Comment 1: *My main concern that must be addressed before the manuscript is published is the method of pore size characterization used in the study. It appears that the researchers used an object identification algorithm to identify individual pores and then used volumes and a shape factor of the identified pores as one of the main tools in characterizing them. I am afraid I have to say that this approach is quite meaningless, and probably some of the lack of pore effects reported in the study is just reflecting the fact that inadequate criteria of pore characterization were used. This approach completely ignores pore diameters and tortuosity - that is, the pore characteristics that are most relevant to their functioning. Say, we identified two pores with approximately the same volumes. One of them could be a thin and long tortuous pore, the other can be a large round cavity. Their functioning in terms of water, air, microbes, SOM decomposition, anything, will be completely different, yet in the classification system of this study they will be lumped in the same size class. While the distance from the pore component of the study is valid and interesting, the components that are based on the object-based pore identification (Figs. 4 and 5) should not be included in the manuscript.*

Response: Our aim was to compare the overall size and shape characteristics of the nine aggregates and we did this with the two sets of analyses presented in the original manuscript. We agree that analyses of both pore diameter and tortuosity could be useful in terms of understanding pore functioning and we have computed these data using: i) the Fiji plugin *AnalyseSkeleton* for tortuosity index (computed as length of pores divided by Euclidean distance between their furthest ends) and ii) pore diameter using the *thickness* function in the *BoneJ* package. We have included an interpretation of these data in the manuscript. We disagree with the reviewer when he/she suggests that the data on pore size and shape should not be included (Figures 4 & 5). We consider these data are essential in the characterisation of the pores in the nine aggregates and we would wish to include them in the final version along with the new information. We have updated our interpretation to include the pore tortuosity and thickness data in relation to soil heterotrophic respiration.

Comment 2: *Minor items: p.3 l. 6 -the part regarding representative volume does*

not seem to fit with the rest of the study.

Response: Inclusion of this reference to representative elementary volumes was in error - it will be removed from the final version of the manuscript.

Comment 3: *p.3 l. 10 - something is missing after 'and'*

Response: The 'and' has been omitted from the final version and this now makes the sense clear.

Comment 4: *The experimental part seems to be very thoroughly conducted. I am curious - what was the need in using glass beads? Not having them would simplify the authors life a lot in terms of creating aggregate masks.*

Response: We included the quartz beads to prevent the aggregates from fragmenting. The greatest risk of this was at the freeze-drying stage, when the aggregates were subject to forces that could cause them to move and fragment inside the vials; and also during transport to and from the synchrotron. We could not select aggregates with diameters that were exactly the same as the vials (fixed diameter) and so any movement of the vial could cause collisions between the dry aggregates and the wall of the vial, causing them to fragment. The quartz beads acted as an inert supporting medium reducing the forces of fragmentation on the aggregates, ensuring their structure was maintained prior to synchrotron X-ray CT scanning. If we repeat this experiment, we may consider a more dense supporting material, such as stainless steel beads, to avoid the problems associated with making masks of the aggregates where surrounding material is of a similar density. However, it is possible that the larger density steel beads could also lead to fragmentation of the aggregates during transit. This needs to be tested further.

Comment 5: *Are all these details in describing how the aggregate masks were created really needed? A lot of the steps talk about in-house R codes or macros and, without those provided as part of the manuscript, this procedure description is not something that anybody from the audience can even try to reproduce*

Response: The stages and macros we refer to in this section could be reproduced

quite quickly in Fiji or R by any other researcher. If we did not provide these details they would not be able to do this and we considered it important that others could reproduce our workflow. We therefore chose to leave these detailed instructions in the final version of the manuscript and provided the scripts as supplementary material (see comment by reviewer 2).

Comment 6: *I think it is unfortunate that the authors decided to aggregate the image data. Why not just use the subsections of the original 3 micron resolution data sets?*

Response: We did not wish to aggregate the data. However, we chose to do so because of the difficulties in analysing the data in terms of computer memory and processing time. To undertake various analyses of the 3D numeric array at the original resolution ($2544 \times 2544 \times ca.$ 1200) was extremely challenging even using a high performance computing cluster. Preliminary tests showed that some analytical steps would take a number of days to complete and given this was to be repeated for 9 aggregates we took the decision to reduce the resolution so we could complete our study in a timely manner. Once reduced in resolution (by a factor of 8) each 3D numeric array was 340 Mb in size which we found to be manageable in terms of reading and writing from memory.

Comment 7: *While I do not see it as a big problem for the current study I believe in future the authors should seriously consider the need to look not just at pores in general, but to keep in mind that depending on their diameters and other characteristics pores can function very differently. The authors expectations regarding pore-emission-SOM relationship.*

Response: No response needed here we believe.

Comment 8: *I agree that scaling CO2 emission by TOC makes sense, but just for "quality" check - was there a positive correlation between SHR and TOC? Because if everything worked as expected there should be one, and it would be nice to hear about it. If there was none, it is also important to report.*

Response: Yes there was a positive correlation between SHR (CO_2 generated) and

TOC with a Pearson correlation of r=0.29. We have reported this in the new version of the manuscript but we do not place too great an emphasis upon it because we consider scaling CO_2 to TOC content to be a more relevant measure.

Comment 9: *The discussion on differences between aggregate and bulk soil findings is a bit simplistic. It is a basic soil science knowledge that density of aggregates is typically greater than soil bulk density (simply put, soil bulk consists of aggregates and large pores among them). Much more interesting would be comparisons of porosity, density, etc. results of this study with literature data that were collected on the same spatial scale (i.e., based on aggregates).*

Response: We do not agree that this discussion is too simplistic. We felt it necessary to explain these differences; not all readers will be familiar with the relationships between aggregate and larger scale bulk density values. The problem with comparing bulk densities (BD) of aggregates (with data captured at a similar scale) is that there are many features that can influence BD (SOC content, texture, mineralogy, differences in soil formation processes) and making clear interpretations concerning them would be problematic. We do not propose to change the final version of the manuscript in this respect.

Comment 10: *In Table 1 and in other places that mention porosity it should be noted that here we are looking at image-based porosity that reflects volume of pores above certain threshold.*

Response: We agree with this comment and will make these changes to the final version of the manuscript.

Comment 11: *Fig.6 - maybe do not show PP, OO, and MM values? They are not informative and without them the differences in other transition groups will be more visible.*

Response: We do not agree that the PP, OO and MM values should be removed from Figure 6. We consider it is helpful to show that the three phase transitions (for each phase) must sum to 1 and their inclusion makes this clear in the Figure. We have not

made this change to this Figure in the final manuscript.

Comment 12: *I have to admit that what is shown on Fig.7 and its relationship to what is shown on Fig.6 eludes me*

Response: Figure 7 shows the individual aggregate transition probability values of the far right panel (OO-OM-OP) of Figure 6 (here expressed as percentages rather than decimal proportions). This change in presentation style may have caused some confusion so we have altered Figure 7 to make this clear by reporting the transitions as decimal proportions in the final version. We have altered the captions to make the relations clearer.

Comment 13: *Figs. 8 and 10 - even though the relationships are not significant, adding regression line, p-value and r2 would be good*

Response: We refer the reviewer to an article on the use and misuse of regression by R. Webster (Webster, R. 1997. Regression and functional relations. *European Journal of Soil Science*, **48**, 557–566). Properly applied, regression is used to derive a predictive relationship of one variable from another or (in strictly limited circumstances) to calibrate a linear functional relationship. The regression line is not a suitable summary of the bivariate relationship between two variables unless one is measured without error. The regression line is therefore not a suitable decoration for the scatter plot. We can calculate the correlation coefficient, and report a p-value for the null hypothesis that is is zero, but this will reflect little more than the small size of our sample.

Comment 14: *I understand the driving for reporting the probabilities as the main outcome of this study from the modeling perspective, but can this probability information be somehow presented in units of actual distances? I believe it would be of interest to greater audience.*

Response: The transition probabilities are reported for one voxel transitions and the scale of the voxels is of side length of 6.6 µm. We only report and discuss these one-step transitions; transitions over larger scales could be computed and presented and we assume this is what the comment is suggesting, but we are not certain. We have

not undertaken these analyses to date and so we do not present them.

Comment 1: *Abstract should focus on the key message in concise form. The last paragraph of the introduction section should be brief with clear objectives. Discussion section should include validity of the experimental approach, justification of results obtained (i.e. porosity, pore shape, SOM volume, accessibility and soil respiration).*

Response: We consider that the original version of the abstract does focus on the key message, and does so concisely with clear objectives. In order to be self-explanatory the abstract requires some context for the study and not just results. The discussion section in our original version of the manuscript focussed on the wider implications of our findings, putting them in the context of other work and exploring options for further analyses of our data. We have included as part of our new discussion section consideration of how we might proceed to determine the location and quantities of finely disseminated organic matter sorbed onto mineral surfaces as suggested by reviewer 1 in other comments below.

Comment 2: *The authors followed largely the staining and scanning protocol published by Peth et al. (2014). The authors haven't provided any experimental data to demonstrate that Os was preferentially taken up by SOM only not adsorbed on mineral matrix of the soil. The authors used aggregates from a Clay soil for their experiment. Low diffusivity of clay soil could preclude the flow of Os vapour to SOM but increase the chance of adsorption of the vapour on clay surfaces. Moreover, Os can also react with clay-SOM complex not only the particulate SOM (POM) in the aggregates. From the Figure 3 it is not at all clear (resolution is too coarse) whether Os adsorbed on mineral matrix or SOM or POM. In my view, much better presentation could be a thresholded image slice showing pores and SOM alongside with greyscale scanned image of that slice. It will be nice to see if the authors could separate the 3D distribution of SOM adsorbed on clay surfaces and POM..*

Response: Peth et al. (2014) demonstrated using the same methodology that Os was preferentially adsorbed to organic matter rather than clay minerals. We do not consider it necessary to repeat the same verification steps as Peth et al. (2014). In addition, as we freeze-dried our samples we consider there is more scope for the Os

vapour to diffuse into finer pores than may have been the case with the Peth et al approach in which small quantities of moisture would have remained in the finest pores following their use of air-drying. The separation/identification of SOM adsorbed on clay surfaces is beyond the scope of this paper and we do not state it as one of our objectives. The osmium retention by clay was addressed in our original manuscript by basing the threshold for organic matter classification – using differences between Os absorption above and below the adsorption edge – on the inferred volumetric organic matter content of the aggregate. This requires that SOM adsorbs Os more than does clay, not that there is no adsorption of Os by clay. See further response to this in comment 5 below. In the final (modified) version of the manuscript we present a thresholded image for the same slice as shown in the original Figure 3, as suggested by the reviewer.

Comment 3: *Another concern, POM and adsorbed SOM both contain carbohydrates, will this affect Os reaction with SOM? I think the methodological approach followed in this work requires a calibration/verification protocol. Authors could use X-ray spectroscopy to verify the SOM distribution they found in an image slice using Os staining and scanning. A standard sample with known distribution of SOM or POM can also be used to verify the method presented in this paper.*

Response: In their paper, Peth et al. (2014) stated: 'We selected osmium as a staining agent as this reacts with unsaturated C-bonds of organic compounds ....including finely disseminated organic matter often absorbed onto clay mineral surfaces and not visible as discrete organic particles (Chenu and Plante, 2006). They showed that they could detect Os-staining of both POM and finely disseminated SOM and validated the method using SEM-based EDX X-ray analysis (see Figures 3 and 5 in Peth et al (2014)). Therefore we do not consider it necessary to undertake another validation of the approach.

