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 1

Comment 1: The manuscript describes the results of an MIR based spectroscopic analyses of three size-based soil organic matter fractions in low C-soils of Australia of varying texture and to test whether MIR spectra can discriminate between mineral and organic functional groups and explore relationships between them. With this focus, the manuscript deals with an important topic that is worth exploring and could certainly provide insights that would help to close data gaps across soil types with respect to carbon storage, stabilization mechanisms and (potentially) carbon turnover. I do think however that the manuscript falls short at several occasions to provide what is to be expected from these objectives and the finally delivered novelty of the analyses is unclear to me.

Authors: Thank you for acknowledging the importance of the research and the potential of soil spectroscopy for improving the characterisation of the C fractions and providing insights into C stabilisation. We agree that there are aspects of our analysis and presentation that we can improve. Below, we provide specific responses to your comments and suggestions. Regarding novelty, as far as we are aware, our study is the first to use MIR spectroscopy to investigate the organic C fractions compositionally (i.e. not via multivariate calibrations), and to show the potential for using the information on the organic and mineral functional groups in the spectra to infer soil organic C stability.

Comment 2: The title speaks of "ecologically distinct" organic carbon fractions, but

it remains unclear what this means for the authors.

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'.

The applied fractionation scheme isolates size classes and not functional pools or fractions. In fact, the terms used in the manuscript (labelling the fractions >50µm as POC) is misleading since these are not pure POC sources.

Authors: We do not completely understand the referee's comment. First, the fractionation procedure that we used is based on methods described and used by others (e.g. Poeplau et al., 2018; Cotrufo et al., 2019), and tested in an Australian context by Baldock et al. (2018, 2013). Our method has some modification, however, in that we use automated sieving, and sonication instead of of a chemical dispersant (see Figure 1 in submitted ms). Second, we were careful with the language, not to confuse the different particle size fractions and the organic carbon content in each the fraction, i.e. in Figure 1, we note that the sieving produces three different particle size fractions, and it is only when we measure the organic C content of each size fraction that we derive the POC and MAOC, which are different in their formation, persistence, and functioning. We understand that different physical fractionation procedures (size or density) have benefits and limitations, and that the separation into the discrete fractions is not perfect—is this what the referee means by 'these are not pure POC sources'. In this case, our results show that using the spectra we could also get indication of the efficacy of the fractionation (see next response)

At the applied sonication power and in the absence of density fractionation, CN ratio assessments or isotopic ratios or mineralization assessments it is unclear what the ecological meaning of these fractions are, nor in how far the fractions really represent POC or rather more stable microaggregates and the C locked inside them (which the authors discuss also a bit). Thus, the interpretation of the data created by this fractionation scheme should be revised.

Authors: We have responded to the 'ecological' comment above and here we will deal only with the dispersion. Of course we understand that adequate dispersion is critical for soil organic C fractionation. Our method uses ultrasonic dispersion because it is one of the more common and efficient methods and is reported to be better than chemical dispersion, e.g. with sodium hexametaphosphate (Just et al., 2021). Sonication energies reported in the literature tend to vary significantly, hence we performed experiments to determine the best power to use (see Supplement). Our aim here was to determine the sonication energy that disrupts the macro- and microaggregates without significantly disrupting the organic-mineral associations. Following the sonication tests, we measured the MIR spectra of each fraction to assess the efficacy of the dispersion. That is, using the relevant absorptions in the spectra, we could determine the applied energy which disrupted the macro- and microaggregates (i.e checked by characterising the reduced absorptions due to clay minerals in the POC spectra) within the POC fractions, whilst not applying excessive energy to break up other silicates (e.g. quartz grains) that could contaminate the MAOC fraction (i.e checked by characterising the absorptions of quartz minerals in the MAOC spectra). Thus, we are confident that our interpretation of the fraction data is correct. In fact, our work also shows how MIR spectra can be used to assess the efficacy of the fractionation. For example, our testing of sonication energy and verification with MIR showed that the applied energy is appropriate for the soils used in this study. We showed these results in the Supplement, but we can of course, either move them to, or more clearly emphasise these results in the main text. We

did not test density fractionation, or isotopic rations, that is true, but we do not believe we need them to address the specific aims of our study. We will however, include measures of CN ratios.

I do find it interesting however to see clear distinctions between the fractions in terms of their qualitative properties that were derived with the MIR, which is a strong indication that "something different" has been isolated here, even though the exact meaning of this for C turnover is unclear. But this is certainly something worth exploring in a revised version.

Authors: Yes, our results show differences in the mineral and organic composition of the three C fractions, both qualitatively (as in the frequencies at which the different functional groups absorb), **AND** quantitatively (i.e. by the size of the absorptions). What this means for C turnover and stabilisation is what we tried to show and discuss. We will improve our articulation of this in the revision.

