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New insights into organic carbon stabilization in soil macroaggregates: An in situ study by optical microscopy and SEM-EDS technique

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(Article begins on next page)

Geoderma

New insights into organic carbon stabilization in soil macroaggregates: an in situ study

--Manuscript Draft--

Manuscript Number:	GEODER-D-20-00730
Article Type:	Research Paper
Keywords:	Macroaggregate size; aggregate thin sections; optical microscopy; SEM-EDS; physical occlusion; organo-mineral interactions
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Order of Authors:	Guidi Patrizia Gloria Falsone Wilson Clare Cavani Luciano Ciavatta Claudio Marzadori Claudio
Abstract:	The purpose of this study was to investigate the in situ characterization of organic matter (OM) within soil macroaggregates, and to assess the relationships between OM characteristics and macroaggregate size indicating different OM stabilization mechanisms. Optical micro-morphological investigations, coupled to SEM-EDS technique, were carried out on thin sections of 4-1 and 1-0.25 mm soil aggregates (coarse and fine macroaggregates, respectively) of four different sites in which soil structure were not disturbed by tillage. Fine macroaggregates were significantly less porous (3.70-6.71% of total porosity) and had higher presence of the finest pore class (<50 mm) compared to coarse macroaggregates (5.93-9.08% of total porosity), independently from sites. The percentage of organic matter forms (OMFs) identified by optical investigation was significant higher in fine (13.54-17.75%) than in coarse (4.19-8.27%) macroaggregates. In particular, fine macroaggregates were richer in red and black amorphous organic forms, which were characterized by the highest values of Al:C, Fe:C and Ca:C molar ratios. These findings suggested thus an accumulation of OM in fine macroaggregates than in coarse macroaggregates occurred. It was probably due to a more efficient OM stabilization in fine than in coarse macroaggregates related to both physical occlusion (lower porosity and smaller pore size) and organo-minerals interaction (higher presence of OMFs characterized by the highest Al:C, Fe:C and Ca:C ratios), The OM exposure index (EI), a measurement of the OM surface exposed to pores and thus potentially available for microbial activity, was unexpectedly higher in fine than in coarse macroaggregates (EI: 0.48-0.79 and 0.25-0.58 mm⁻¹ in fine and coarse macroaggregates, respectively). However, the accessibility of OM defined by the EI seemed to facilitate neither the oxidative transformation nor the damage of enzyme activities, being the EI positively related to C:N ratio (r=0.66), negatively to d₁₃ C values (r=-0.74) and positive to the geometric mean of the five assayed enzyme activities related to C-cycle (r=0.79). Therefore, even more potentially exposed, in fine macroaggregates the OM was not accessible to microorganisms due to the effective physical occlusion, and thus both accumulation of few transformed OM and maintenance of functionality related to C-cycle occurred. The OM stabilization in macroaggregates thus involved both physical occlusion and organo-metals/mineral phase interactions processes. Both these processes are often related to microaggregates rather than macroaggregates. Our findings thus seem to provide a new insight for studying the potentiality of OM stabilization and C sequestration in soil macroaggregates.

Suggested Reviewers:	Hiroko Nakatsuka hn206788@nodai.ac.jp he is an expert in soil micromorphology applied to SOM dynamics
	Nadia Vignozzi nadia.vignozzi@crea.gov.it She is an expert in soil micromorphology applied to soil porosity
	E. Azizsoltani nhonarjoo@khuisf.ac.ir She/he is an expert in soil structure
	Maria del Carmen Gutierrez-Castorena castor@colpos.mx She is an expert in quantification of soil components in thin sections
	X. Peng xhpeng@issas.ac.cn He is an expert in C storage in soil aggregates

Dear Prof. Kögel-Knabner,

Please do find here a manuscript proposal " New insights into organic carbon stabilization in soil macroaggregates: an *in situ* study" by P. Guidi, G. Falsone, C. Wilson, L. Cavani, C. Ciavatta and C. Marzadori.

A previous version was submitted to Geoderma (GEODER_2020_432) and, after its rejection, we would like to submit a substantially revised version of our manuscript which would be considered as a new submission. Thank you to give us this permission.

According to the reviewers' suggestions, the present version has been completely reorganized, especially the Results and Discussion have been separated in two different sections, in the Introduction our hypothesis has been specified, the M&M section has been improved to explain more in detail the procedures used (also adding a supplementary Figure S2 describing the OM forms classification), and in the Conclusion section we have added a Figure (Figure 6) in order to present a conceptual scheme on the dynamics of macroaggregates and OM stabilization as the take home message of the study. Please find more details below in our reply to reviewers' comments.

Any communication about the manuscript should be addressed to:

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Manuscript is not submitted elsewhere. Manuscript has not previously been published.

Sincerely,

Gloria Falsone

Ref: GEODER_2020_432

Title: New insights into organic carbon stabilization in soil macroaggregates: an *in situ* study

Journal: Geoderma

Dear Dr. Falsone,

Thank you for submitting your manuscript to Geoderma. I regret to inform you that your paper is not acceptable for publication. We have completed the review of your manuscript and a summary is appended below. The reviewers have advised against publication of your manuscript and I must therefore reject it at this time. For your information and guidance, any specific comments explaining why I have reached this decision and those received from reviewers, if available, are listed at the end of this letter.

You have the option of resubmitting a substantially revised version of your paper, which would be considered as a new submission. If you decide to do this, you should refer to the reference number of the current paper and include a cover letter which explains in detail how the paper has been changed or not, in reply to the Editor and Reviewer comments.

Thank you for giving us the opportunity to consider your work.

Kind regards,

Professor. Koegel-Knabner
Editor
Geoderma

Comments from the editors and reviewers:
-Reviewer 1

-

Manuscript number GEODER_2020_432

New insights into organic carbon stabilization in soil macroaggregates: an in situ study

General comment:

The manuscript presents findings on relationships between pore characteristics and properties of macroaggregates. Coarse and fine macroaggregates have been collected from topsoil from two topographies and under contrasting vegetation. Different techniques have been used to evaluate pore characteristics and composition of macroaggregates. Overall findings of the study are within the scope of Geoderma and should be of interest for its readers. However, I believe that the MS must be revised, mostly the R&D section, so to most effectively transfer the findings of the study to its' readers.

Q: The Introduction justifies the relevance and importance of the topic. It can be shortened in places (see below).

A: Done (see reply to specific comments)

I raised minor comments, mostly grammar and clarity. The rest of the MS can be benefitted by such improvements too.

Q: I propose to add the key question and the hypothesis of the study in the end of the Introduction, so the reader understands what to look for as the main question and foundation of the research. In its current format, after reading the Introduction, the reader would still remain unclear what is the purpose of the research, and what to look for before reading through the rest of the MS.

A: As suggested, we added at the end of the Introduction the main question (“We would furthermore test if the hypothesized relationships between OM characteristics and macroaggregate size were similar among different sites (i.e., sites which differed in climate and management) or if it exists a certain size-effect transgressing the environmental key properties).

Q: The M&M needs further justification and clarity, specifically references to support the approach and methodology (see below M&M).

A: Done (see reply to specific comments)

Q: The R&D is the section that by far needs most improvements.

The Results and Discussion has been drafted in a very narrative tone and mostly describing findings such as that for a Result section. Authors have converted the data into Tables and Figures, but the connection between the findings and their interpretation (backed by the literature), in a format that relates the findings together, and also the findings to the literature for a mechanistic understanding of aggregate dynamics is largely missing. In some parts the authors have achieved this though. For example, L332-341. There is much more space in the R&D section to combine the findings into a coherent piece that easily conveys the key messages to the reader. For example, EI property has been related to C/N, $\delta^{13}\text{C}$ natural abundance, and activity of five indicative soil enzymes, in separate figures, but this all needs to be captured in a conclusive way (preferably based on a multivariate statistic approach), so to serve as one of the take-home messages of the study.