Comment 4: *Authors presented that SOM occupied >50% of total aggregate volume, although %SOM was 4-7%, which is very difficult to grasp and warrant a validation of the approach used.*

2

Response: The approach we developed to estimate the volume of organic matter in each aggregate was based on sound physical principles and accurate estimates of constants (such as the density of mineral matter (2.65 g cm^{-3})). The reviewer does not state on what basis he considers this approach to be flawed and without further detail we contend that our approach is justified and does not require further validation.

Comment 5: *Authors also need to present concentration of POM and SOM on silt+clay particles in their aggregates to justify the 3D distribution of SOM.*

Response: Some of this was also addressed in response to comment 2. We consider this to be beyond the scope of our paper. To our knowledge such an analysis has not been undertaken before and would require careful development in terms of the approach. We would need to identify an upper threshold size/volume/shape for sorbed organic matter and rules governing the size and shape of neighbouring mineral particles to select SOM on clay or silt particles. We have not modified our manuscript in this respect.

Comment 6: *Authors also need to present a thresholded image and greyscale scanned image to demonstrate their stepwise approach of image segmentation.*

Response: We have updated Figure 3 to show the thresholding, stepwise approach in the revised version of the manscript.

Comment 7: *Authors need to describe how the pores and stained SOM separated during phase segmentation of the image slices. Since the volume of SOM was calculated by subtracting volume of mineral phase from total volume of soil solid phase, accuracy of the image thresholding is very important.*

Response: We gave a detailed description of the segmentation of the image slices in the original version of the manuscript. We first separated the pores from the solid phase using a two component mixture algorithm. We then computed the the volume of organic matter and the differences in the adsorption values above and below the osmium adsorption edge to differentiate organic matter from mineral phases. The use of image thresholding based on the two-component mixture algorithm has a clear

theoretical basis for its application.

Comment 8: *Authors also referred 2.65 g cm^{-3} as bulk density of the mineral matter but should be written as particle density of the mineral particles. Moreover, the term density of organic matter is much preferable than 'bulk density' of organic matter.*

Response: We agree with this comment and we have amended the final version of the manuscript to reflect this.

Comment 9: *The figures presented in the article are not clear enough to show the distribution of pore geometry in the aggregates. The naming of 9 aggregates in Tables and Figures is not clear..*

Response: We believe this comment is directed at Figures 4 and 5. We needed a way to summarize the features of the pores (size and shape factor) and we consider that the boxplots presented do this effectively. We have undertaken further analyses of pore tortuosity and thickness/diameter and we present these new data in the final version of the manuscript (see response to comment 1 by reviewer 1).

Comment 10: *A graph with multiple lines showing pore volume against pore diameter in different aggregates, I think would be much more informative than presenting Figure 4 as boxplots.*

Response: This analysis is not possible based on the outputs from the 3D objects counter function from the BoneJ package which is routinely used for pore analysis. We consider that the boxplots in Figures 4 and 5 are informative as they summarize the data for each aggregate. We have also computed pore diameters for all 9 aggregates in response to comment 1 of reviewer 1 and we include these data in our modified version of the manuscript.

Comment 11: *Figure 5. Is it possible to extract images of different pore shapes of aggregates using threshold pore images? Authors can use threshold images to demonstrate the variation in pore shape and then distribution of different shapes in aggregates.*

Response: We described how we applied the 3D objects counter function in BoneJ (see section 2.5.4) to extract pore volume and surface area to compute pore shapes

for each pore structure from a regular block within each aggregate. This is based on the pore:solid phase threshold images. Our aim here was to summarise the overall features of the pore size and shape for each aggregate so they could be compared and we consider that this was achieved effectively.

Comment 12: *Figure 6: Authors should focus on transition between SOM and pores. I feel it would much better if the authors could translate transition probability values in a form understandable for wider audience.*

Response: We do focus on the transitions between organic matter and pores in Figure 7 (see next comment). We describe how to compute transition probabilities in our Methods section and we consider that the majority of readers would be able to understand this based on the mathematical notation which is not particularly complex.

Comment 13: *Not clear why Figure 7 is included in the text*

Response: Figure 7 presents, in a more detailed form than Figure 6, the transition probabilities between organic matter centred voxels (O) and the other phases. We consider this plot is useful as the reader can see clearly how these important properties, which have never been computed at the aggregate scale before, vary between the nine aggregates. Note we have improved this Figure based on a comment by reviewer 1.

Comment 14: *Figure 8 and 10: Dull scatter plots, a simple regression equation with R2 value can covey the massage.*

Response: See our response to Reviewer 1's comment 13. We are sorry that the reviewer finds the scatter plot dull, but the use of regression lines for decoration (except in some circumstances which do not apply here) is statistically unsound. We do include the correlation coefficient, however. We have not changed the final version of the manuscript in this respect.

Comment 15: *If possible calculate pore connectivity from the dataset and plot it against SHR.*

Response: We consider this to be beyond the scope of the current paper but could be addressed in a subsequent analysis.

Comment 16: *Table 3: not clear why this table is needed. Authors need to present variogram model graphs showing the spatial variability of SOM in the aggregates. The graphs are more informative than the presented box plots in Figure 9.*

Response: We disagree with the reviewer on this point. We chose to focus on the range parameter of the variogram models because this is the main feature of the spatial variation. We considered that an effective way to summarize the range data for each of the nine aggregates and three phases was by presenting a boxplot of the data and we consider that these present the data very effectively. Individual models for each region of each aggregate would confuse the reader in our view. We have not changed the final version of the manuscript.

Comment 17: *Authors incubated aggregate samples in 37C for 24 hours and then measured the CO2 concentration of the headspace. The temperature was bit high to measure soil respiration and I suppose it gradually made the aggregates dry over 24 hours, which would affect the respiration rate.*

Response: Based on the literature we considered 37 °C to be an appropriate temperature for incubation. As the vials were sealed during the incubation phase we do not expect the soils would have dried substantially over this period.

Comment 18: *The authors wrote in many instances they used custom wrote scripts/macros in R and Fiji without presenting the codes. Authors may present the codes in supplementary material of the manuscript.*

Response: Yes we can provide these as supplementary materials.

References:

Chenu, C., Plante, A.F., 2006. Clay-sized organo-mineral complexes in a cultivation chronosequence: revisiting the concept of the 'primary organo-mineral complex. European Journal of Soil Science 57, 596-607.

Peth, S., Chenu, C., Leblond, N., Mordhorst, A., Garnier, P., Nunan, N., Pot, V., Ogurreck, M., and Beckmann, F. 2014. Localization of soil organic matter in soil aggregates using synchrotron-based X-ray microtomography, Soil Biology and

Biochemistry, 78, 189 194.

To: Steven Sleutel

From: Barry Rawlins - lead author

Date: 27th Sept 2016

Dear Steven

My co-authors and I have re-processed the data based on an assumed density of organic matter of 1.4 g cm^{-3} which was one of the main criticisms of the original version. This approach is detailed in the new version of the manuscript. By doing so we created a completely new partition of the three phases (mineral, pore and organic matter) in each of the nine aggregates. There is evidence to suggest this was effective because we now observe a strong negative correlation ($r = -0.98$) between aggregate bulk density and porosity. I am confident this has significantly improved the paper which we now think is ready for further consideration. In revising the new manuscript we addressed all the other comments made in the two referee reports that we referred to in our original responses. I have also responded to each of your topical editor comments below:

Comment: *My major concern is the one as R2Comm4: The volume % of organic matter in the studied aggregates was unusually large. But much seems to be related to the spatial resolution of 3µm. To most readers it would appear that the organic matter fraction was strongly overestimated by the adopted Os-staining procedure and that OM densities of 0.4g cm-3 are extremely low. Usually adopted values are 1.4 (e.g. Chenu et al., 2006 EJSS). Is this discrepancy explained by the simple fact that you are considering bulk densities of OM? This would imply that much of the aggregate volume classified as OM in fact includes air inside sub-resolution pores as well. So the question remains how much of the aggregate volume was approximately overestimated to be either mineral or organic because pores <3 µm were misclassified as being OM or mineral matter? This needs to be addressed still in the manuscript and it should be*

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stipulated that the OM densities in table 1 represent apparent bulk densities.

Response: We have re-processed the data using an OM density of 1.4 g cm^{-3} and we think this has significantly improved the accuracy of the estimates for the three phases. We therefore no longer present OM density values in Table 1.

Comment: *I concur with the authors that no new proof needs to be presented for the validity of the Os-staining procedure (R2Com2 & 3). Nevertheless the authors should acknowledge that Os-staining of OM sorbed onto mineral particles could have resulted in misclassification of voxels that are partly OM and partly mineral matter as being entirely organic. There is no way of confirming this because such edge effects are only visible at finer spatial resolutions. This knowledge should, however, be taken along when critically assessing the quantified volumes of organic phase and OM densities. Most likely the OP and MP values are also strongly underestimated as compared to the physical reality in which many sub-resolution pores are in contact with both organic and mineral phases, especially so in clay soil. So again the computed probabilities are estimates based on CT-images, which also only apply to the spatial resolution at hand: The probability of OM to be in contact with a pore >6.6 μm is 0.02-0.03. The actual OP at finer resolution is likely much larger. This needs to be properly explained in the discussion and certainly in Figure 7s caption.*

Response: We have included a note in the caption to the Figure which displays the transition probabilities (now Figure 6) that these estimates must be understood at this resolution and that the values might be different at finer scales. We also refer to this in the Results and Discussion sections.

Comment: *The value of calculating semi-variogram ranges for all three soil phases is less apparent. I found Fig. 9 overloaded and table 3 redundant. There is really no need to present semi-variogram ranges for the mineral and pore phases so please remove these from Fig. 9 and omit Table 3. You could indicate the median range values (in μm) within Fig. 9.*

Response: We have removed the range estimates for mineral and pore phases from Table 3. However, contrary to your comment, we felt that it is useful to present

the ranges for all three phases in (new) Figure 8. We think readers would want to understand how the variations differ among the three phases within aggregates at these fine scales and we comment on this in the Results and discussion section in relation to the vast majority of variation occurring at scales of <250 µm.

Comment: *The statement The transition probabilities between OM-centred voxels and adjacent pore voxels a measure of OM accessibility were both small and of limited variation (probabilities between 0.02 and 0.03) for all aggregates and there was no clear relationship between accessibility and the magnitude of SHR. In the conclusions stands a bit isolated. So what is your conclusion now: is it still worthwhile calculating transition probabilities? What about calculating these for transitions in future from OM to specific pore size classes*

Response: The transition probability statements have been updated to reflect the new values reported in the revised version.

Comment: *The statement shorter length scales of OM variation leads to greater frequencies of microsites and greater CO2 production through microbial respiration and we present preliminary evidence to support this relationship. in the conclusion is interesting. In practice a larger range could represent the presence of more larger particulate OM particles, with many OO transitions over considerable distance (even milimeters). If aggregates contain much particulate OM the OM variation scale / respiration relation could also be inverse: fresh organic substrate particles encapsulated in aggregates (e.g. plant litter) may be more labile, resulting in a higher soil respiration than in a soil aggregate with an equal amount of OM, which is instead mineral-bound. I would be happy if the authors could comment on this in their discussion.*

Response: On re-processing the data – using a OM density of 1.4 – we no longer observe the significant relationship between length scale and SHR. We do not therefore consider it appropriate to comment on the potential relationships between length scale, size of organic matter in aggregates and their magnitude of SHR because this would be only speculative.