Comment 3: In general, I find it a bit difficult that there is so much inferences made from the spectra on both organic and inorganic soil components while no alternative assessment methods for organic or mineral features is provided. I am not saying that I do not trust the MIR data and the interpretation of it (the group is one of the leading groups worldwide in this field). But given the fact that (i) no real quantification of the identified groups of organic molecules or minerals present in the different fractions is made, (ii) that three very different soil samples are compared to each other and (iii) some interpretation of possible causal links between the different organic and mineral elements are made it remains speculative what the actually importance of the postulated stabilization mechanisms may be. This could be fixed by cross-checking the data with XRD spectroscopy, or simply calculating some element ratios (Al/Si, for example) for the cases of the mineralogy. For the organic phases I would recommend similar measures when it comes to interpreting C quality and stability.

Authors: The referee's primary concern seems to be the validity ('trust') of the MIR spectra for characterising the clay minerals and organic functional groups in the soil

samples. FT-IR spectroscopy is a well-established quantitative method long used in physical and analytical chemistry because molecules absorb radiation at specific frequencies and have their own unique spectra. Soil MIR spectroscopy integrates signals from soil's minerals and organic matter (and water adsorbed or present in mineral structures) (e.g. Nguyen et al., 1991; Madejová et al., 2017; Volkov et al., 2021; Calderón et al., 2013). The spectra provide information on the molecular composition of the soil, and research has shown that soil spectra responds to variation in that composition (Calderón et al., 2013; Wetterlind et al., 2022). XRD analysis will not provide any additional or better information on clay mineralogy than FT-IR spectroscopy (Kasprzhitskii et al., 2018; Fernandes et al., 2020), the technique is significantly more complex and it is not necessarily any more 'quantitative'. We also do not agree that elemental ratios, often used as proxies for mineralogy, will provide any more or better information on clay mineralogy that FT-IR. FT-IR characterises mineralogy directly (Madejová et al., 2017). The information on the organic functional groups is a little more complex because in the double bond region of the MIR between 2000-1500 cm-1 there is some overlap with mineral absorptions—note that we discuss this in the manuscript. Note also that organic compounds assigned to frequencies in MIR spectra have been correlated with organic compounds derived using ^{13}C NMR (Wetterlind et al., 2022). Thus, since MIR spectra can discriminate between the different organic C fractions and a spectrum holds information on the mineralogy and the organic functional groups we postulate that it can be used to infer C stabilisation.

We understand that the presentation and interpretation of our results was somewhat unclear, so we propose to improve the results by condensing Figures 4–6 in to a single plot to easily compare the relative absorbances of key organic and mineral assignments between the C fractions and between soils. This will allow us to more clearly synthesise these findings and interpret the understanding gained. We will also improve Figure 7 by adding colour and shape to the points to denote its soil and C

fraction, and improving the axis titles and figure caption to be more descriptive and accurate for the plot. We will also improve the discussion on how we could use MIR to infer stability.

Comment 4: The applied dry combustion technique seems to cut-off the organic C phases after heating at 400°C and then 900°C for inorganic phases. There are many forms of organo-mineral associations that do remain stable at 400°C, and so some verification would need to be provided that those forms are net present here. The inorganic phase could also be assessed by other methods, if acidification of the samples prior to heating at higher temperatures than 400°C is not an option.

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 5: The whole topic of potential interaction (in the introduction listed as a focus of this study) seems to be covered by Figure 7 and the correlations it presents. Not to speak that correlations are always problematic for deriving causality, but I am also lost in what I am supposed to learn from this figure. The caption also does not match the axis labels and it is unclear how I can see the three different soils in there. So for me, the topic of interactions between organic and mineral phases is not really covered in this manuscript in a satisfying way.

Authors: We will elaborate and better explain the relationship between clay mineral and organics measured in the distinct C fractions of the soils in our results. We were careless with some of the figure captions and will improve that of Figure 7. Although the correlation vs. causation comment is rather clichéd, we understand the referee's point and we will clarify. We propose that the use of MIR in the manner we have

throughout the whole study is how we can infer C stability of soils, not only the correlation of clay minerals to organic C absorptions. 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 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.

Comment 6: Similar, the discussion does seem to rely a lot on correlative interpretations of the data, but it is not well linked to the different panels and tables that are presented in the manuscript. For me it was hard to follow the reasoning in there. To follow some of these arguments, we would need at least some sort of table or figure that shows the quantities of the different mineral and organic phases that are compared and linked here.

