dilution of POC is limited. We will add this point to our results section on the recovery of the C fractions.

The logical consequences of this for the MIR analyses of the fraction is also presented in Line 167-174 and further discussed in the discussion. But as this is expected, it is not clear why the authors make this to the main part of the discussion. The authors do not provide any further discussion or any prove of what they actually separated by using other proxies such as d13C or C/N of the fractions determine plant derived or decomposed organic matter in the different fractions. This is especially critical considering the authors intention to study "ecologically relevant" pools.

Authors: At lines 167–170 we describe the shared features of the C fractions present in all three soils. We disagree with the referee's statement that this makes up the main part of our discussion. In the discussion we reiterate in one sentence "...absorptions of clay minerals in the POC fraction may suggest the presence of aggregates that did not break during dispersion...". We agree that with d13C we could trace the C fractions origin. However, this was not measured as part of this study. We can provide C/N for the fractions to aid with our interpretation of the organic components of the C fractions, we will add this to our results.

Comment 6: The introduction gives a quite broad overview of several fractionation methods. However, some this seems rather to extensive and also some aspects are not fully correct. For example: Line 37-38: Doe the authors refer here to TG-DSC? This is not clear. The Cited paper is using Py-GC/MS which is more or less an evolving gas method and not a DSC. As far as I know, the thermal energy was modelled in the study by Sanderman and Grandy (2020). Please check this and also consider that there are more thermal methods

Authors: Yes Sanderman and Grandy (2020) used Py-GC/MS, we will add more papers which consider the use of other thermal methods including TG-DSC. For example Krahl et al. (2023) and Williams et al. (2018) both used TG-DSC to infer the composition/stability of C fractions. We will add this detail.

Line 39: Physical fractionation can involve both, particle and density separation and in combination. Further, the whole setting and rational of this study is not clear from the introduction.

Authors: Agreed, physical fractionation involves both density and particle separation. We will clarify this in the introduction. In relation to the setting and rationale of the study we believe we did not summarise the gap in the literature well before presenting our aims in our introduction. We will add this to the introduction to improve the readability and clarify the rationale and novelty of our study.

Line 57-59: This is stated because the study is performed on Australian soils? This is not clear from the introduction nor from the material and methods.

Authors: Line 57–59 we state that for Australian mineral soils that are abundant in iron oxides and low in C, NMR is impractical. We state this here because it will lead well to our research gap and why we have chosen these soils and our methodology. We suggest removing "In Australia" as it implies detail that has not yet been discussed as the reviewer notes.

Comment 7: Table 1 it is not clear how the pH, TN and clay were measured

Authors: In the revision, we will describe the methods used to derive this analyses and will also add the CEC to this table. Clay content and CEC were estimated with the MIR spectra based on a soil and spectral library of our lab. We developed spectral predictions tools in line with Soriano-Disla et al. (2014). The predictions for CEC and clay content are accurate, with a ρ_c of 0.94 and 0.95 respectively. We measured pH in a 1:5 soil to 0.01M $CaCl_2$ and H_2O, with a pH meter. We measured total nitrogen with an elemental analyser (Elementar VarioMAX).

Comment 8: Table 2 and 4 seem to be repetitive.

Authors: Table 2 was meant to highlight the spectral regions and possible assignments that are presented in the investigation of the C fractions spectra. Whereas Table 4 is used to identify the important frequencies for the separation of the C fractions with the CVA analysis. We agree that they are repetitive, and suggest to remove Table 2 as this does not provide any additional information that is not in Table 4. Following the comments of reviewer 1– We will move Table 4 to the Supplement as the information contained is useful for those interested in the possible assignments, but not directly relevant to our study.

Comment 9: Table 2 and 3 partly present the same parameters but different values for the TOC of the sand. Please clarify and avoid repetition. The fraction presentation of organic C (%) is also misleading here for the fractions. This is the

actual TOC that is in each fraction rather than the fraction organic C, if I understand correctly. Please clarify this and also consider to present the relative share of each fraction on TOC.

Authors: Table 2 presents the features we used to investigate the mineral and organic composition of the C fractions, and Table 3 is the organic C content (%) of the soils and their C fractions. These two tables are different and the information in them does not overlap. However, if the referee meant Table 1 and Table 3, yes, the TOC is the same in both. Table 3 presents the C content of the isolated fractions. We show the TOC of the whole soil and C fractions here so the reader can determine the recovery of our fractionation methodology. In the results we report that the fractionation procedure had on average >98% recovery. Readers can then inspect the table to determine each individual soil recovery. There is a typo in Table 1 which has lead to different values for the sand soil between Table 1 and Table 3, thank you we will correct so that they are the same.

Comment 10: Line 90-95: Do I understand correctly that the authors consider the TOC400 value as the total C? This would result in a large underestimation of organic carbon as a significant fraction is more stable than a combustion at 400°C. This is not a full combustion but only one defined thermal fraction.