Comment: *R1Com1. I agree to referee 1s main comment. The provided pore shape*

factor F on its own does not well quantify phenomena like tortuosity. I also follow the authors in that data presented in Figs. 4 and 5 should not be omitted.

Response: We have undertaken new analyses (since the original version) and present data for pore diameter (distribution) and tortuosity in the new version of the paper.

Comment: *R1Com4 please add a sentence explaining the purpose of beading the aggregate in glass beads*

Response: We have added this sentence to the new version.

Comment: *R1Com5 I appreciate the authors initiative to as far as possible enable reproduction of their work by detailing the workflow of the CT volume processing. However, section 2.5.1 was pertinent because of the specific packing of aggregates in quartz beads. The relevance of this section may be small in future experiments with other ways of packing soil samples. So I would ask the authors to move the stepwise listing presented in 2.5.1 to supplementary material.*

Response: We have moved the stepwise description to supplementary material file.

Comment: *R2Com15 ok with authors response, but could this point not be added to a perspective for a future relevant avenue?* **Response**: We have added a sentence on this in the new version.

Three dimensional soil organic matter distribution, accessibility and microbial respiration in macro-aggregates using osmium staining and synchrotron X-ray CT

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Abstract. The spatial distribution and accessibility of organic matter (OM) to soil microbes in aggregates – determined by the fine-scale, 3-D distribution of ~~organic matter~~OM, pores and mineral phases – may be an important control on the magnitude of soil heterotrophic respiration (SHR). Attempts to model SHR at fine scales requires data on the transition probabilities between adjacent pore space and soil OM, a measure of microbial accessibility to the latter. We used a combination of osmium staining and synchrotron X-ray CT to determine the 3-D (voxel) distribution of these three phases (scale 6.6 μm) throughout nine aggregates taken from a single soil core (range of organic carbon (OC) concentrations 4.2-7.7 %). Prior to the synchrotron analyses we had measured the magnitude of SHR for each aggregate over 24 hours under controlled conditions (moisture content and temperature). We test the hypothesis that larger magnitudes of SHR will be observed in aggregates with i) shorter length scales of OM variation (~~i.e. more frequent, and possibly more finely disseminated, OM and a larger number of~~ more aerobic microsites) and ii) larger transition probabilities between OM and pore voxels.

After scaling to their OC concentrations, there was a six-fold variation in the magnitude of SHR for the nine aggregates. The distribution of pore ~~volumes, pore shape and volume normalised surface area~~ diameters and tortuosity index values for pore branches were similar for each of the nine aggregates. The Pearson correlation between aggregate surface area (normalised by aggregate volume) and normalised headspace C gas concentration was both positive and reasonably large ($r =0.44$), suggesting the the former may be a factor that influences SHR. The overall transition probabilities between OM and pore voxels were between ~~0.02 and 0.03, significantly~~ 0.07 and 0.17, smaller than those used in previous simulation studies. We computed the length scales over which OM, pore and mineral phases vary within each aggregate using 3D indicator variograms. The median range of models fitted to variograms of OM varied between ~~178 and 487~~ 38 and 175 μm ~~. The linear correlation between these median length scales of OM variation and the magnitudes of SHR for each aggregatewas −0.42, providing some~~ and were generally larger than the other two phases within each aggregate, but in general variogram models had ranges <250 μm. There was no evidence to support ~~our hypothesis. We require a larger number of observations to make a statistical inference~~the hypotheses concerning scales of variation in OM and magnitude of SHR; the linear correlation was 0.01. There was ~~no~~ weak evidence to suggest a statistical relationship between voxel-based OM:pore transition probabilities and the magnitudes of

aggregate SHR ~~. The solid-phase volume proportions (45-63 %)of OM we report for our aggregates were surprisingly large by comparison to those assumed in previous modelling approaches. We suggest this requires further assessment using accurate measurements of OM bulk density in a range of soil types.~~

(r=0.12).

1 Introduction

In soil heterotrophic respiration (SHR) microbes utilise the carbon in soil organic matter (SOM) as an energy source, releasing gaseous CO_2 which accumulates in the soil at significantly larger concentrations than in the atmosphere (**?**). Ultimately this excess CO_2 is released to the global atmosphere. It is important we understand the processes that determine variations in the magnitude of SHR because it influences the flux of carbon dioxide (CO_2) from soils to the atmosphere, an important part of the global carbon cycle with major implications for global climate change (**?**). The turnover of SOM is also an important control on the cycling of other macronutrients, notably nitrogen.

It has been suggested that it is essential to understand the influence of microscale intra-aggregate heterogeneity of soil properties to ensure that organic matter (OM) mineralisation can be modelled effectively (**?**). The majority of soil microbial communities reside in pore networks within soil aggregates which are three-dimensional (3-D) agglomerations of mineral particles, varying in size, that form a hierarchy (**?**) with small, micro-aggregates (<250 μm) forming larger, macro-aggregates (> 250 μm). Soil aggregates consist of complex mixtures of SOM, mineral particles, pore space, microbes and moisture. The accessibility of SOM to microbial communities (substrate availability) is determined by the distribution of pores (**??**) which also also determines water potential and the flux of oxygen. Soil matric potentials vary over short scales due to the varying size of pores, with microbes concentrated at the interfaces between air and water. Decomposition rates of SOM may therefore be influenced by moisture content (**?**)), pore size and location within an aggregate (**?**), and also by temperature and substrate quality (**?**), and microbial properties (**?**).

The majority of SOM utilised by soil microbes, the former both as large individual particles and more finely disseminated material associated with minerals, occurs both on the surfaces of, and within, soil aggregates (**?**). In controlled laboratory experiments, the magnitude of SHR has been shown to vary considerably between soil aggregates (**?**). It has been suggested that the location of OM within soil aggregates may be a significant factor governing the magnitude of OM mineralisation in soil (**?**). Microsites for aerobic SHR occur where SOM and pores are adjacent to one another in soil aggregates, but to date their frequency and spatial distribution have not been established within macro-aggregates. If a large proportion of intra-aggregate SOM is occluded by minerals so that microbes cannot utilise it, there will be fewer interfaces between SOM and pores and the magnitude of SHR for such aggregates may be smaller than those in which SOM is more accessible (a larger proportion of pore:SOM interfaces). In a recent study, **?** asserted that soil structure may be of limited importance in determining rates of SHR at the scale of the soil core. The authors created soils with differing structural properties (undisturbed versus disaggregated and sieved) and showed that after the structural peturbations had dissipated, there were no significant differences in SHR rates for

both native and added soil carbon. However, the observed increase in rates of SHR following disturbance also implies that soil structure does exert an influence on rates of microbial SOM minerlisation.

Another feature of soil aggregates that may influence the magnitude of SHR is the size and distribution of its SOM including particulate organic matter (**?**). Consider two aggregates, with the same concentration of SOM, removed from a single soil core. In the first aggregate the SOM consists of small, finely disseminated material that occurs frequently over short length scales, whilst in the second there are fewer, larger particles of SOM with larger distances separating them. We hypothesise that in the former aggregate there will likely be a larger number of microsites leading to a greater magnitude of SHR compared to the latter. This hypothesis could be tested by determining the magnitude of SHR in such aggregates if it were also possible to subsequently determine the length scales over which the SOM is distributed in these aggregates. ~~The~~ Although the statistical methods are well established for doing so, there are currently few laboratory methods for establishing the 3-D spatial variation of ~~SOM, mineral and pore phases in aggregates can be investigated using: i) geostatistics and ii) determining whether there are Representative Elementary Volumes (REVs)where properties are computed for increasing scales and in which smaller volumes are nested within larger volumes~~intra-aggregate OM distribution (see below).

To date, relatively few experimental approaches have been applied to determine: i) the accessibility of soil OM in macro-aggregates, and ii) whether the accessibility and of SOM in soil aggregates exerts a strong influence on microbial SHR at the macro-aggregate scale. This is in part because scientists have lacked methods for fine (<10 µm) scale 3-D discrimination between minerals and SOM within aggregates. Approaches to date have generally been limited to mapping SOM in two dimensions (**?**), or in 3-D within smaller regions of aggregates (**?**). In a recent study, **?** used a combination of X-ray computed tomography (CT) and scanning electron microscope images to map soil chemical composition, including soil carbon, at a resolution of 15.8 µm in a small block of soil (side length of 1 cm). An alternative approach was recently demonstrated by **?** where differences in X-ray absorption above and below the osmium (Os) edge — using synchrotron beamline X-ray computed tomography (CT) – was used to discriminate between OM, and mineral phases in aggregates of around 2-3 mm diameter, at a resolution of 9.77 µm.

In this paper we report the results of applying and extending the Os-staining and synchrotron X-ray CT method developed by **?**. Specifically we establish the 3-D distribution of mineral, SOM and pore space throughout nine macro-aggregates from a single soil core at fine (6.6 µm) length scales. To our knowledge such data have never been analysed geostatistically to determine the 3-D length scales over which SOM, minerals and pores vary both within and between aggregates. In so doing we establish the magnitude of any structural differences between the nine aggregates. Prior to the synchrotron X-ray CT analyses we measured the magnitude of SHR of each aggregate in the laboratory by measuring headspace CO_2 concentrations after incubating the aggregates in separate vials, having controlled for both temperature and moisture content. We have determined the accessibility of SOM within each aggregate by computing the transition probabilities ~~of~~ between adjacent SOM and pore ~~voxles~~voxels, and also ~~transition probabilities between the other phase combinations using the 3-D voxel classification.~~ the diameter and tortuosities of the pore networks. We used the SOM-pore transition probabilities as an index of SOM accessibility to determine whether it is strongly related to the magnitude of SHR for each aggregate, the latter scaled by total organic carbon (TOC) content. We also computed the length scales over which SOM varies in each aggregate and compared this to their

magnitudes of SHR, again scaled by aggregate TOC content. We discuss the implications of our findings for empirical and modelling studies which aim to (respectively) quantify and simulate the magnitude of SHR at the macro-aggregate scale.

2 Materials and methods

2.1 Aggregate samples, preparation and respiration measurements

95 An intact, cylindrical core of soil (diameter and length 50 mm) was collected from a field that had been under pasture for more than 15 years (British National Grid reference metres; Easting 463374, Northing 331992) in Keyworth (near Nottingham, UK). The upper edge of the soil cylinder was inserted (at a depth of 5cm below the top of the mineral soil horizon) into a vertical soil face that had been exposed with a spade (the lower level of the cylinder was at a depth of 10cm below the top of the mineral horizon). The parent material of the soil at this site is a mudstone and the soil is a Luvisol (**?**) in texture-class
100 Clay (**?**) based on a particle size analysis of material from a soil core collected from a location adjacent to the sampling site. After return to the laboratory (at room temperature) the intact core was removed from its container and placed on a plastic sheet. The core was broken apart by hand to separate large aggregates along natural fracture surfaces and also those formed by fine roots. This procedure yielded aggregates of different sizes. We selected a subset of 9 aggregates which had shapes that were approximately either cubes or spheres with side length or diameter (respectively) of approximately 5-6 mm. We visually
105 inspected each aggregate and rejected any which appeared to be dominated by a large single particle (e.g. a stone fragment).