A limited number of references have been cited in the discussion, making it more like a reporting the results rather than incorporating findings of the study by former literature and add to our current knowledge. In fact, if the reader disregards reading through the R&D section, and focuses only on data presented in Tables and Figures, they would have not missed much. Apart from that, I would like to see some discussion on relating findings to dynamics of aggregate turnover and aggregate porosity in relation to management/disturbance, etc. There should be reasons that authors have selected samples from different topographies and vegetation histories. I understand that the focus of the study was aggregate dynamics, but assuming that it occurs in larger context, i.e. whole soil, some basic measurements of the soil as a whole would have been appropriate. Such as total C and C/N as basic proxies of soil OM content and its decomposition status. This would help the authors to relate the aggregate and soil properties, for example as a function of management.

A: The R&D section has been completely reorganised also in agreement with the rev#2's suggestion. Now, the Results and Discussion are in two separated section. In the Discussion section we improve our data interpretation backed by literature. The discussion of the data in relation to site conditions is now reported. See also reply to rev#1's specific comments.

Q: The Conclusion section follows the same pattern in terms of being narrative rather than combining findings and relating findings into a big picture of aggregate and C dynamics. It is rather a repeat of former statements elsewhere in the MS. I recommend to add a visual demonstration that conceptualizes findings as the take home message of the study. How different attributes of porosity, OM status and soil properties ($\delta^{13}\text{C}$, C/N, indicative enzymes) are related based authors' findings, without too much generalizing the findings.

A: We now insert a figure (see Figure 6) reporting the conceptual scheme of the dynamics of macroaggregates and OM stabilization. Additionally, the Conclusion section has been improved in accordance with the suggestion.

Q: There are few but highly relevant published pieces that support findings of the study. These may further help the authors to back up/incorporate their findings to expand the current knowledge on the topic:

- Toosi, et al., 2017. Effects of management and pore characteristics on organic matter composition of macroaggregates: evidence from characterization of organic matter and imaging. European Journal of Soil Science.
- Sarker et al. 2018. Linking organic matter chemistry with soil aggregate stability: Insight from ¹³C NMR spectroscopy. Soil Biology and Biochemistry.
- MY Quigley et al., 2018. Influence of pore characteristics on the fate and distribution of newly added carbon. Frontiers in Environmental Science.

A: The references have been inserted. Thank you for the suggestions.

Specific comments.

Q: The 1st paragraph is too general and can be merged with the 2nd paragraph in a smaller section so to shorten the message for the reader, assuming that the importance of SOM is known for the reader.

A: As suggested, the two paragraphs have been merged and summarized, assuming that the importance of SOM and the contrasting effects of human activity are known for the reader

L62: change “the flow of .. to the soil” to “the flow of C from, within, and to the soil”

A: Done. Thank you for the suggestion

L70: “as being due either to its chemical conformation or to its physicochemical protection”, change to as being due to its chemical structure and its physicochemical protection”

A: Done

L72: “stressed” change to “stressed”

A: We changed “stress” into “stressed”

L74: “organic C stabilization” change to stabilization of organic C”

A: Done

L76: “to the decline of microbial activity due to reduced diffusion of oxygen into aggregates”, change to ” to reduced microbial activity due to a lower diffusion of gasses into and within aggregates”

A: Done. Thank you for the suggestion

L84 “sites”, change to “environment”

A: Done.

L88: “because fresh plant residue”, change to “because living fine roots and fresh plant residue”

A: Done. Thank you for the suggestion

L81 to L94: the two paragraphs need to be merged together and significantly shortened. Note that the mechanisms of aggregate formation have been extensively described elsewhere in the literature and is not necessary to be narrated in the Introduction.

A: As suggested, we merged and shortened the two paragraphs

L100: “requires... studied”, change to “demands in-depth investigation”

A: Done

L102: “OM in the undisturbed”, change to “OM in an undisturbed”

A: Done

L103: “in situ important characteristics”, change to “in situ characteristics”

A: Done

L104: “and that within the macroaggregates the processes leading to the long-term soil OM stabilization begins (i.e.,”, change to “and that the processes leading to the long-term soil OM stabilization begins within the macroaggregates (i.e.,”

A: Done

L:134-135. Is the range for macroaggregates 0.25-1 and 1-4 in line with the literature? Where is the ref. for this separation in the MS?

A: We have now inserted in the references for the lowest size limit of macroaggregates both in the text and in the references list

M&M:

L138: “Aggregate thin sections (2.8 x 4.8 cm) were”. Is this correct, cm?!

A: We changed into 28 x 48 mm.

Section 2.2. this whole section lacks adequate information, for example how were slicing and stabilization of the slices conducted, any reference, instrument, etc. This section doesn't share enough information so to allow other researchers follow the preparation procedure protocol based on the given information.

A: This section has been improved describing more in detail the preparation procedure.

Section 2.5: what was the basis of the GMea approach for determining enzyme activities? What was the selection criteria for the measured enzymes (vs. other commonly assayed extracellular enzymes)? Any reference/s to support?

A: This section has been improved

Q: What was the method for measurement of OM (see Table 3 and elsewhere) Loss on Ignition, etc.?

A: Organic C of aggregates, expressed as g C kg^{-1} aggregate (see now recalled Table 2), has been measured on ground coarse and fine macroaggregates by dry combustion, as indicated in section 2.5. The organic matter forms, expressed as %, have been measured by image analysis of coarse and fine macroaggregate thin sections, as indicated in section 2.3.2.

Section 2.6:

Q: L233 change “one-way ANOVA analysis of variance” to “one-way ANOVA”

A: Done

Q: L234: here and elsewhere: change “ANOVA analysis” to “ANOVA”

A: Done

Q: L236: “Differences in the molar ratio (Al:C, Fe:C and Ca:C) among organic forms and between coarse and fine macroaggregates were tested by the one-way ANOVA analysis.” The methodology for supporting this is missing. Move them from end of Table 2 to here.

A: There was a misunderstanding because the molar ratio (Al:C, Fe:C and Ca:C) referred to macroaggregates thin sections and, as indicated in section 2.4, it has been determined from the EDS analysis data. We now better specify it also in section 2.6

Q: why the measurements of Al, Fe, and Ca were conducted? References to justify the relevance of these measures are missing.

A: The measurements of Al, Fe and Ca were conducted in order to determine the Al:C, Fe:C and Ca:C molar ratio. As indicated in section 2.4, we took into account the Al:C, Fe:C and Ca:C molar ratios as indicators of organic-metals/minerals interactions. The reference Falsone, G., Wilson, C.A., Cloy, J.M. Graham, M.C., Bonifacio, E., 2014. Relating microfeatures of organic matter to C stabilisation: optical microscopy, SEM-EDS, abiotic oxidation. Biol. Fertil. Soils 50, 623-632 has been inserted here and in the list of references.

Results and Discussion

Q: L348-249: I am unclear how the total porosity of aggregates based on the method used by the authors can be related to the “good soil structural conditions”. Doesn’t look like a robust conclusion from the data.

A: We compared our data to those reported by Pagliai et al (1998) which evaluated soil structure by image analysis and fixed <10% values as indicator of physical degraded (compacted) soils. In any

cases, in our study the intraggregates pores have been measured, thus lower value than soil porosity derived by inter and intra-aggregate has been expected. We have thus deleted it.

Q: L263: organic C concentration needs to be reported based on mg kg⁻¹, as correctly reported in Table 3 and elsewhere. Needs to be corrected in the text accordingly.

A: We prefer to report organic C concentration as g kg⁻¹, as currently accepted in the international literature.

Q: L264: “The organic C content of fine and coarse macroaggregates was characterized by a high variability mainly attributable to different soil managements.”.

While management is a strong attributor to the differences mentioned, other factors such as vegetation (input and fine root activity) may have also contributed to the differences.

A: we agree with the reviewer that other factors than soil management may affect the aggregate properties. We use the term “soil management” effectively in leading confusion way. We prefer therefore to change “soil management” in “site”, also in agreement to the main question added at the end of the Introduction (see also previous reply, please).