Authors: We agree that the presentation of the results and the discussion can be improved and we will do so. We will reformulate Figures 4–6 in to a single figure to more coherently show the the variation in the key organic and mineral assignments of the different fractions and soils. This will improve the readability of the results, which will more easily link our findings to the discussion of functional groups present in each soil and C fraction and their potential origin. We are not completely sure what is meant by 'correlative interpretations', we assume this is in relation to the use of absorption strength to infer the abundance of compounds in the sample. As we mentioned in an earlier comment, the use of MIR to characterise organic and mineral composition is well established and here we apply those concepts to the C fractions.

Comment 7: In general, I think the discussion needs to flash out and make a very strong case for the novelty provided by this study, since many of the relationships that are described are well known for many years. Also some of the claims (for example that the spectra can be used to infer stability, line 236) need to be much better substantiated with actual experimental data on those soils or similar soils.

Thus I think that also the conclusions that MIR can in C stabilization with minerals is not well supported.

Authors: Although the technologies that we use (physical fractionation and FT-IR) are not themselves new, our investigation of the mineral and organic composition of the distinct soil organic carbon fraction and the relationships between the mineral and organic functional groups of the MIR spectra is novel. We will improve the discussion and emphasise the novelty of our research. We will also further elaborate in the discussion on the use of the spectra to infer stability, and the limitations of our study. We will add discussion on future work, where we can further test the ideas presented here.

Comment 8: Not much is explained in terms of rationale behind the three selected soil types. Yes, they are on the lower end of SOC and have varying texture, but what are the other factors that explain those? Are they all equally weathered? From same or different climate zones? Same or different land use or geological background? Same soil depth? Same soil types? These things matter in order to understand the absence or presence of organic and mineral features, and also how C is stabilized.

Authors: The three soils are all from cropping areas with varying clay content. Whilst not all-encompassing, the three soils are representative of highly weathered soils over 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. This will include, soil type, climate zone, annual rainfall and total nitrogen (%), and CEC.

Comment 9: Recent literature has shown quite clearly that C stabilization depends quite clearly on reactive metal phases in soils, while the clay content across soil types (and clay types) is often not very helpful to understand its role in C stabilization. Have you thought about this here? I could not find something on this issue in the discussion or the data description.

Authors: Thanks for the comment. Yes, of course, we have thought about this. We

only partly agree with the comment. Certainly, the literature shows that metal oxides are important in the stabilisation of C (Li et al., 2023; Wagai et al., 2020)—we agree. However, it is well known that clay content and mineralogy are also important for stabilising C in soil (Six et al., 2002; Hassink, 1997; Churchman et al., 2020). Therefore, we don't agree with the comment that clay content and type don't play a role in C stabilisation. For instance, it is well-understood that soils rich in 2:1 clay minerals, e.g., smectites, illites, have a greater ability to stabilise C than 1:1 clay minerals, e.g., kaolinites (see cited references above), due to their higher specific surface areas and cation exchange capacities. Other layer silicate or phyllosilicate minerals that are important components in the sand and silt fractions in our soils have also been found to influence C stabilisation (again, see cited references above). We will improve descriptions in the Methods section and in the Discussion, we will emphasise the above with relation to what we can see in the FT-IR spectra.

Comment 10:Given the size and prominence of Figures 4-6 I find them a bit underused. In particular the panels from which not all are cited. I think more focus here on what you want to highlight (and why) might be helpful for the readers. You could use additional graphical amendments for example to illustrate the figures in a better way (just as you did for Figure 2, which I found very helpful)

Authors: We will reformulate Figures 4–6 in to a single figure. The headings of the panel would easily show the reader the region that has been isolated (quartz, clay minerals, CH_2, etc.). Readers would then be able to easily compare the differences between the C fractions within a soil, but also the same C fraction across the three soils. As mentioned in an earlier comment this will more coherently show the the variation in the key organic and mineral assignments of the different fractions and soils. We will then directly link our findings to the discussion of functional groups present in each soil and C fraction and our interpretation of their likely origin.

Comment 11: You may want to revise Table 4 – what does it help you to list all

these positions? Wouldn't it make more sense here to provide a more condensed version that does provide some context in terms of what the (ecological or biogeochemical) importance of those assigned features may be? Like it is currently, it is just an overview table which information I forget as soon as I stop looking at it.

Authors: Table 4 is a summary of the key frequencies and their functional assignments used to discriminate the C fractions. We will move this table to the Supplement. Readers interested in this information would be able to find our summary there instead.

Minor comments: Two of the soil types are abbreviated (SL and SCL) while the third one is called Sandy soil or sometimes Sand (in tables). I find this confusing please revise. Table 1 and Table 3 in parts duplicate data for TOC. However, for the sandy soil the TOC value is different between both tables. Please explain.

Authors: Thank you, we will ammend accordingly and revise the document to ensure the abbreviations are used correctly and consistently throughout

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