We weighed each aggregate in a pre-weighed and labelled weighing boat and then placed each on a saturated 1 bar pressure plate (Soil Moisture Equipment Corp, Santa Barbara, CA) for 20 minutes so that the moisture content of each aggregate would increase markedly. We then set the pressure plate to 0.5 bar (-50 kPa) for 5 hours so that each aggregate had the same moisture content, slightly less than field capacity. After removal from the pressure plate the aggregates were placed inside
110 a filter insert (Costar (R) Spin-X Centrifuge Tube, Sigma-Aldrich, UK) which had been partially filled with 500 μm quartz beads (Sigma Aldrich, UK). A further small quantity of quartz beads were ~~placed on top of the aggregate~~ added to ensure each ~~was surrounded by beads~~ aggregate was surrounded and the insert (including quartz beads and aggregate) was re-weighed. We added quartz beads to prevent the aggregates from fragmenting at either the freeze-drying stage or during transport to and from the synchrotron.
115 The filter inserts were then placed inside a glass headspace vial (Thames Restek, Pennsylvania, USA) each of which had been filled with an equal quantity of quartz beads to reduce the volume of air in the vial. A crimping tool was used to seal the headspace vials with an aluminium seal and the vials were placed in an incubator at 37° C for 24 hours. After removal of the vials from the incubator, the concentration of CO_2 in the headspace of each vial was determined by removing a subsample of gas using a syringe and injecting it into a gas chromatograph (porapak column Q 80/100 mesh in Agilent GC 7820) which had
120 been calibrated with CO_2 standards of 100, 500, 1000 and 2000 mg l^{-1}. We subtracted the background concentration of CO_2 (400 mg l^{-1}) from each headspace analysis to give the excess CO_2 due to respiration. The septa and seals were then removed from the headspace vials and the aggregate and filter holder placed into a Costar (R) Spin-X Centrifuge Tube (total volume 2 ml; see Figure 1). The un-capped centrifuge tubes were placed in a freeze drying unit for 48 hours until any moisture in the
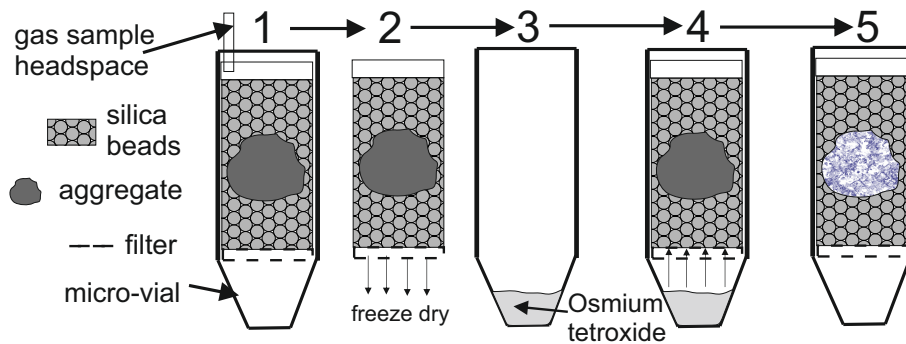
Figure 1. Schematic diagram showing a series of five steps in the treatment of the aggregates in their vials: 1) headspace gas removal, 2) freeze-drying, 3) & 4) staining with OsO_4 and 5) scanning in the synchrotron beamline.

vials had been removed completely. By removing all moisture from the aggregates we wanted to ensure that the OsO_4 would
125   diffuse completely through the soil aggregate pore space. The filter inserts were then reweighed so that the dry mass of each aggregate could be calculated.

2.2   Osmium staining of soil organic matter

The aggregates (each inside a filter insert and centrifuge vial) were placed inside a fume cupboard so that osmium tetroxide (OsO_4) could be used to stain the OM in each of the soil aggregates (**?**). A set of strict health and safety procedures were
130   adopted due to the hazardous nature of OsO_4 but we do not describe these in detail here. Half a millilitre of OsO_4 was pipetted into the bottom of each centrifuge vial and the vials sealed with caps. Each was left for 48 hours inside the fume cupboard during which time the Os diffused through the base of the filter, through the glass beads and Os was adsorbed preferentially by the carbon bonds in the OM. A schematic diagram showing the main steps in this procedure is shown in Figure 1.

The filter inserts were then removed and wiped clean and the top of each filter insert was sealed using caps and Araldite
135   resin. Each filter insert was then fixed to bespoke stainless steel supports using Araldite resin so that the filters could be placed into a synchrotron beamline.

2.3   Synchrotron X-ray CT analysis

Each of the nine aggregates inside the filter inserts were scanned using synchrotron X-ray CT at the Diamond Light Source (Harwell, UK) using the I12 beamline. Each aggregate was scanned at three energy levels: 53, 73.2 and 74.4 keV, the latter
140   were determined to be just below and above the K-absorption edge for Os, based on initial scanning of an osmium standard material. The 53 keV energy level provided an effective means of separating the solid and pore phases. The images for each horizontal slice through the aggregates were reconstructed yielding a set of 32-bit .tif files in which each pixel has an adsorption value for each energy level, and each pixel represents a 3-D voxel with side length of 3.3 μm.

2.4 Total Organic Carbon content of aggregates

145 After the aggregates had been scanned in the beamline they were carefully removed from their vials and their TOC content estimated using a Elementar Vario Max C/N analyzer at 1050 °C. Prior to measurement any inorganic carbon was removed from the aggregates by adding HCl (5.7 M), then dried at 100 °C for 1 hour. The limit of quantification for TOC for a typical 300 mg sample was 0.18%

2.5 Synchrotron X-ray CT data processing

150 All the synchrotron X-ray CT data was processed using the same protocol described here. The first procedure was to subtract the absorption values from the 73.2 keV energy level (below the Os absorption edge) from the images created from the 74.4 keV energy level (above the Os absorption edge; see **?**). This was undertaken using a script written in the R (**?**) and we subsequently refer to the resulting data and files as 'diffedge' (difference at the absorption edge). An example of the differences in absorption values above (74.4 keV) and below (73.2 keV) the Os absorption edge for one soil aggregate image slice example is shown in Figure 1. Note that the absorption values are generally larger above the Os edge, below the 1:1 line in Figure 2.
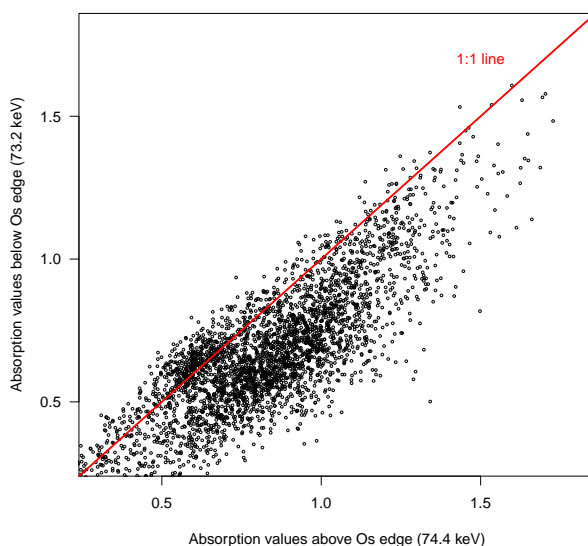
Figure 2. Raw absorption values above and below the Os absorption edge for one soil aggregate slice from the synchrotron X-ray CT beamline.

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6

2.5.1 Creating masks for aggregate slices

Prior to analysing the synchrotron data at the three energy levels it was necessary to create masks of the aggregate outlines so that pixels outside the aggregate could be excluded from the analysis. Where quartz beads (with similar density to soil material) occurred adjacent to aggregates in the synchrotron images, those pixels within the beads were replaced with background absorption values using the software package VG Studio Max prior to creating masks for these images. To create the masks we used a multi-stage procedure using scripts in the R environment and the image processing package Fiji (**?**) ~~: map the original 32-bit image into an 8-bit .bmp image and then apply a procedure for outlier removal written as an R script that was applied to all image slices of each aggregate. crop the image to extract a smaller sub-image that contains the aggregate using a macro written for Fiji. using a macro written in Fiji we segmented each image using statistical region merging (with parameter Q=between 10 and 25). apply binary segmentation to each image using the adjust threshold function to extract as much of the aggregate as possible. using the binary image we then used the 'fill holes' function in Fiji which provides a mask of the aggregate that contains cracks. to fill in the cracks we wrote a majority filtering code in python and applied it several times until the cracks in the aggregate masks were completely filled.~~ described in the supplementary material file.

Each mask was saved as an 8-bit .tif file. We wrote an R script using the raster package (**?**) to crop all the original images (nine aggregates each with two sets of images: i) 53 keV, and ii) the diff edge images) using the masks for each associated image slice, setting all the values outside the cropped region to a constant value.

2.5.2 Reduction of raster resolution

The original synchrotron image files comprised 2544×2544 pixels with each pixel measuring 3.3 µm and and between 1400 and 2600 slices in each aggregate image stack. We found that it was challenging to process these data using large 3-D numeric arrays so we chose to reduce the resolution of the image stack by 50% in each dimension. By doing so we achieved an 8-fold reduction (i.e. a 2-fold reduction in each dimension) in the size of the numeric array. Aggregation was carried out from top to bottom for each of two neighbouring layers (e.g. layer 1&2, 3&4, ...). The aggregation process was carried out in three steps. All these steps were applied to the images from the 56 keV energy level and the diffedge files. First the masks were applied to each file and adjacent layers were aggregated horizontally (2-D) by a factor of 2 using the aggregate function in the raster package (**?**) using the mean function to compute the mean of the layers. Then the two horizontally aggregated layers were averaged vertically yielding a matrices of dimension 1272×1272. Any averaged outlying values greater than 10 were set to a value of 10. The matrices were combined into a 3-D numeric array with the third (vertical dimension) equal to the half the number of slices in the original data. These processing steps were undertaken using an MPI computer cluster.

2.5.3 Segmentation of solid and pore phases

~~We undertook exploratory analysis of the data in each horizontal slice of the 3-D numeric array at the 56 keV energy level. We plotted the frequency distribution of the absorption values and observed two distinct but overlapping distributions, one with a smaller mean value (pores) than the other (solid phase) . To segment the solid and pore phase distributons~~ An initial approach

to segment solid and pore phases in each aggregate slice based on a two-component mixture algorithm were inconsistent with realistic assumed values for organic matter particle density (**?**) . So we used: i) our measurements of total organic matter, ii)

190 the aggregate volume computed from the X-ray CT scans and, iii) assumed values for the mineral particle and organic matter densities, to compute the proportion of pore space in each aggregate using the following approach.