Tables

Table 3. the title for the table is somehow vague. I suggest to combine the first two sentences and make it more understandable for the reader.

For “plots” in tables, give a brief description for each plot ID, so the tables read stand-alone and the reader doesn’t need to refer to the text for understanding what each ID refers to.

A: The table caption has been modified according to the suggestion. We however prefer to specify on which type of samples the measurements have been conducted, being total porosity and organic matter forms percentage performed on macroaggregate thin section while organic C concentration on ground macroaggregates. We now move it at the bottom of Table 2. Here, also the description of each sites has been inserted.

Figures:

Q: Fig. 3. Provide info regarding what the symbols are ,i.e. mean/median, 75% percentile/STDER/STDEV, etc.

A: We have included this info in the figure caption

-Reviewer 2

-

Title : New insights into organic carbon stabilization in soil macroaggregates: an in situ study

General comments

The study aims to increase our knowledge on soil organic matter (OM) persistence by focusing on in situ characterization of OM within soil macroaggregates, and relationship between OM characteristics and macroaggregate size.

The issues are relevant because stabilization processes of soil OM is a critical thematic to understand C sequestration in soil and agrosystems. The methods seems relevant and the results are numerous. Despite these strengths, I have identified some weaknesses that should be addressed prior to publication.

Q:- First, the "materials" section is not sufficiently informative. The presentation of the soil profiles is a bit incomplete. As the study is limited to very specific situations, it is important that the specificities and properties of the soils and the samples are well presented.

A: A more detailed soil description has been reported, also in accordance with the to the rev#2's specific comments (see the replies below)

Q:- Second, the "methods" section needs to be better enhanced. The current text raises too many questions before addressing the "results" section. This weakens the authors' argument because it casts doubt on the measurements.

A: The Methods section has been improved. Please see the reply to the rev#2's specific comments

Q:- Third, the "results" section needs to be better enhanced so as to be as close as possible to the figures and tables presented. The different results are sometimes difficult to relate to each other.

- Finally, it is imperative to separate the presentation of results from considerations of any interpretation or discussion.

A: The R&D section has been separated and both Results and Discussion section have been completely rewritten. Please see the reply to the rev#2's specific comments

I recommend that authors strengthen the materials and methods section and separate results and discussion sections because this manuscript does not seem to meet the publication standards of a journal such as Organic Geochemistry.

Specific comments and suggestions

1. *Introduction.*

- The introductory section is particularly clear. The ideas are presented in a simple and documented manner. The arguments are well developed step by step and the issues are clearly presented. Questions, objectives and hypotheses are well formalized.

1. *Materials.*

- The USDA soil taxonomy is not necessarily the most common one, especially at the sub-order level. If the authors insist on this classification, perhaps it would be advisable to specify the order (Inceptisols?) and the character that guided the choice of these sub-orders.

A: In the text we now refer to soil order (Inceptisol).

- The presentation of soil profiles is very limited, while the information on vegetation is most precise. The nature of the substrate is implied, although it can play an important role for poorly developed soils. The sampling depth is indicated (0-20 cm) but neither the type of horizons nor their structure is specified.

A: We now specify that 0-20 cm soil layer corresponded to A horizon.

- Table 2 appears to compile means and standard deviations, but the number of samples for each category is not specified. The presentation of the sites suggests pseudo-replicates (on the same site), not independent soil profiles.

A: Table 2 (now moved in supplementary materials and recalled Table 1S, see reply to next comment) reported data on two independent soil profiles opened in each site (M-OW, M-AA, P-

FF and P-NF). In fact, two plots have been selected in each site, and then in each plot one pit has been dug. Each sample has been collected in all the pit width (ca. 0.3 m). Thus in each site we collected two sample from the 0-20 cm soil layer. Now, we better explain it in the M&M section.

- The methodological information provided below Table 2 is out of place and incompleted. It seems preferable to integrate them in the section « Materials and Methods » under a paragraph "2.2 physico-chemical properties" and to detail the analytical procedures.

A: Table 2 gave information on fine earth fraction and not on studied aggregates. Because our study focused on aggregates, and not on fine earth, in order to avoid any misunderstanding we prefer to move it into supplementary materials as Table 1S.

1. *Methods*

- Q: Device and equipment models could be specified for all analyses. These technical aspects may be of interest to some readers.

A: Done

- Q: The authors indicate that « conventional descriptions allowed them to identify the area of interest that, in this study, corresponded to the intra-aggregate area ». This choice is undoubtedly justified, but it is difficult to evaluate without precise explanations.

A: The identification of intraggregate area as area of interest was coherent with the research aim. We now specify it in M&M section.

- Q: L153. I don't understand what "image analysis" of "pores" and of "organic matter parameters" means. Isn't the expression "for image analysis of intra-aggregates porosity and particulate organic matter" more appropriate?

A: Thank you for the suggestion. We change into “for image analysis of intraggregates porosity and organic matter”

- Q: Has the procedure to study porosity already been used or is it an original development? The lack of bibliographical reference justifies the question. As I understand it, the pore size is assessed in 2D. As the observations are made on a limited number of aggregates, the question of the representativeness in 3D of this measurement in 2D may arise. All elements consolidating this methodological aspect would be welcome.

A: The porosity has been measured according to Falsone et al. (2014), The reference has been inserted in the list of references.

For thin section preparation, at least 25 and 50 single aggregates have been randomly kept from coarse and fine macroaggregates, respectively. These amount of aggregates corresponded to about two spoons of samples, which is thus a sufficient amount able to guarantee the representativeness of our 2D measurement being higher, or at least comparable, to the amount of samples generally used in the main physico-chemical 3D characterization. Further, in order to gain in representativeness of our samples, the aggregates of each macroaggregate size class have been gently mixed before the randomly selection of the single aggregates for the thin section preparation. Now we better specify it.

- The criteria for optical classification of organic particles are given but not illustrated. A figure for assessing these criteria could be added, perhaps in supplementary materials. The current illustration (S1) is not informative in this respect.

A: We have inserted a new figure (S2) describing the procedure followed for organic features classification

- Is the Exposure Index (EI) a methodological development or has it already been used? The authors conclude paragraph 2.3.3 by stating that "the EI gives information [...] on the

potential physical exposure of organic matter to the microbial activity". Is this an assumption, hypothesis or conclusion of previous studies?

A: The EI has never been used before to our knowledge. The total length of the contact perimeter normalized by the total area of organic form in our opinion can represent a good assessment of the potential physical exposure of organic matter to the microbial activity, being the organic matter availability to microorganisms dependant on the exposed surface to pores (Young et al., 2008).

- Q: The text begins by stating that SEM-EDS analysis was performed "on the aggregate thin sections". Then it is specified that "the microanalysis was carried out for organic features at least on 100 points for each thin section". Figure 2 shows calculated results for each particle class. The procedure used needs to be clarified. The processing of measurements also raises some questions (cf infra).

A: We have now described the procedure more in detail

- Q: The paragraph on enzymes could be separated from the paragraph on geochemical measurements.

A: Done

- Q: What is the margin of error considered for optical quantification? Analytical errors must be specified for geochemical measurements.

To exclude any electronic noise, the minimum size for detecting features by optical quantification was set at 100 μm^2 .

- Q: The sentence "All data treatments were carried out using R (R core team, 2019)" is not sufficient. The package used should be specified.

A: We have specified that *agricolae* package has been used.

1. **Results**

- Why state that "the total porosity is" below the lowest limit of good soil structural condition", when afterwards it is stated that "because of sample preparation, the voids between aggregates (inter-aggregates) was not measured". I think it would be more accurate to refer to "intra-aggregate porosity" rather than "total porosity" or "total detectable porosity ». Is the accuracy to the second decimal place reasonable with respect to the method of measurement? Since the analytical error is not specified, it is difficult to judge.