Authors: We did not explain the method well and therefore, the referee misunderstood the method used. We will clarify. Note that the determination of the TOC derives from a combustion at 900C following the combustion of inorganic compounds. This methodology has been used previously to determine TOC and TIC (Mörchen et al., 2019; Natali et al., 2020) without the need for acid pretreatment which can bias the TOC results (Serrano et al., 2023).

Comment 11: Line 95: why does the TOC and TIC measurements result in distinct C fractions. Please revise this.

Authors: Thank you for picking that up. We agree that the wording is confusing

Comment 12: Line 104-105: What calibration do the authors mean here? I assume this corresponds to the background correction. Further, I assume that the authors performed a correction for H2O and CO2 interference. Please clarify.

Authors: Yes the reviewer is correct this relates to the background correction, which relates to the calibration of the spectrometer. We perform a background measurement at the beginning of each measurement plate with a gold standard. The CO_2 and H_2O corrections are then performed in line using the Bruker software. We will clarify this in the method section.

Comment 13: Line 110-113: It is not clear to me why the authors performed a baseline correction and then SNV and SG correction. It is normally performed the other way around with baseline correction as the last step. What is the rational here? Also, did the authors perform a re-sampling prior to the SNC and SG?

Authors: The referee is referring to the preprocessing steps we undertook before we ran the CVA analysis. We offset corrected the spectra, then interpolated the spectra to 8 cm−1 prior to the baseline with a quartic polynomial and SG. Thank you, we mistakenly omitted this and will clarify this in the methods section.

Comment 14: Line 113-114: It is not clear what are the 27 observations when the replicate spectra were average and why is it only 425 wavenumbers?

Authors: The 27 observations are three soils with three separate fractions, replicated three times, coming to 27 samples. The spectra were interpolated to 8 cm−1 before the CVA analysis, hence 425 wavenumbers. Thank you, the interpolation of the spectra is missing from this section, we will add this to the revised manuscript.

Comment 15: Line 136-138: "To gain a better understanding of the features of the C fraction in relation to the whole soil, we multiplied the absorption values at each wavenumber by the proportion of each fraction in the whole soil." Is not clear to me

and I am not convinced that it is a correct step to divide the absorbance by a quantitative fraction proportion. Please explain the rational here.

Authors: The spectra of the C fractions contain the information on the relative abundance of the molecules in each fraction. Here we were interested in the relative abundance of those molecules in the whole soil, similar to how we correct TOC values for the C fractions to determine composition of whole soil C. So to give an indication of the relative abundance of the molecules from the C fractions in a whole soil sample we multiplied the absorption at each frequency by the proportion of each C fraction in the whole soil. To improve clarity, we will add this to the methods section.

Comment 16: Figure 2: I like the presentation in general. However, it is misleading that the different soils are presented and at the same time the general absorbance bands are explained. The bands for minerals, organics and the assignments are true for all soils because they are true for MIR spectra in general.

Authors: We believe that the presentation of the bands for the minerals, organics, and the assignments is key to visualise for readers not familiar with MIR spectroscopy. As we will be removing the tables with wavenumber assignments this figure becomes crucial to the readability of this paper. However, we agree with the referee that these assignments are true for all soils, not just those in each panel. We will add to this to the figure caption to avoid confusion.

Comment 17: The Table 3 and Figure 2 are rather general description of the soils. Especially, Figure 2 presents rather the expected spectra for the three different soils

Authors: We don't completely understand this comment. Figure 2 presents the spectra of the whole soils sample for the three different soils where we can make inference on some of the features present in the whole soils. Whereas Table 3 present the organic C (%) data for each of the soils and their C fractions. This table also shows the recovery of our fractionation procedure.

Comment 18: Section 3.3 and Figure 7. I cannot fully follow the approach here and

why the shown correlations would present a mechanism of stabilization. It would also be beneficial to separate here into the soils and fractions in the visualization. Especially for Fig. 7b it is not clear if these three clusters are the soils of fractions. Please clarify the general use of this correlation.

Authors: We agree with the reviewer that separating the soils and fractions in the visualisation would be beneficial. We will add colour and shape to the points to denote its soil and C fraction, and improve the axis titles and figure caption to be more descriptive and accurate for the plot.

We did not explain this section well, we will include clarification in a revised version. This figure alone doesn't present a mechanism of stabilisation, rather in combination with earlier findings of the spectra of C fractions, their unique mineral and organic components we can begin to infer C stabilisation of the soils we tested based on our current understanding of C stabilisation. We agree that we need to further elaborate on the interaction between organic and mineral components within the spectra. We will add detail to our discussion to clarify that inference of possible associations and stability is based on all of the results presented, and a complete use of the MIR spectra, rather than what we have presented in Figure 7.

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