We computed the mass of mineral matter (M_m) by subtracting the mass of OM (M_{om}; two $\times$ TOC content; (**?**)) from the total mass of the aggregate. We assume a particle density of the mineral particles of (D_m) to be 2.65 g cm^3 (**?**) so we calculated the volume of mineral material (V_m) in each aggregate ~~slice we wrote an R script using the mixtools package (?),~~

195 ~~specifically utilising the *normalmixEM2comp* function which is a fast algorithm for two-component mixtures of univariate~~ ~~normal distributions. The algorithm requires starting values of the model parameters which, in this case, are the mean and~~ ~~variance of~~ as:

$$V_m = \frac{M_m}{D_m} \tag{1}$$

We assume an OM density of 1.4 g cm^{-3} (**?**) . We computed the volume of OM (cm^{-3}) by dividing the mass of OM by its

200 density.

$$D_{om} \tag{2}$$

205 In doing so we were able to determine the proportions of OM and mineral volumes in the ~~data values for the~~ solid phase, and the volume of pore space (V_p) as the difference of their sum from the total aggregate volume (V_{ag}):

$$V_p = V_{ag} - (V_{om} + V_m) \tag{3}$$

We computed for each aggregate the number of voxels equivalent to the total volume of pores. We then computed the threshold adsorption value at 56 keV that equated to the transition from pore (smaller adsorption) to solid phase (larger adsorption) and

210 assigned each voxel a pore ~~and solid phase , and their relative proportions. We assigned each pixel to either pore~~ or solid phase ~~based on the class with the largest posterior probability density under the mixture model~~classification based on whether its values was above or below this threshold. Using these classes we created a series of .tif image files storing the two-fold solid:pore classification, with a third class value for pixels outside the aggregate.

2.5.4 Porosity, surface area, ~~pores sizes~~ pore diameter and ~~shapes~~tortuosity index

215 We used the images from the segmentation of solid and pore phases to compute a series of physical properties for each aggregate. Total porosity and surface area on planar/volume images were calculated using a bespoke Win32 computer program

minkowski.exe that includes estimation algorithms published in **?**. The bespoke software was originally developed and verified by Alasdair Houston at SIMBIOS (Abertay University) as part of a research degree programme. ~~We used the *3D Objects counter* plugin (**?**) for Fiji to compute the volume of each pore in the maximum regular block of voxels that could be extracted~~

220 ~~from each of the nine aggregate arrays. We also used the surface area and pore volume outputs from *3D Objects counter* to compute the pore shape factor (F) as:~~

$$F = \frac{Ae}{A}$$

~~where A_e is the surface area of a sphere with a volume equal to that of the pore and A is the measured pore surface area (**?**). A value of 1 for the F parameter represents a sphere, whilst smaller F-values refer to more irregular or elongated pore shapes.~~

225 **2.5.5** ~~**Separation of mineral and organic matter phases**~~

~~Prior to the measurement of TOC, we had removed all the water from the aggregates by freeze-drying so we can compute the mass of mineral matter (M_m) by subtracting the mass of OM (two × TOC content; (**?**)) from the total mass of the aggregate. We also know the bulk density of mineral matter (BD_m) in soil to be 2.65 g cm^3 (**?**) so we calculated the volume of mineral material (V_m) in each aggregate as:~~

230 $$V_m = \frac{M_m}{BD_m}$$

~~We were then able to compute the volume of OM by subtracting the volume of mineral material from the total volume of the solid phase (mineral organic matter; section 2.5.3) that was computed using the Gaussian mixture model. In this way we were able to determine the proportions of OM and mineral volumes in the solid phase. Given that we know the volume of OM (V_{om}) in each aggregate and its mass (M_{om}; total aggregate mass minus the mineral mass), we can compute the bulk density of the~~

235 ~~OM (BD_{om}) in each aggregate as:~~

$$V_{om}$$
$$($$

~~In doing so we were able to check that the bulk density values for OM were consistent with previously published data,~~

240 ~~taking into account any differences in moisture content. To achieve our objective of separating~~ Pore diameters were computed using a combination of the BoneJ (**?**) plugin for Fiji and a bespoke program which uses the using the method of Maximal Inscribed Balls (**?**) to convert the BoneJ mask thickness map into pore diameters. We used the Fiji plugin *AnalyseSkeleton* (**?**) to determine the tortuosity index (TI) of pore branches within each aggregate, computed as the length of the pore branch divided by the Euclidean distance between their furthest ends.

245 **2.5.5 Separation of mineral and organic matter phases**

To separate all the solid phase voxels into either mineral or OM classes we used: i) the diffedge data values for each aggregate, and ii) the proportions of mineral and OM volumes in each aggregate. Those voxels with the largest values in the diffedge numeric array for each aggregate were assigned an OM classification, and the proportion of solid phase voxels assigned as OM was the volume proportion of OM in each aggregate (see Table 1). The other solid voxels were assigned a mineral classification.
250 We reclassified the orignal mineral and pore classified arrays for each aggregate (section 2.5.3) into a three-fold classification (mineral, pore and OM).

2.6 Statistical and geostatistical analyses

2.6.1 Transition probabilities

We assume that the critical locations in soil for microbial respiration are the interfaces between (voxels of) OM and pore
255 space (**?**). We ask the question: ~~how many such voxel interfaces are there per unit volume of soil? We define an OM/pore interface voxel as an OM class voxel from which one can make a one-step transition to a porevoxel. For the present we ignore direction of transition. The (transition) probability that a voxel is at~~ given that a particular voxel corresponds to OM, what is the probability that a neighbouring voxel corresponds to pore? Consider a pair of neighbouring voxels. The probability that they constitute an OM/pore interface ~~is~~ can be written $P(OM,pore)$. If the probability that a ~~pair of adjacent voxels are~~
260 ~~OM and pore, which we refer to as $P(OM|pore)$~~randomly selected voxel corresponds to class OM is written as $P(OM)$ then $P(OM,pore) = P(pore|OM)P(OM)$, where the first conditional probability is the probability that a randomly selected neighbour of an OM voxel corresponds to pore space. We call it the transition probability from OM to pore. ~~We computed transitions probabilities for an arrangement where we consider the 26 voxels around a central OM voxel . Consider a cube with side length of 3 voxels, giving a total of $3^3 = 27$ voxels in total, with $27 - 1 = 26$ transitions from the central voxel.~~ We use the
265 transition probability between OM and pore as a quantitative measure of OM accessibility. If one normalizes the respiration rate of an aggregate relative to its total organic content (effectively allowing for differences in $P(OM)$ between them), then it may be hypothesized that differences between the aggregates with respect to this response depend on the transition probability $P(pore|OM)$.

We computed transition probabilities for the 3-D numeric arrays in which each voxel was one of four classes: 0=mineral (M),
270 1=pore ($pore$), 2=organic matter (OM) and 9=mask. The total of the three transition probabilities (~~$P(pore|pore), P(M|pore)$ and $P(OM|pore)$~~ $P(pore|OM), P(M|OM)$ and $P(OM|OM)$) is one. We wrote a script in R that progressed from the from top to the bottom of the numeric array, starting from the second layer and ending at the penultimate layer. For every iteration three neighbouring layers were used (one above, a central layer, and one below) avoiding the outermost rows and columns of the 3-D array in the analysis. All three layers were simultaneously shifted by one pixel around their initial position
275 in the x and y directions, while for every offset combination the voxel classification was queried and concatenated with the classification of the original non-shifted central layer which shared the same spatial location (x and y). The class comparisons always originated from the central voxel to ~~either the six main facing voxels or the~~ the voxels of the surrounding complete $3 \times$

3×3 array subset. For the ~~six main face voxels we computed transition probabilities for all nine phase combinations, including transitions to and from the same phase. For the~~ 26 neighbouring voxels, we ~~only~~ considered transitions from the central OM voxels to the neighbouring 26 voxels. In addition to the voxel class, we also recorded the direction of transition ~~was~~ because: i) this was necessary to remove the combination where the central layer was compared with the non-shifted central layer, and ii) in future analyses (not in this paper) we may wish to undertake directional analyses of transitions. We computed the frequency of each class combination (transition) for each layer comparison and in a final step the frequency of each transition and layer comparison was computed.

In a recent study, **?** used the proportion of OM-centred voxels which had at least one transition to a pore voxel in adjacent voxels (in our case there were 26) as a measure of OM accessibility, and so we computed these proportions for our nine aggregates. To distinguish this additional measure of accessibility from the transition probabilities, we refer to these former values as the minimum threshold OM-pore proportions.

2.6.2 Indicator variograms and variogram models

To understand the length scales over which the three phases vary, we computed 3-D indicator variograms (**?**) for each phase and for each aggregate using scripts written in R with the *gstat* package (**?**). Using the 3-D numeric arrays in which each voxel had been classified as either mineral, organic matter, pore or mask (exterior) we chose a random starting point within the numeric array of each aggregate and selected a cube of voxels with ~~side length~~ 200 voxels in each dimension around this point. We then checked that less than 10% of the voxels were classed as mask. If mask values accounted for more than 10% of all voxels, a new random starting point was selected until this condition was met. We converted this 3-D array into a *gstat* object (x, y and z coordinates plus the voxel class), and excluded the exterior voxels. To compute indicator variograms for each phase we recoded the phase classes so that a single phase took a value of one, and the other phases were set to zero. We then randomly selected a subset of 50 000 voxels with which to estimate the indicator semi-variances for each phase at a series of increasing lag intervals up to a maximum of 250 voxels. We plotted a set of indicator variograms for each phase and fit a range of single authorised variogram models to them (**?**) . In all cases the exponential model gave the best fit and so we computed and recorded the parameters of the exponential model fitted to each set of indicator semi-variances. For the model range parameter, we recorded the effective range which in the *gstat* package is three times the theoretical range reported. We repeated this procedure 50 times for each of the three phases in each of the nine aggregates to ensure that we encompassed the variation inside each aggregate. We note that each indicator variogram may not be independent because in some cases the starting points may be sufficiently close for the 3-D arrays of ~~side length~~ 200 units in each dimension to overlap.

11

3 Results and their interpretation

3.1 Aggregate properties and respiration rates

A set of properties determined for each of the nine soil aggregates are summarised in Table 1. There was an approximate two-fold variation in the quantity of TOC in the nine aggregates (range 4.18-7.7%), whilst the magnitude of respiration (based on the excess CO_2 concentration scaled to the TOC content) varies by more than a factor of 6 (range 10.5-65.9 µg C mg C^{-1}). There was ~~an approximate three-fold~~ a positive correlation between the magnitude SHR and TOC with a Pearson correlation of r=0.29. We do not place too great an emphasis upon this because we consider scaling the SHR TOC content to be a more relevant measure. There was an approximate two-fold variation in total porosity between the aggregates (range ~~4.4-11.1~~21.8-45.1%) but no clear relationship between total porosity and the magnitude of respiration scaled by TOC content.

At the scale of the soil core we have observed strong statistical relationships between topsoil bulk density and the square root of TOC for this soil type in a local cultivated field (**?**), but there was no similar relationship for our nine aggregates taken from a single core. ~~In addition~~However, there was ~~no strong~~ a strong negative statistical relationship between total porosity (based on averaging from the scan resolution of 3.3µm) and bulk density ~~. The bulk density values for the nine aggregates (range 1.29-1.83 g cm^{-3}) were generally larger than would be predicted from a pedotransfer function that uses the TOC content of soil cores (?), but this can be explained by the markedly larger porosity values (20-45%) that we reported at the soil core scale (?) from a cultivated field on the same soil type~~($r = -0.98$) for each of the nine aggregates. There was ~~limited~~ a substantial, four-fold variation in the surface area of the aggregates after it ~~has~~had been normalised by aggregate volume (range ~~10.1-15.6~~16.7-69.6 mm^2 mm^{-3}; see Table 1). The Pearson correlation between surface area normalised by aggregate volume and normalised headspace C gas concentration was both positive and reasonably large (r =0.44) suggesting the the former may be a factor that influences the latter.