A: We prefer to delete this paragraph, because it did not add any relevant findings (see also reply to rev#1)

- Is sampling design adequate to measure a possible effect of management practices? If the samples are all linked to the same plots and therefore interdependent, I am not sure that the conclusion is so obvious because the measurements then could characterize a sampling station and not a practice.

A: We agree with the reviewer that the use of the term "soil management" led confusion. We prefer therefore to change "soil management" in "site", also in agreement to the main question added at the end of the Introduction (see also replay to rev#1 comments).

- L268 to 271. A reminder of the objectives is not necessary in a results section, which should remain very formal.

A: As suggested, the Results section has been completely rewritten. The sentence has been thus delete from the Results section

- L272 to 276. What is claimed is not clear from the data.

A: As suggested, the Results section has been completely rewritten

- Q: L278 to 289. Interpretation or discussion of the results should not be found in a section entitled "results".

A: As suggested, the Results section has been separated from the Discussion ones.

- Q: I am not convinced by the results presented in Figure 1. The comparison of a distribution over 3 classes with one to 4 classes influences the ANOVA results. Are the results still significant if the class > 200 is omitted in the macro-aggregates and then absent in the micro-aggregates

A: We agree with the rev#2 that the lack of the pores > 200 um in the fine macro-aggregates did not allow to compare it between fine and coarse macroaggregates. For these reason, in Figure 1, for pores >200 um the comparison between coarse and fine macroaggregates has not been performed. In order to clarify it, we changed “-“ into “n.d.” (not determined) in Figure 1.

- It is a regret that the figures 2 and 3 do not show the results for the different plots. This makes it difficult to relate these results to the following figures.

A: Figs 2 and 3 showed the results according to the aggregate size. We prefer to maintain this type of presentation which is the same that the following figures (figs 4 and 5), We believe that it should allow to the reader to make some comparisons and gain in results readability.

- Q: I'm confused by the results of figure 2. All three graphs have a common structure that could be related to the C concentration in each particle class. Isn't it possible to relate the measurement of C to another element, relatively stable and little involved in organo-mineral interactions (Si?) to appreciate the variation of C independently of the other 3 elements? Why didn't you standardize the ratios so that the standard deviations for each element can be compared?

A: The element composition of each organic matter form has been measured by EDS analysis. As indicated in M&M section, the microanalysis data was normalized to 100%, giving a semi-quantitative measure of elemental concentrations. Thus elemental molar ratios are discussed in this work rather than absolute concentrations. Additionally, using elemental molar ratios any C resin effect has been avoided. We decide to use Fe, Al and Ca because they are elements involved in organo-,mineral interactions, rather than others little involved, and thus the Al:C, Fe:C and Ca:C molar ratios can be use as indicators of the degree of organic-metals/minerals interactions.

- Q: Paragraphs 3.3 and 3.5 do not meet the publication standards.

A: The whole Results and Discussion section has been now spitted in two separated sections as suggested in the General Comments. The two paragraphs, therefore, are completely revised.

1 **Title**
2 **New insights into organic carbon stabilization in soil macroaggregates: an *in situ* study**

3
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15

ABSTRACT

The purpose of this study was to ~~study-investigate~~ the *in situ* characterization of organic matter (OM) within soil macroaggregates, and to assess the relationships between OM characteristics and macroaggregate size indicating different OM stabilization mechanisms. Optical micro-morphological investigations, coupled to SEM-EDS technique, were carried out on thin sections of 4-1 and 1-0.25 mm soil aggregates (coarse and fine macroaggregates, respectively) of four different sites in which soil structure were not disturbed by tillage. Fine macroaggregates were significantly less porous (3.70-6.71% of total porosity) and had higher presence of the finest pore class ($<50\ \mu\text{m}$) compared to coarse macroaggregates (5.93-9.08% of total porosity), independently from sites. The percentage of organic matter forms (OMFs) identified by optical investigation was significant higher in fine (13.54-17.75%) than in coarse (4.19-8.27%) macroaggregates. In particular, fine macroaggregates were richer in red and black amorphous organic forms, which were characterized by the highest values of Al:C, Fe:C and Ca:C molar ratios. These findings suggested thus an accumulation of OM in fine macroaggregates ~~than in coarse macroaggregates occurred. It was~~ probably due to a more efficient OM stabilization, ~~in fine than in coarse macroaggregates~~ related to both physical occlusion (lower porosity and smaller pore size) and organo-minerals interaction (higher presence of OMFs characterized by the highest Al:C, Fe:C and Ca:C ratios), ~~than in coarse macroaggregates occurred.~~

The OM exposure index (EI), a measurement of the OM surface exposed to pores and thus potentially available for microbial activity, was unexpectedly higher in fine than in coarse macroaggregates (EI: 0.48-0.79 and 0.25-0.58 mm^{-1} in fine and coarse macroaggregates, respectively). However, the accessibility of OM defined by the EI seemed to facilitate neither the oxidative transformation nor the damage of enzyme activities, being the EI positively related to C_d:N ratio ($r=0.66$), negatively to $\delta^{13}\text{C}$ values ($r=-0.74$) and positive to the geometric mean of the five assayed enzyme activities related to C-cycle ($r=0.79$). ~~We hypothesized~~ ~~I therefore-that~~, even more potentially exposed, in fine macroaggregates the OM was not accessible to microorganisms due to the effective physical occlusion, and thus both accumulation of few transformed OM and maintenance of functionality related to C-cycle occurred.

41 The SOM stabilization in macroaggregates thus involved both physical occlusion and organo-metals/mineral
42 phase interactions processes. Both these processes are often related to microaggregates rather than
43 macroaggregates. Our findings thus seem to provide a new insight for studying the potentiality of OM
44 stabilization and C sequestration in soil macroaggregates.

45

46 **KEYWORDS**

47 Macroaggregate size; aggregate thin sections; optical microscopy; SEM-EDS; physical occlusion; organo-
48 mineral interactions.

49

1. INTRODUCTION

The largest amount of organic C in terrestrial ecosystems is in the soil and it is three times the amount of C in the atmosphere and four times that in the biota (Janzen, 2004). ~~soil contains about 1,500–2,000 Pg of organic C immobilized in various soil organic matter (OM) pools, down to a depth of 1 m. The persistence of soil OM pools is an important factor to balance the flow of C through atmosphere, biota, and soil reservoir.~~ The persistence of this high amount of organic matter (OM) in soil depends on many factors including land use, edaphic factors and climate (Smith et al., 2008), ~~and can be.~~ ~~The natural flows of C between environmental reservoirs have been~~ altered by human activities, which can indeed have contrasting effects. ~~On one hand, several actions have been promoted to enhance the capacity of soils to sequester C with positive impacts on global climate change and food security (Lal, 2004a; Lal, 2004b; Lal et al., 2015). On the other hand, many activities, generally related to intensive agriculture, such as change from forest to agricultural land use, reduction of the agricultural rotations, failure of the C output reintegration, can cause losses of organic C resulting in a worsening of soil ecosystem services (Lal, 2004b; Bai et al., 2018; Baude et al., 2019).~~

One of the objectives of the current soil science research is to model, in a reliable way, the flow of C from within, and to the soil in order to allow the assessment of the different soil properties and management practices applied. To date, one of the main difficulties in reaching this goal is given by the lack of sufficiently detailed knowledge on the processes that govern the persistence of the soil OMOM (Schmidt et al., 2011).

Several authors consider that, for a mechanistic understanding and modelling of soil SOM decomposition and stabilization, it is crucial to improve knowledge on processes such as occlusion of organic matter within aggregates and sorption of organics onto mineral surfaces (Kögel-Knabner et al., 2008; ~~Heckman et al., 2018; Poeplau et al., 2018~~). Conant et al. (2011) proposed a conceptual model defining the resistance of soil SOM to decomposition as being due ~~either~~ to its chemical ~~conformation-structure or and to~~ its physicochemical protection. The former referred to the de-polymerization process, the latter to adsorption/desorption on mineral surface and aggregate turnover. Recently, Wiesmeier et al. (2019) stressed the role of physical protection within aggregates for soil OM persistence, stating that physical protection,

75 and therefore the aggregation process itself, must be considered as an important mechanism for stabilization
76 of organic C-stabilization.