Figure 3 shows distribution of the OM, pore and mineral phases in one aggregate slice. Using our approach to computing the volume of ~~mineral and~~organic matter in each of the aggregates (section 2.5.5)~~it is noteworthy that the organic matter comprises a large proportion of the solid volume (48.5-63.4%). Also, the dry organic matter bulk density values (following freeze drying) are larger (range 0.35-0.81 g cm^{-3}) than those estimated by **?** from soil cores (0.207-0.311 g cm^{-3}) over moisture contents ranging from field capacity (−~~, it exhibits a two-fold variation between 10 ~~kPa) to a water potential of −20 kPa. The value of 0.81 g cm^{-3} for aggregate number 76 is anomalous and we cannot account for it based on the other physical properties we report in Table 1.~~ and 19.5%.
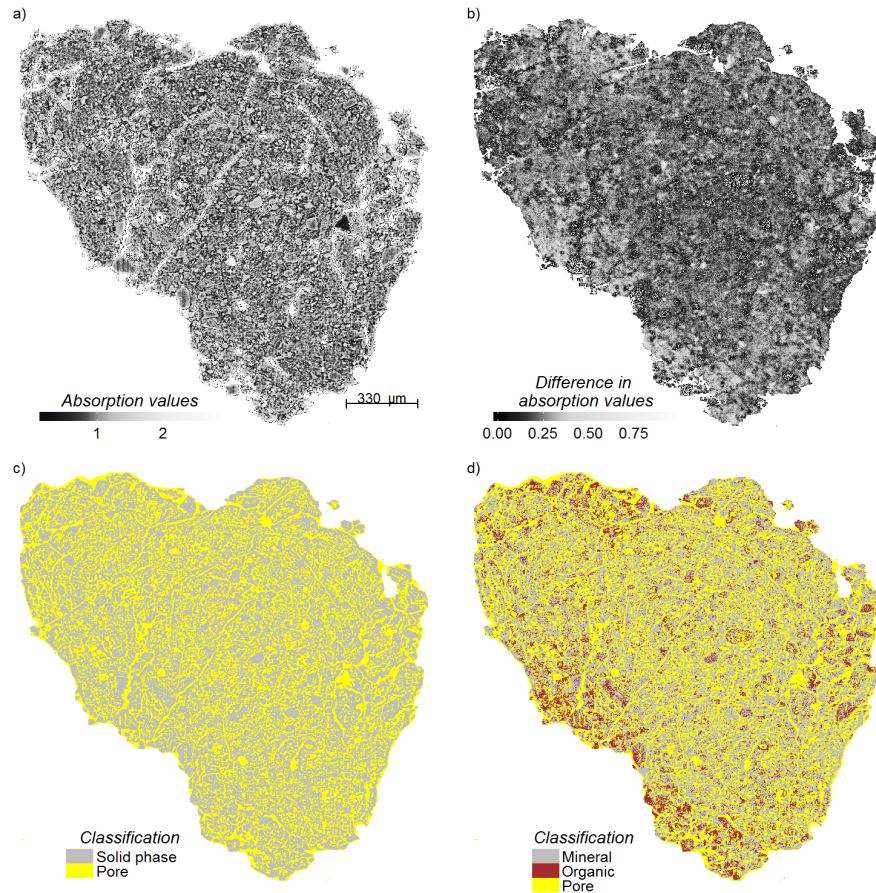
Figure 3. ~~An example horizontal~~ Horizontal cross-section through one ~~of the~~ aggregate showing: a) the raw absorption values at the low energy (~~number 4056 keV~~)~~showing~~, b) the difference in absorption between the two larger energy scans, c) the distribution of solid and pore phases (determined using 3a) and d) the distribution of mineral, pore and organic matter ~~phases~~determined using both 3b) and 3c).~~Movies showing sequential images of each aggregate slice will be posted as supplementary material to the manuscript.~~

Table 1 Physical properties of the nine soil aggregates and quantities of CO_2 released by the aggregates from soil heterotrophic respiration during incubation (see text).

Aggregate number	37	40	43	49
Dry mass (mg)	106	118	177	103
Aggregate volume (mm^3)	73.7	70.2	137.3	70.8
TOC (%)	6.81	5.27	7.06	5.53
Dry bulk density ($g\,cm^{-3}$)	1.43	1.68	1.29	1.46
[a] Porosity (%)	~~11.1~~ 39.4	~~7.72~~ 30.7	~~7.29~~ 45.1	~~8.88~~ 39.6
[b] Mineral mass (mg)	91.2	105.5	152.2	91.7
[c] ~~Dry OM bulk density ($g\,cm^{-3}$) 0.44 0.50 0.35 0.38 0.45 0.42 0.47 0.40 0.81~~ OM volume (%) [d]				
[~~d~~c] Mineral volume (%)	~~51.1~~ 13.9	~~61.4~~ 12.6	~~44.6~~ 13.0	~~53.8~~ 11.5
[~~e~~d] Moisture loss (freeze-drying) (%)	23.5	18.1	27.8	21.3
[~~f~~e] Surface area (mm^2)	~~1042~~ 3462	~~706~~ 2085	~~1397~~ 2299	~~895~~ 4929
Surface area/agg. volume ($mm^2\,mm^{-3}$)	~~14.1~~ 47.0	~~10.1~~ 29.7	~~10.2~~ 16.7	~~12.6~~ 69.6
[~~g~~f] Excess headspace C concentration ($mg\,kg^{-1}$)	76	108	238	350
[~~h~~g] Normalised C gas concentration ($\mu g\,C\,mg\,C^{-1}$)	10.5	17.3	19.1	61.5

[a] solid:pore thresholds computed using ~~two-component Gaussian mixture model from adsorption values at 56keV (see text)~~ aggregate volume, and assumptions for organic matter and mineral particle density. This is an image based porosity estimate based on averaging values at the scanning resolution of 6.6µm.

[b] Mineral mass computed from total aggregate mass − mass of organic matter (2× TOC).

[c] ~~Organic matter (OM) bulk density ($g\,cm^{-3}$) computed using Equation 3.~~ Expressed as a proportion of the solid volume (excludes pore space) and assumes a mineral density of 2.65 $g\,cm^{-3}$.

[e,d] The mass proportion (%) of moisture lost between mass of field-moist aggregates and freeze-dried mass (the latter after changes in moisture introduced through the pressure plate). We cannot compute volumetric moisture content ($cm^3\,cm^{-3}$) because the freeze-drying procedure also removes moisture from both pore space and organic matter.

[f,e] Surface area was computed using a bespoke programme (see text).

[g,f] A background CO_2 concentration of 400 mg kg^{-1} was assumed.

[h,g] Headspace C gas concentration normalised by the TOC content of each aggregate.

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3.2 ~~Porosity, surface area, pore sizes~~ Pore diameters and ~~shapes~~ tortuosity index values

Figure 4 shows ~~the variation in pore volumes~~ how the pores of differing diameter (μm^3) contribute to the total pore space in each aggregate, computed for the regular ~~regions~~ blocks extracted from each of the ~~nine aggregatesin the form of a boxplot. For the smaller pores (~~aggregates. Pores with diameters of <~~30 000 22 and 22-30 μm^3 ~~) the distributions are very similar (median value range 10924-12074 m^3) , whilst there are a greater number of large pores (>100 000 m^3)~~in some aggregates compared to others. The pore shape factor (F) distribution is also similar across all the~~ account for the vast majority (82-89%) of the total pore space in each aggregate. Although aggregate 40 appears to be anomalous in Figure 4, with a notably smaller porosity than the other aggregates, because this approach was applied to a subset of the aggregate volume (a regular three-dimensional region with no external feature space), in this case we believe it is not representative of the total aggregate pore space (a value of 30.7% reported in Table 1) which was computed using the entire aggregate.

The total number of pore branches in each aggregate for which TI values were calculated for the nine aggregates ranged from 247699 to 562655. There is little variation in the overall distribution of TI values for the nine aggregates (Figure 5), ~~with some aggregates having a greater number of the most elongated pores (F < 0.2) than the others. Overall we infer that both pore size and elongation are relatively similar for each aggregate and therefore are unlikely to account for significant differences in the magnitudes of SHR we observed.~~ each having mean values between 1.29 and 1.31, an each having a small number of more tortuous pores (TI>2) which are shown as outliers in Figure 5. Given the overall similarities between both pore diameters and TI values for each of the nine aggregates we have not made any comparison with their magnitudes of respiration as we do not consider it would prove meaningful.

3.3 Transition probabilities

~~Figure 6 shows the variation in the overall transition probabilities from a central voxel to the adjacent 26 faces for the nine aggregates. As expected, the largest overall transitions are between the same phases with overall probabilities of between 0.64 and 0.94. The smallest transition probabilities are from mineral and organic phases to the pore phase (range 0.01-0.05) which reflects the smaller total proportion of pore voxels compared to the two other phases. Although on average the proportion of organic voxels is larger than the mineral voxels (see Table 1), the median transition probability from pore to mineral (0.17) is larger than the median transition probability from pore to organic (0.11).~~

~~Boxplot showing the transition probabilities between a central voxel of organic matter and the 26 neighbouring voxels for each of nine (numbered) aggregates. The three transition types are: i) OO=organic matter to organic matter, ii) OP=organic matter to pore and iii) OM=organic matter to mineral. Note how the scales are truncated to a restricted region of the ternary diagram.~~

~~Figure 7 shows the overall~~ The transition probabilities from each organic matter ~~central~~ centred voxel to the neighbouring 26 voxels for each of the nine aggregates ~~(in~~ is shown in Figure 6, within a restricted region of a ternary diagram~~). The~~. These transition probabilities are estimated for this voxel resolution (6.6 μm). We know that some voxels may be misclassified
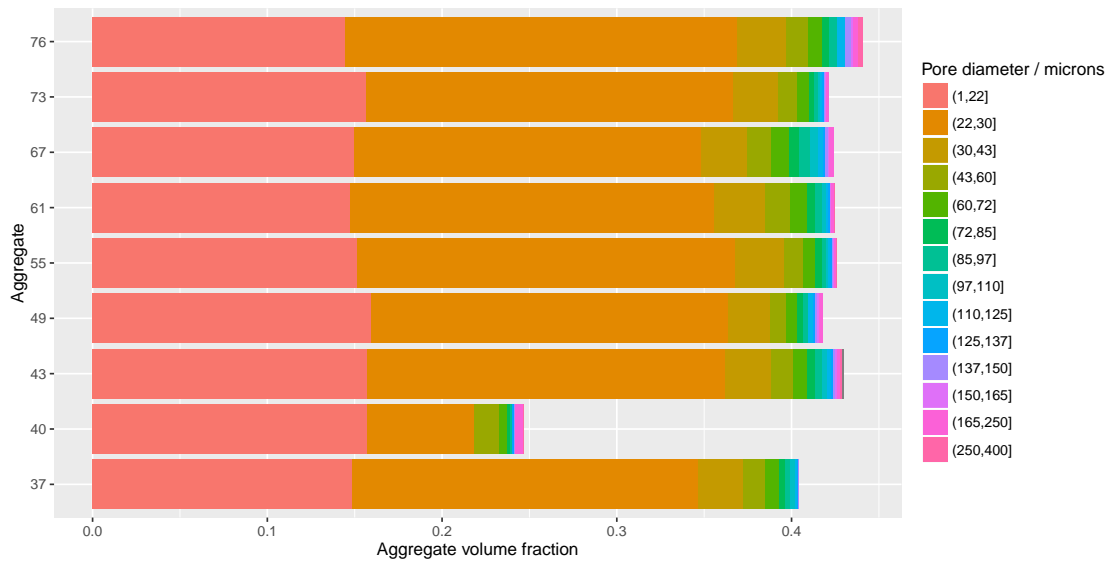
Figure 4. Distribution of pore ~~volumes~~ diameters (μm^3) ~~in~~ expressed as a proportion of total volume of a regular 3-D region extracted from each of the nine aggregates.

because they represent a mixture of OM and mineral phases and more accurate classifications may only be possible at finer
380   scales.