77 The physical protective capacity of aggregates to soil OM is related to the spatial separation of substrate and
78 microorganisms, as well as to reduced the decline of microbial activity due to reduced a lower diffusion of
79 gasses oxygen into and within aggregates (Mikutta et al., 2006; Six et al., 2002). Furthermore, organo-mineral
80 associations acting in soil SOM protection can be considered as structural units of soil aggregates and
81 nanoparticulate fractions of the smaller aggregates themselves (Totsche et al., 2017), and are, therefore,
82 strictly related to the aggregate formation process.

83 Six et al. (2000) postulated that SOM stabilization is based on microaggregate (<0.25 mm) formation within
84 macroaggregates (>0.25 mm), with C in microaggregates stabilized and sequestered for the long-term.

85 Macroaggregates would instead provide minimal C physical occlusion (Six et al., 2004). The efficiency of
86 macro- and microaggregates in soil OM stabilization is due to the different mechanisms that generate
87 aggregates of different size, as extensively described (e.g., Six et al., 2004). However, the role of
88 macroaggregates is essential in soil OM stabilization; macroaggregates being important sites-environment
89 where both organic C is preferentially accumulated and microaggregate formation occurs (Six et al., 2000;
90 Gioacchini et al., 2016).

91 ~~The efficiency of macro- and microaggregates in soil OM stabilization is due to the different mechanisms that~~
92 ~~generate aggregates of different size. Soil aggregation begins because fresh plant residues induce aggregate~~
93 ~~formation by stimulating microbial activity and consequent releasing of microbial derived bindings agents.~~
94 ~~These processes form macroaggregates rich in organic C (Six et al., 2004). SOM decomposition, however,~~
95 ~~proceeds in macroaggregates, and plant residues or particulate organic matter become encrusted with~~
96 ~~mineral particles and microbial by-products in macroaggregates. This forms the core of smaller aggregates~~
97 ~~within the larger ones, increasing soil OM stabilization by organ-mineral interactions in microaggregates (Six~~
98 ~~et al., 2004).~~

99 In addition to the aggregate size, the extent of the C transformation and stabilization in aggregates can be
100 influenced by the network of the intra-aggregate pores, and by the OM exposure to the pore surface

101 (Ananyeva et al., 2013). The exposure of the OM to the pores surface can influence the contact with the
102 gaseous and biotic phase of the soil, two fundamental factors in the transformation OM processes. We
103 suggest thus that the localization of the OM within the aggregates is an aspect that needs to be taken into
104 account and ~~requires demands in-depth investigation to be more widely studied.~~
105 Optical micro-morphological investigations of soil aggregate thin sections allows researchers to localize soil
106 OM in ~~the an~~ undisturbed physical space within aggregates and, coupling them with SEM-EDS analysis, to
107 investigate *in situ* ~~important~~ characteristics of SOM. Considering that C preferentially accumulates in
108 macroaggregates, and that ~~within the macroaggregates the~~ processes leading to the long-term soil OM
109 stabilization begins within macroaggregates (i.e., the microaggregates formation begins within macro-
110 aggregates), we believe that a study of OM properties within macroaggregates can provide new insights into
111 the understanding of the processes of organic carbon preservation into soil aggregates.
112 For this, optical micro-morphological investigations, coupled SEM-EDS technique, of thin sections of
113 macroaggregates of different size (4-1 mm coarse macroaggregates, 1-0.25 mm fine macroaggregates) were
114 carried out to study *in situ* OM properties. ~~We investigated four soils from sites that differed in key drivers~~
115 ~~of OM persistence, such as climate and management (Wiesmeier et al., 2019).~~ In order to increase our
116 knowledge on soil OM persistence, the current research examined these microfeatures in soils characterized
117 by different site conditions in two mountain and plain areas in the Northern Italy.
118 Specifically, this study focused on (i) the *in situ* characterization of soil organic matter within coarse and fine
119 macroaggregates from soil in different site conditions, and (ii) the existence of relation between OM
120 characteristics and macroaggregate size suggesting different-specific OM stabilization processes. We
121 investigated four soils from sites that differed in key drivers of OM persistence, such as climate, soil
122 properties and management (Wiesmeier et al., 2019) because we would test if the hypothesized relationships
123 between OM characteristics and macroaggregate size were similar among different sites (i.e., sites which
124 differed in climate, soil properties and management) and thus if a certain size-effect exists transgressing the
125 environmental key properties.

126

2. MATERIALS AND METHODS

2.1. The study area

In this study we investigated both mountain and plain ~~sites-areas~~ of different altitudes in the Emilia Romagna region (Northern Italy). The mountain ~~site-area~~ was located at Monzuno in the Appennine mountain, while the plain ~~site-area~~ was at Cadriano in the Po Valley (Table 1). The soils in the mountain ~~site-area~~ formed on limestone-marl and pelitic-sandstone stratifications, ~~and are ascribed to Eutrodepts (Soil Survey Staff, 2014),~~ while those in the plain ~~site-area~~ develop on conoids, i.e. sedimentary bodies consisting of a clastic sediment accumulation. ~~Both soils are ascribed to Inceptisols (Soil Survey Staff, 2014); as evinced from the Regional Soil Survey Service database and Haplustepts (Soil Survey Staff, 2014) occur~~ (Regione Emilia Romagna, 2018). The climate of the mountain ~~site-area~~ is characterized by mean annual temperature of 11.6°C and mean annual precipitation of 967 mm, while in plain ~~site-area~~ by 12.9°C and 645 mm, respectively.

In both ~~sites-areas~~ we selected two ~~plots-sites~~ on the basis of soil management (Table 1), avoiding agricultural ~~areas-sites~~ subject to annual tillage operations that would strongly affect soil aggregation (Bronick and Lal, 2005). In the mountain ~~site-area~~, we thus selected a 16-yrs old oak wood (M-OW) and a 5-yrs old alfalfa (M-AA.). In the plain ~~site-area~~ we investigated an experimental walnut grove of the cv. Lara in place since 2001 selecting one fertilized area (P-FF) receiving 90 kg urea ha⁻¹ y⁻¹ as granular urea and one non-fertilized control area without urea distribution (P-NF). In each ~~plotsite~~, two ~~different plots have been selected and~~ pits ~~wide about 0.3 m~~—were dug. ~~From each pit, in a representative area and the 0-20 cm topsoil-soil layer corresponding to A horizon~~ was collected ~~from each pit~~. The main physico-chemical properties of the fine earth of 0-20 cm topsoil were reported in Table ~~1S2~~.

All soil samples were air dried at room temperature and sieved in order to separate two different macroaggregate ~~(>0.25 mm; Six et al. 2000; Tisdall and Oades, 1982) size classes~~fractions: coarse macroaggregates (4-1 mm) and fine macroaggregates (1-0.25 mm).

2.2. Soil aggregate thin sections preparation

The aggregates in both each macroaggregate classes have been gently mixed and at least 25 single aggregates and 50 single aggregates have been randomly kept for the preparation of thin section of the coarse and fine macroaggregate, respectively. The method for preparing thin section was based on Takeda (1988) and Tippkötter et al. (1986). Blocks of aggregates were obtained by impregnation of aggregates samples with polyester resin. The blocks have been then cut along a diameter plane, shaven out and glued to the slide. The slices were thinned to a standard thickness of 30-40 µm, using the Logitech precision lapping machine. The slices were further reduced to few µm and hand-polished by rubbing the slices on paper coated using the birefringence colours of the minerals as indicated in the Michel-Levy paper which reports the birefringence colours of individual minerals according to their thickness. Finally, aggregates thin sections (28 x 48 mm) were polished using diamond paste. The slides were not cover-slipped since the organic microfeatures in these thin-sections were to be analysed for their elemental composition by scanning electron microscope (SEM) equipped with an EDS probe.

~~Aggregate thin sections (2.8 x 4.8 cm) were obtained from the two different macroaggregate fractions size classes by impregnation of aggregates samples with polyester resin. The aggregates in each macroaggregate class have been gently mixed and at least 25 single aggregates and 50 single aggregates have been randomly kept for the preparation of thin section of the coarse and fine macroaggregate, respectively. Coarse macroaggregate thin sections contained at least 25 single aggregates, while fine macroaggregate thin sections contained at least 50 single aggregates. The slides were not cover-slipped since the organic microfeatures in these thin-sections were to be analyzed for their elemental composition by scanning electron microscope (SEM) equipped with an EDS probe.~~

2.3. Optical micromorphology observations, image analysis of pores and organic components in the aggregate thin sections

Conventional descriptions of thin sections were made at 40X following the guidelines of Stoops (2003) and Fitzpatrick (1980). To achieve our research aim, in this study, area of interest in each thin section in this study corresponded to the intra-aggregate area. In coarse macroaggregate thin sections, from 9 to 16 single

178 aggregates were analysed for each sites, while ~~for in~~ fine macroaggregate thin sections from 23 to 41
179 aggregates were investigated. Measurements on aggregates close to the edge of the thin sections or having
180 inside/near artificial bubbles were avoided. Optical observations have been carried out using a polarised
181 microscope Olympus BX50.
182 For image analysis of intra-aggregates porosity and organic matter~~For image analysis of intra-aggregates~~
183 ~~pores and organic matter parameters~~, high-resolution images were captured at 40x using a digital camera,
184 and connected to a computer equipped with an images frame grabber. Captured images were then available
185 for computerised analysis carried out by AnalySIS v 510 (Olympus Soft Imaging Solutions GmbH) image
186 analysis software. Image analysis provides quantitative information from the scanned image.

187

188 2.3.1. Total porosity and pore size distribution

189 To measure pores, multiple images of the same representative aggregates were taken under both plane (PPL;
190 Figure S1-a) and crossed polarized light (XPL) at 0.5 and 15° (Falsone et al., 2014). –This was necessary to
191 distinguish between pores and quartz, since both were translucent under PPL. These images were additively
192 combined and the result inverted. The inverted images were multiplicatively layered with a natural light
193 image to produce a composite binary image in which minerals were readily distinguished from voids, with
194 minerals and soil matrix represented by black pixels and pores by white pixels (Figure S1-b). To exclude any
195 electronic noise and difficulties in removing quartz, the minimum size for detecting pores was set at 100 μm^2 .
196 The pores were classified according to four different size classes (Zhou et al., 2012; Pagliai et al., 2004): <50,
197 50–100, 100–200, >200 μm , on the basis of their equivalent diameters. The total surface of pores and the
198 surface of each pore classes were measured. The percentage of total porosity (total porosity %, i.e., total
199 surface of pores/surface of investigated area) and pore size distribution (% of <50, 50–100, 100–200, >200
200 μm ; i.e., surface of each pore class/total surface of pores) were thus calculated.

201

202 2.3.2. Total surface of organic matter forms (OMFs) and their distribution

203 Under PPL and XPL conditions, the organic forms were identified and categorized as being either organ or
204 amorphous in form (Babel, 1975; [Figure S2](#)). Once classified according to their form, organic components
205 have been further described according to the extent of their decomposition following the classification
206 proposed by Fitzpatrick (1993) where amorphous forms were strongly decomposed organic fragments, and
207 were further described by their colour, with change in colour from yellow to red and black indicating greater
208 decomposition due to oxidative and microbial processes (Bullock et al., 1985; [Figure 2](#)). A manual delimitation
209 of each organic component has been provided using image analysis software within PPL images (Figure S1-
210 c). Images were thus segmented selecting for organic fragments, and the total area of organic fragments and
211 the area of each class of organic features was measured.

212 The percentage of total surface of organic forms (organic matter forms %, i.e., surface of organic forms/
213 surface of investigated area) was calculated. The distribution of different organic components recognised (%
214 of organs and amorphous forms, classified according to their decomposition degree and colour, respectively)
215 was also calculated (i.e., surface of each organic form/surface of investigated area).

217 2.3.3. *Organic matter-pores contact: the exposure index (EI)*

218 The images obtained by organic components analysis was exported and stacked upon the binary pore image
219 thereby forming a map showing the distribution of organic matter in relation to soil pores. It was thus possible
220 to identify the surface of organic matter in contact to the pores and to measure the length of contact
221 perimeter between the two features. Then, the total length of the contact perimeter (in mm) was normalized
222 by the total area of organic form (in mm²), in order to obtain a measure of the proportion of the organic
223 matter surface in contact to the pore. For each sample, an index, called exposure index (EI; mm⁻¹) was
224 calculated. The EI gives information about the organic matter-pores contact, and therefore on the potential
225 physical exposure of organic matter to the microbial activity (Young et al., 2008).

227 2.4. *SEM-EDS analysis on the aggregate thin sections*

228 Polished thin sections were analysed using an environmental scanning electron microscope (SEM) and
229 elemental data were collected by energy-dispersive spectroscopy (EDS) detector using ZEISS SEM systems
230 (EVO MA15) linked to an Oxford Instruments INCA X-max detector with an 80-mm² SDD. For this work, the
231 instrument setup was: low vacuum conditions (>30 kPa), accelerating voltage of 5-20 keV, process time of
232 5.0, working distance of 8.5 mm, spot-size between 500-560. EDS analysis was performed at high
233 magnifications (500-1000x). ~~The microanalysis was carried out for organic features at least on 100 points for~~
234 ~~each thin section. The microanalysis was carried out for the detected organic features (Figure S2) in coarse~~
235 ~~and fine macroaggregates. About 50 points were scored for each organic feature.~~ Data was normalized to
236 100%, giving a semi-quantitative measure of elemental concentrations. Thus elemental molar ratios are
237 discussed in this work rather than absolute concentrations. Additionally, using elemental molar ratios any C
238 resin effect has been avoided. The ratios are thus being interpreted relative to one another rather than being
239 presented as actual soil ratios.
240 In this work we took into account the Al:C, Fe:C and Ca:C molar ratios as indicators of the degree of organic-
241 metals/minerals interactions (Falsone et al., 2014).

243 2.5. ~~Chemical and biochemical~~ Aggregate properties measured on aggregate fractions separated by sieving
244 In order to check the relationships between the features measured *in situ* on aggregate thin sections and
245 chemical and biochemical aggregate properties, the organic carbon, total nitrogen, $\delta^{13}\text{C}$ signature and
246 enzyme activities related to carbon cycle have been measured.

248 2.5.1. Organic C, total N and $\delta^{13}\text{C}$

249 On each aggregate fraction separated by sieving, the total organic C ($\text{g C kg}^{-1}_{\text{aggregate}}$) and total N ($\text{g N kg}^{-1}_{\text{aggregate}}$)
250 concentration were determined on finely ground aggregate subsamples (ground to <0.5 mm) by dry
251 combustion (CHNS-O Elemental Analyser 1110, Thermo Scientific GmbH, Dreieich, DE). The relative
252 abundance of C stable isotopes was determined by continuous flow- isotope ratio mass spectrometry (CF-

IRMS) using an isotopic mass spectrometer Delta V advantage (Thermo- Finnigam, DE). The values were then expressed as $\delta^{13}\text{C}$, as deviation in parts per thousand compared to the universal reference standard.