    With the exception of aggregate 61, the largest overall transition probability in each case is from:to the organic phase (range ~~0.66-0.75)whilst the overall~~ 0.35-0.58). The range of organic to mineral phase transitions were between ~~0.22 and 0.31~~0.32 and 0.49. In terms of OM accessibility, the OM-pore transition probabilities are ~~small transition (range 0.02-0.03) which indicated that only a~~ generally smaller (range 0.07-0.17) than transitions to the other two phases, indicating that a relatively small
385   proportion of all OM voxels are accessible to soil microbes. The values of our alternative measure of OM accessibility~~was~~, the proportion of OM-centred voxels with at least one adjacent pore voxel (the minimum threshold OM-pore proportions), are shown in Table 2. These values range from ~~13.1 to 19.0~~0.13 to 0.19% showing that the frequency distribution of the numbers of adjacent pore voxels is positively skewed, with a larger frequency of voxels having only one OM-pore transitions compared to larger numbers of OM-pore transitions around a central OM voxel~~(overall transition probability range 2.2-3.1 %) . There was~~
390   ~~no significant relationship~~. The Pearson linear correlation coefficient (r) between the magnitude of SHR (rescaled to aggregate TOC content) and the transition probabilities from a central OM voxel to a neighbouring pore voxel ~~(Figure 8~~was 0.12, which is not particularly strong (see Figure 7).

3.4 Indicator variograms and models fitted to them

~~Figure 9~~ Figure 8 shows the variation of the effective range of the exponential models fitted to the indicator variogram semi-
395   variance estimates for the 50 randomly selected blocks for the three phases in each of the aggregates. Selected statistics of the
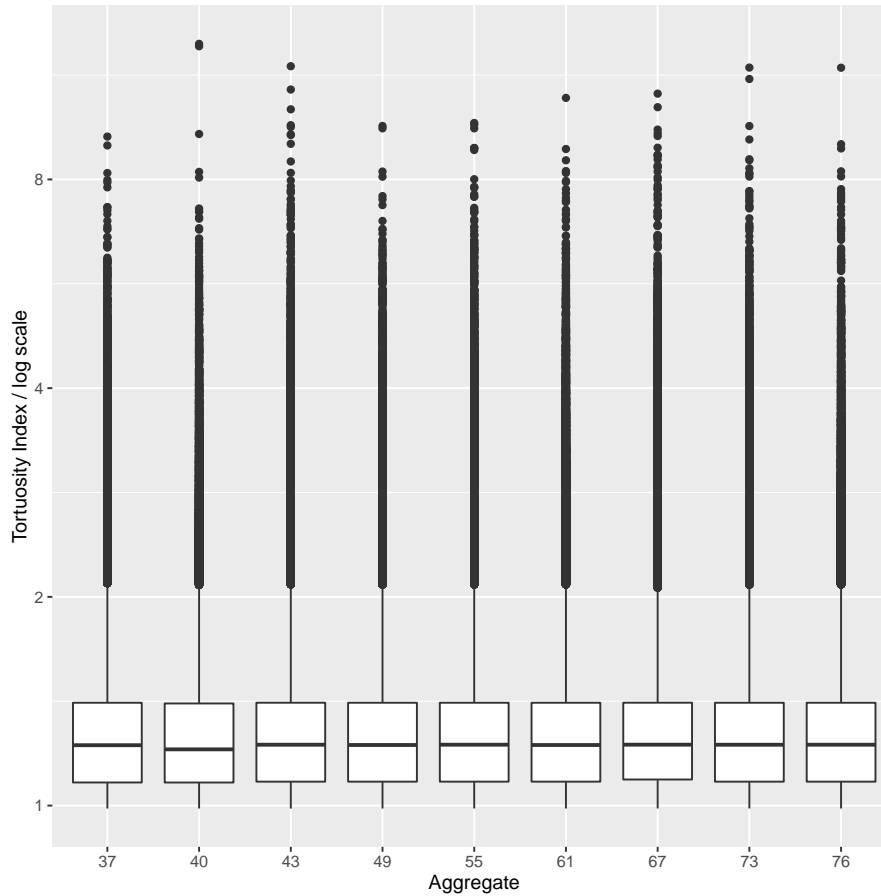
Figure 5. ~~Variation in the pore shape factor~~ <u>Distribution of tortuosity index</u> (~~F~~<u>dimensionless</u>) ~~in a regular region extracted from~~ <u>for</u> each of the nine aggregates.

Table 2 - The proportion of minimum threshold voxels (at least one OM-centred voxel has an interface with an adjacent pore voxel) in each soil aggregate.

Aggregate	37	40	43	49	55	61	67	73	76
Proportion (%)	~~16.0~~0.16	~~17.2~~0.17	~~13.1~~0.13	~~16.4~~0.16	~~13.8~~0.14	~~15.1~~0.15	~~15.0~~0.15	~~19.0~~0.19	~~14.3~~0.14

effective range values <u>for OM</u> are shown in Table 3. ~~In seven of the aggregates the median effective range of porosity is less than the mineral and organic matter phase effective ranges, whilst in two of the aggregates (40 49) the pore phase has a larger effective range. In three of the aggregates (49, 55~~

<u>With the exception of aggregate</u> 61~~) the interquartile range (IQR) of the effective range values are generally smaller than the~~

400 ~~other aggregates. The IQR of the effective range of the mineral and organic matter phases are generally larger than the pore phase, and the median effective range values are also generally more variable than the pore phase (Figure 9)~~, <u>the length scale</u>

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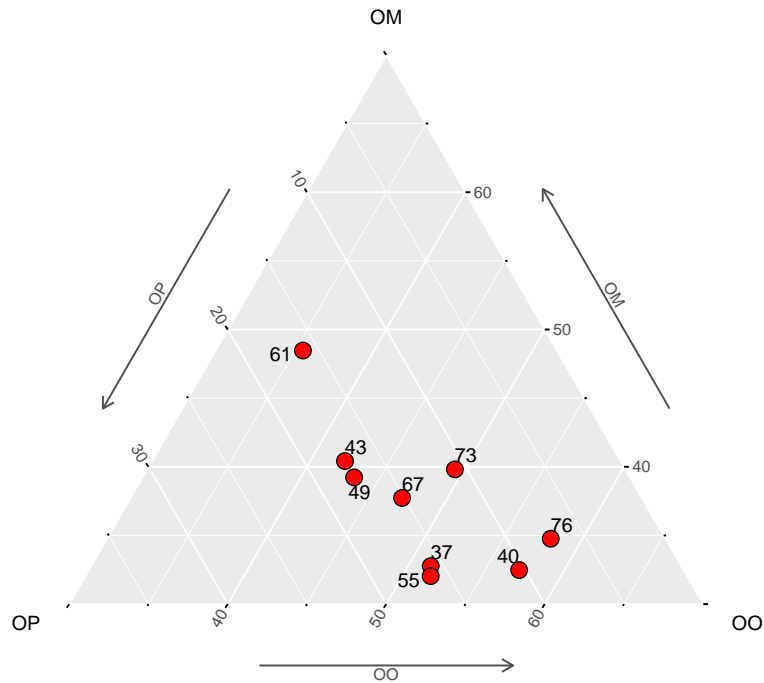
Figure 6. Ternary diagram showing the overall transition probabilities (%) between a central <u>organic matter</u> voxel (side length 6.6 μm) and 26 adjacent voxels (see text) for each of the nine aggregates (labelled). The ~~nine~~ transitions are between <u>organic matter and</u> each of the three phases (O=organic matter; P=pore, M=mineral). For example, OO=organic matter to organic matter. ~~Note the~~ <u>The</u> truncated axes show only a sub-region of the ternary diagram. <u>The osmium-staining of OM sorbed onto mineral particles may have resulted in misclassification of voxels that are a mixture of OM and mineral matter. We cannot account for this because such effects could only be reconciled at finer ($<$6.6 μm) spatial resolutions.</u>

<u>(range value) over which organic matter varies is significantly greater than for both mineral and pore phases (Figure 8) in all aggregates. In most cases the inter-quartile range for OM is generally large when compared to that of both mineral and pore phases.</u> Given the considerable ~~differences~~ <u>variation</u> in the length scales of OM variation between the nine aggregates <u>(median</u>
405 <u>range value 38-175 μm^3)</u>, we considered there may also be equivalent differences in the frequency of microsites for microbial respiration, with sites occurring more frequently where OM varies over shorter length scales. In Figure ~~10~~ <u>9</u> we show the median of the effective model ranges (Table 3) of OM matter plotted against the TOC normalised respiration values from the laboratory measurements (Table 1). ~~Although there are only nine pairs of observations~~<u>However</u>, there is ~~evidence of a negative~~

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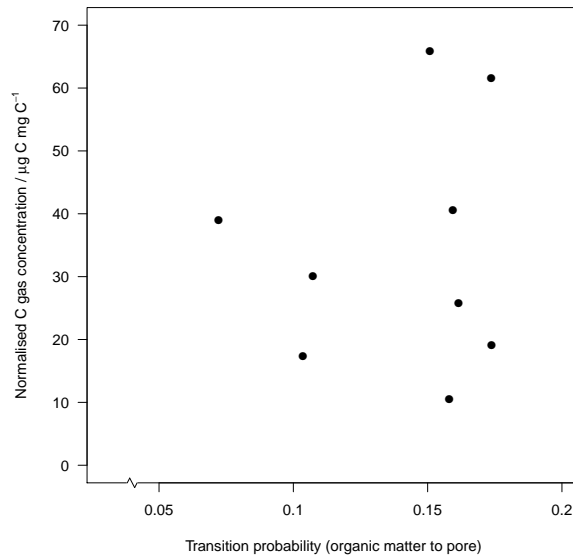
Figure 7. Scatterplot showing the transition probabilities between a central voxel of organic matter and 26 neighbouring voxels versus the magnitude of respiration (normalised to aggregate TOC content).