2.5.2. The geometric mean of enzyme activities (GMea)

The geometric mean of the assayed enzyme activities (GMea) was used as a comprehensive index of soil quality in order to compare enzyme activities in coarse and fine aggregates (Liu et al., 2013). For each aggregates class the geometric mean of the assayed enzyme activities (GMea) was calculated as:

$$GMea = \sqrt[5]{\beta - GLU \cdot \alpha - GLU \cdot N - AG \cdot \beta - XYL \cdot \beta - CEL} \quad (1)$$

where β -GLU, α -GLU, N-AG, β -XYL and β -CEL were β -glucosidase, α -glucosidase, N-acetyl β -glucosaminidase, β -xylosidase, β -cellobiosidase, respectively. These enzyme activities were chosen on their relevance in for C cycle (Liu et al., 2013, Qin et al., 2010). The activity of these five extracellular hydrolytic enzymes was determined using MUF conjugates at final concentrations (Microplate fluorometer infinite200, TECAN, Männedorf, CH), ensuring substrate saturating conditions in according to Giacometti et al. (2014).

2.6. Statistical analysis

Differences in the micromorphological features (porosity, organic forms, EI) between coarse and fine macroaggregates (size factor) were checked by the one-way ANOVA analysis of variance. Within each aggregate class, the ANOVA analysis was carried out on pores and organic forms data considering the soil management (WO, AA, NF and FF) as factor.

Differences in the molar ratio (Al:C, Fe:C and Ca:C), determined from EDS analysis, among organic forms and between coarse and fine macroaggregates in thin section were tested by the one-way ANOVA analysis.

The assumption of ANOVA was tested by Shapiro-Wilks test for normality and data distribution and Levene test for homogeneity of variances.

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278 The relationships between EI micromorphological property and both the chemical and biochemical
279 properties measured on the aggregates were evaluated using the Pearson's correlation coefficient.
280 The threshold used for significance in all statistical tests was set at 0.05. All data treatments were carried out
281 using R [agricolae package](#) (R core team, 2019).
282

283 3. RESULTS AND DISCUSSION

284 3.1. Porosity

285 ~~Table 3 shows the percentage of total porosity in the coarse and fine macroaggregate thin sections.~~
286 ~~Aggregates in general showed total porosity lower than 10%, and thus below the lowest limit of good soil~~
287 ~~structural condition (Pagliai et al., 1988). Because of sample preparation, the voids between aggregates~~
288 ~~(inter-aggregates) was not measured. In our study the total porosity therefore refers to the intra-aggregate~~
289 ~~porosity, justifying the low values of total porosity.~~

290 The total detectable porosity, measured in the intra-aggregates [space](#), ranged from 5.93 to 9.08% and from
291 3.70 to 6.71% in coarse and fine macroaggregates, respectively ([Table 2](#)). Porosity varied significantly based
292 on size factor ($p < 0.001$), and the fine macroaggregates were less porous than the coarse ones ([Table 2](#)).
293 Within each aggregate class, ~~among sites no differences in the management factor did not significantly affect~~
294 ~~intra-aggregate porosity were found.~~

295 Figure 1 (a-b) shows the pore size distribution (PSD) in the different aggregates. The PSD was significantly
296 influenced by the size factor ($p < 0.01$; Figure 1-c). The pores $> 200 \mu\text{m}$ were only present in coarse
297 macroaggregates, while in fine macroaggregates pore $< 50 \mu\text{m}$ predominated. Fine macroaggregates,
298 therefore, were significantly less porous and showing finer porosity compared to coarse macroaggregates.

299 Between ~~management sites factor~~, no differences in the PSD were found (Figure 1-c).
300

301 3.2. Organic C concentration and organic matter forms (OMFs)

302 The concentration of organic C in aggregates, measured on ground samples, varied from 6.6 and 49.6 g kg⁻¹
 303 and from 7.8 and 52.4 g kg⁻¹ in coarse and fine macroaggregates, respectively (Table 23). The organic C
 304 content of fine and coarse macroaggregates was therefore characterized by a high variability, but -mainly
 305 attributable to different soil managements. However, despite the high variability made the size factor not
 306 significant, it was possible to observe a tendency for which the accumulation of C significantly increased
 307 decreased passing from the coarse to the fine macroaggregates within each site (p always <0.01; Table 2).
 308 One of the objectives of our work was to characterize soil organic matter (OM) fractions according to their
 309 specific physical location within the fine and coarse macroaggregates (in situ). Technically this type of
 310 investigation was performed through optical investigation of aggregate thin sections which allowed us the in
 311 situ identification and quantification of organic matter forms (OMFs). The percentage of OMFs, measured on
 312 aggregate thin sections, varied from 4.19 to 8.27 and from 13.54 to 17.75% in coarse and fine
 313 macroaggregates, respectively (Table 23), confirming that accumulation of organic matter was higher in fine
 314 than in coarse macroaggregates (p<0.001). The percentage of OMFs on macroaggregates thin section and
 315 organic C concentration measured by dry combustion on ground aggregates showed thus a significant similar
 316 trend (r=0.567, p<0.05). The two methods used to quantify the OM content in the aggregates were thus
 317 correlated, supporting the use of optical micro-morphological parameter as an indicator of the OM present
 318 in the aggregates.
 319 Table 3 shows that the amount of OMFs was higher in fine macroaggregates, with significant effects of size
 320 factor (p<0.001). In our opinion, the lowest porosity and smallest pore size in fine macroaggregates (Table 3)
 321 could allow a higher persistence of OM in finer than in coarse macroaggregates. The effect of pores network
 322 on soil OM stabilization has been observed by several authors. Kravchenko and Guber (2017) reported
 323 experimental evidences indicating pores of 30-90 µm in size as drivers in processes of organic carbon
 324 decomposition. Ananyeva et al. (2013) showed that abundance of 40-70 µm pores was negatively correlated
 325 with levels of organic carbon in macroaggregates, suggesting that aggregates with great amount of such
 326 pores poorly protected organic matter. Yang et al. (2019) suggest that total porosity has a significant role,
 327 increasing soil aggregate organic carbon respiration. With regard to our data, we suggest that the highest

total porosity could have favored a greater degradation of organic matter in coarse macroaggregates, and that the greatest percentage of pores <50 µm in fine macroaggregates, according to cited authors, could have contributed to organic matter storage within them (Table 3 and Figure 1). Therefore, the physical occlusion would be more efficient in fine macroaggregates than in coarse ones.

3.3. OMFs distribution and Al:C, Fe:C and Ca:C molar ratios ~~localization vs. stabilization processes~~

Optical micro-morphological technique allowed us to identify different categories of OMFs, specifically organs, yellow amorphous, red amorphous and black amorphous, by *in situ* image analysis technique (Table 4). Fitzpatrick (1993) suggested that red and black amorphous organic matter are the end-product of organic residues transformation, while organs consist of plant residues containing cells and represent thus less transformed soil OM. As shown in Table 4, ~~the~~ OMFs detected on thin sections were differently distributed between aggregates (Table 3): the organs have been detected only in coarse macroaggregates of mountain sites, while the organic amorphous forms have been found both in coarse and fine macro-aggregates. ~~The lack of organs in the plain area evidenced difference according sites, which was confirmed also for amorphous forms (p<0.05). However, independently from the sites, according to the size factor, the fine macroaggregates were clearly the richest in red and black organic amorphous forms (p<0.001 Table 4), suggesting that OM accumulated in fine macroaggregates (Table 3) was mainly due to the accumulation of organic amorphous forms pools.~~

Coupling the optical analysis to the SEM-EDS technique, it was possible to perform a semi-quantitative measurement of the element concentrations for each identified OMFs class. In particular, Al:C, Fe:C and Ca:C molar ratios of each OMFs class in coarse and fine macro-aggregates were determined (Figure 2). The morphologically recognised OMFs showed different values of molar ratios. In particular, red and black amorphous forms were characterized by the highest values of Al:C and Fe:C (p<0.05) and black amorphous forms had also the highest values of Ca:C molar ratios (p<0.05). This occurred both in coarse and fine macroaggregates. ~~The Al:C, Fe:C and Ca:C molar ratios reflect the interaction of OMFs with the soil mineral phase and high molar ratio values are chemical indicators of OM stabilization in these amorphous forms within the aggregates (Brown et al., 2000). Our data thus suggested that OM stabilization processes by~~

interaction between OMFs and minerals occurred in the macroaggregates. Furthermore, among OMFs, the OM stabilization by mineral interactions was mainly attributable to red and black OMFs (Figure 2) that preferentially accumulated in fine macroaggregates (Table 4). Thus, the increasing of OM in fine macroaggregates should be ascribe to a more efficient OM stabilization by both physical occlusion and organo-mineral interactions than in coarse macroaggregates.