~~relationship between respiration rate and length scale of OM variation~~ <u>no linear relationship between these values</u> (Pearson linear correlation <u>(</u>r = ~~−0.42). We consider this evidence to support our original hypothesis concerning microsites and length scale of OM variation, but we need a larger set of observations to make a statistical inference.~~ <u>0.01; Figure 9).</u>

We could not find other published studies which have reported the relative magnitude of unaccounted for short-scale (6.6 μm) variation of mineral<u>, pore</u> and OM phases in three dimensions within soil aggregates, and these values may be useful for similar studies. This unaccounted for variation (nugget variance) is the variance of analytical error plus variation that occurs at scales shorter than the sampling resolution. The magnitude of nugget variance is often expressed as a proportion of the nugget plus sill variance (the variance at the range of the fitted model). Across all aggregates, the ~~mean and~~ median proportions of nugget variance for the ~~mineral and organic phases was 0.56 (56%) whilst the mean and~~ <u>organic matter phases varied between 0.47 and 0.73, whilst the range of the</u> median proportions of the pore ~~nugget variance was smaller, 0.23 (23%)~~<u>0.0-0.09) and mineral (0.0-0.37) nugget variance were smaller.</u>

4   Discussion

<u>In our previous analysis (**?**) , we reported what we now recognise were unrealistically small values for OM particle BD which gave rise to small total porosity values (4.4-11.1%) in each aggregate. Using the modified approach we report here, there was</u>

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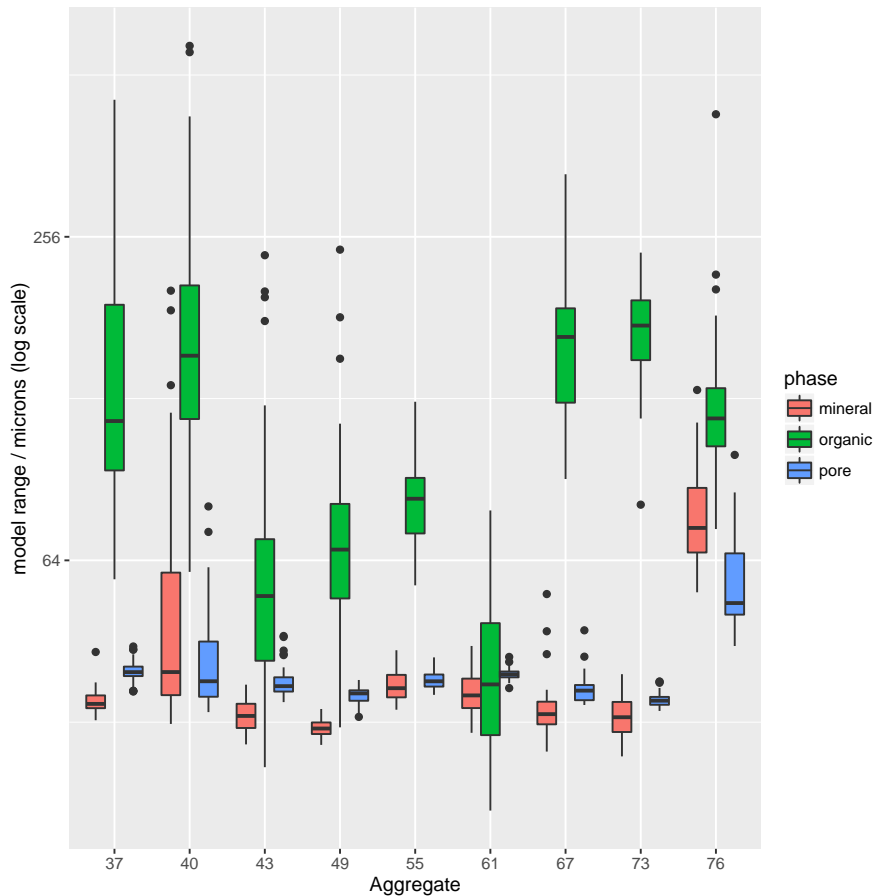
Figure 8. Boxplot showing variations in the model range estimate for exponential models fitted to indicator variograms of the three phases (mineral, soil organic matter and pore space) for each of the nine aggregates. The semivariance estimates were computed from a subsample of 50 000 locations from 50 separate blocks (each measuring $200 \times 200 \times 200$ voxels) within each aggregate.

a very strong correlation (r=0.98) between total soil porosity and aggregate BD for our nine aggregates, which suggests this approach was considerably more successful than the previous one.

425   We have reported what we understand is the first data showing complete 3-D macro-aggregate scale distributions of OM, pore and mineral phases at fine (6.6 µm) scales, plus the length scales over which they vary and the transition probabilities at interfaces between these phases. These data could be used to test both existing and new models which aim to account for small (aggregate) scale variations in SHR (**??**). To fully understand the processes governing SHR in the soil aggregates we studied it is necessary to quantify the distribution of OM and pore space at scales of less than 250 µm, the scale below which the majority

430   of variation in the these key soil properties occurs.

    Our analyses could be used to improve quantitative estimates of the accessibility of both particulate (> 50 µm) and finer ~~organic matter~~ OM in soil aggregates. Our analysis showed that OM voxels which had an overall transition probability to a

Table 3 - Selected statistics of the effective exponential model ranges (μ m) fitted to the indicator variograms of organic matter voxels for each of the nine aggregates.

Aggregate	25th percentile	median	75th percentile	sd
37	~~316~~ 94	~~458~~ 116	~~647~~ 191	~~229~~ 109
40	~~217~~ 117	~~289~~ 154	~~2681~~ 208	~~1607~~ 118
43	~~222~~ 42	~~301~~ 55	~~407~~ 70	~~519~~ 46
49	~~182~~ 54	~~193~~ 67	~~207~~ 82	~~18~~ 36
55	~~358~~ 72	~~388~~ 83	~~419~~ 91	~~51~~ 16
61	~~159~~ 30	~~178~~ 38	~~195~~ 49	~~30~~ 15
67	~~255~~ 126	~~330~~ 167	~~465~~ 188	~~700~~ 56
73	~~410~~ 151	~~487~~ 175	~~581~~ 195	~~211~~ 32
76	~~370~~ 104	~~449~~ 118	~~503~~ 134	~~1325~~ 55
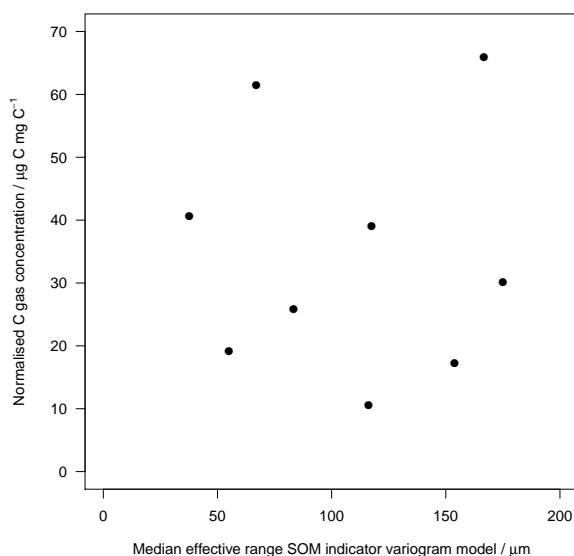
Figure 9. Scatterplot of median model range estimate for exponential models fitted to indicator variograms of organic matter versus headspace CO_2 gas concentration normalised to the carbon content of the aggregate.

neighbouring pore voxel (our criteria of accessibility) of between ~~0.02 and 0.03~~ 0.07 and 0.17. In their simulation study, **?** used proportions of accessible particulate OM voxels (those with at least one neighbouring pore voxel) of between 20 and 100 %

435 (range of OM contents 1.4-7 %). Using the same metric, we estimated that accessible OM voxels account for a substantially smaller proportion (range ~~13.1- 19~~ 13-19 %) of the total quantity of OM in each of the nine aggregates. In their ~~simulation~~ study **?** placed POM at the pore:solid interface which likely accounts for the larger particulate OM accessibility proportions

they report and this approach may require modification if more realistic ~~POM~~ OM accessibility proportions are to be simulated. However, estimated organic matter to pore transition probabilities may be larger at finer X-ray CT resolutions (<6.6 μm) where any misclassification due to edge effects can be resolved.

We plan to extend our analysis further using the approach proposed by **?** by: i) quantifying the distribution of particulate organic matter (length scales > 50 μm) and, ii) computing a statistic of pore connectivity for each aggregate and plotting it against SHR, and, iii) identifying those pores which are connected to the exterior of each aggregate, providing a direct pathway for the diffusion of gas to and from sites of intra-aggregate SHR. These data could be used to assess whether these ~~two~~ factors are strongly correlated with the magnitude of SHR. It would also be helpful to estimate the distribution of water-filled pores (**?**) at the suction (-50 kPa) used in our experiment as this would determine which sites of microbial respiration may have been anaerobic, influencing the magnitude of SHR.

~~We note that the OM volumes (%) computed for our aggregates were large (range 44.6-63.4%) given their TOC content (range 4.2-7.5 %). In a recent modelling approach (?), the authors estimated various physical properties of soil aggregates including OM volumes of between < 1 % and 3.35 % for TOC contents of between 0.7 and 3.5 % (assuming 50 % of OM is organic carbon based on ?). If we compare these TOC contents and OM volumes reported by ? with those we report, the former are significantly smaller. After a comprehensive search we could only find values for OM bulk density reported by ? from soil cores and we suggest this property requires further investigation across scales to ensure that realistic values are used to estimate soil bulk density and OM volumes in studies of soil aggregates~~In subsequent work we will also attempt to determine the location of finely disseminated OM sorbed onto mineral surfaces. In doing so we would need to identify an upper threshold size (volume and shape) for sorbed OM, and rules governing the size and shape of neighbouring mineral particles.

We reported that for a set of nine aggregates there was some evidence that ~~shorter length scales of OM variation were associated with larger magnitudes~~ greater surface area of the solid phase (both mineral and organic matter phases) had a reasonably strong positive correlation ($r=0.44$) with the magnitude of SHR, having scaled the respiration rates to TOC content. This relationship requires further investigation using macro-aggregates with a wide range of TOC concentrations and textures to determine whether this relationship is statistically significant, and ~~whether it supports our interpretation relating to the more frequent~~ how it might be interpreted in relation to frequency of occurrence of microbial microsites. ~~We might expect aggregates from more finely textured soil, in which greater quantities of OM are preserved (?), to have shorter scales of OM variation than those soils of coarser texture.~~

In their study, **?** used air-dried soil aggregates whilst we chose to freeze-dry the aggregates in our experiment to maximise absorption of osmium onto organic carbon bonds. It would be useful to compare the results of both approaches to determine whether the drying process has significant implications for the extent of Os absorption throughout an aggregate. This could be achieved by comparing the magnitude and spatial distribution of Os using energy dispersive X-ray analysis linked to a scanning electron microscope, the method used by **?** to validate their original approach.

5   Conclusions

We have shown how a combination of synchrotron X-ray CT, osmium staining and TOC measurements can be used to successfully quantify the 3-D distribution of OM, pore and mineral phases throughout soil macro-aggregates at fine scales (6.6 μm). The magnitude of SHR which we measured for each of nine macro-aggregates (controlling for moisture content and temperature) varied by a factor of six whilst their TOC contents varied by less than a factor of two. Certain features of the pores, the distribution of pore diameters their tortuosity indices in the nine aggregates were very similar. After normalising for aggregate volume, there was a four-fold variation in the aggregate surface areas and this had a reasonably strong linear correlation ($r=0.44$) with the magnitude of SHR (after scaling to TOC content). This relationship needs further investigation using a greater number of soil samples with a wider range of surface areas (soil textures) and OM concentrations.

The transition probabilities between OM-centred voxels and adjacent pore voxels – a measure of OM accessibility – varied between 0.07 and 0.17 and were generally smaller than the transitions to the other two phases. We believe these are the first data to quantify 3-D macro-aggregate OM accessibility at fine scales and could be used to help parameterise models of OM mineralization. There was a weak linear correlation ($r=0.12$) between OM accessibility (transition probability between pore and OM) and the magnitude of SHR. There were substantial differences in median length scales (median ranges 38-175 μm) over which OM varied between aggregates based on models fitted to their indicator variograms. Studies of the processes governing SHR in soil macro-aggregates need to be undertaken at fine scales (less than 250 μm) because this is the upper threshold of the length scales over which mineral phases, pores and OM are likely to vary.

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