3.4. *Exposure Index (EI) OM chemical and biochemical characteristics vs. potential exposure of OM*

The chemical properties of OM in macroaggregates was also investigated using the C/N ratio and the $\delta^{13}\text{C}$ signature to provide information about its degree of transformation. The biochemical properties have been instead measured by five enzyme activities related to carbon cycle.

The C/N ratio is an indicator of the whole organic matter pool turnover (Bronick and Lal, 2005), and a high value of C/N suggests the presence of OM with low transformed status. During the process of organic decomposition, isotopic carbon fractionation occurs leading to enrichment in ^{13}C due to oxidation of ^{12}C by microorganisms (Feng, 2002). Consequently, lower values of $\delta^{13}\text{C}$ (more negative) correspond to less oxidized organic matter (Angers et al., 1997). The C/N ratio and the $\delta^{13}\text{C}$ signature of fine and coarse macroaggregates are significantly correlated ($r = 0.675$, $p < 0.01$; Figure S2), confirming the data convergence related to the degree of OM transformation.

Oxidative processes generally drive the transformation of soil OM and they may be influenced by the degree of exposure of the OM to pores interfacing with the gaseous and biotic phase (Geisseler et al., 2011). For this reason, an exposure index (EI) of OM was calculated from the aggregate thin sections, and the relationships between EI and both C/N and $\delta^{13}\text{C}$ values have been investigated. We interpreted the EI as an index of the potential physical exposure of organic matter to microbial activity (Young et al., 2008).

The EI values (Figure 3) varied from 0.25 to 0.58 mm^{-1} in coarse macroaggregates and from 0.48 to 0.79 mm^{-1} in fine macroaggregates, being significantly higher in fine macroaggregates ($p < 0.05$). Figure 4 showed the relationships between EI values and both C/N ratio and $\delta^{13}\text{C}$ values. Specifically, EI values was positively correlated to the C/N ratio and negatively to the $\delta^{13}\text{C}$ values. Both observed relationships indicated that high

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values of EI, typically associated with fine macroaggregates, was related to OM form with lower degree of transformation. Therefore, the accessibility of OM defined by the EI, higher in fine macroaggregates (Figure 2), does not appear to facilitate the processes of oxidative transformation of the soil OM. This could be due to higher OM stabilization in fine macroaggregates than in coarse ones, because of higher physical occlusion in fine macroaggregates, which reduces OM accessibility to microorganisms through lower porosity and smaller pore sizes (Table 3 and Figure 1) and to higher OM minerals interaction due to a larger amount of OMFs interacting with metals/mineral phases (Table 4 and Figure 2).

The EI was interestingly significantly positively correlated also to to note the significant positive correlation between EI and GMea (Figure 5).

This would suggest that the highest OM stabilization occurring in fine macroaggregates allowed the conservation of C cycle functionality related to enzyme activities.

The apparent discrepancy between great degree of OM exposure and high physical occlusion in fine macroaggregates, in our opinion should be explain through the origin of fine macroaggregate themselves. Organic residues must initially be accessible (i.e., exposed) to microorganisms in order to form fine macroaggregates in coarse ones, in agreement with Six et al. (2004) who described how the transformations of the OM represent the driving processes for the formation of soil aggregates. Our hypothesis was that at least part of the OM inherited its current localisation in fine macroaggregates from its initial accessibility in coarse macroaggregates. The rapid transformation that initially involved these OM fractions actually favoured OM interaction with the mineral fraction in fine aggregates leading also to very effectively physical occlusion.

4. DISCUSSION

4.1 The effect of macroaggregate size on OM characteristics

One of the objectives of our work was to characterize soil organic matter (OM) fractions according to their specific physical location within the fine and coarse macroaggregates (*in situ*). Technically this was performed through optical investigation of aggregate thin sections which allowed us the *in situ* identification and

404 quantification of organic matter forms (OMFs). The quantification of OM was also performed on ground
405 samples by the well-standardized dry combustion method, which provides the quantification of the whole
406 organic C in disturbed samples without any distinction among different forms. The quantification of OM
407 content in the aggregates obtained by the two methods (i.e., the content of OC in ground aggregates and the
408 presence of OMFs detected on aggregate thin section) showed similar trend and the data was significantly
409 related, further supporting the use of optical micro-morphological parameter as an indicator of the presence
410 of OM in the aggregates.

411 The *in situ* quantification of OMFs showed that fine macroaggregates were richer in organic matter than
412 coarse ones. Organic C accumulation in small aggregates is often reported (Tisdall and Oades, 1982; Six et al.,
413 2000; Six et al., 2004), and in general this C-enrichment refers to microaggregates (i.e, aggregate <0.25 mm).
414 Our findings thus showed that C accumulation can occur also in small macroaggregates of 1-to-0.25 mm size
415 class. Additionally, our data showed that OC accumulation in fine macroaggregates was coupled to a decrease
416 in porosity. In fact, the effect of macroaggregate size was also observed in the aggregate porosity, with the
417 lowest porosity and the smallest pore size in the fine macroaggregate class. In our opinion, thus, the lowest
418 porosity and smallest pore size in fine macroaggregates could enhance the persistence of OM. The effect of
419 pores network on soil OM stabilization has been in fact observed by several authors. Toosi et al. (2017)
420 demonstrated by their long-term experiment that, in natural succession system, the abundance of specific
421 size classes of pores affected OM decomposition and thus its chemistry in macroaggregates. Kravchenko and
422 Guber (2017) reported experimental evidences indicating pores of 30-90 μm in size as drivers in processes of
423 organic carbon decomposition. Ananyeva et al. (2013) showed that abundance of 40-70 μm pores was
424 negatively correlated with levels of organic carbon in macroaggregates, suggesting that aggregates with great
425 amount of such pores poorly protected organic matter. Quigley et al. (2018) agreed that pores of 40–90 μm
426 size range are associated with quick organic C decomposition, while pores <40 μm tend to be associated with
427 C protection.

428 Yang et al. (2019) suggest that total porosity has a significant role, increasing soil aggregate organic carbon
429 respiration. With regard to our data, we suggest that the highest total porosity could have favored a greater

430 degradation of organic matter in coarse macroaggregates, and that conversely the greatest percentage of
431 pores <50 µm in fine macroaggregates, according to cited authors, could have contributed to organic matter
432 storage within them. Therefore, in our study the physical occlusion would be more efficient in fine
433 macroaggregates than in coarse ones.

434 The effect of size classes of macroaggregates on physical occlusion seemed to transgress that of the site
435 conditions, being both porosity and OMFs amount similar within fine and coarse macroaggregates. This
436 finding was quite unexpected, because site conditions (climate, parent material, soil texture, soil OM, etc.)
437 are considered as key factors in the aggregation process (e.g., Saker et al., 2018; Bronick and Lal, 2005).
438 However, in our study a certain site effect has been detected on organic C content of ground aggregates.
439 Thus, even if clearly the aggregate size strongly affected the physical occlusion of OM, we can not completely
440 excluded a specific site effect on OM stabilization. The OMFs distribution in fact differed among sites: in the
441 aggregates from the soils located in the plain areas organs completely lacked while they were present in the
442 coarse macroaggregates of soils in the mountain areas. Fitzpatrick (1993) suggested that organs consist of
443 plant residues containing cells and represent thus less transformed soil OM. The presence of less transformed
444 soil OM in mountain areas was indeed in agreement with the well-known slowing down of organic matter
445 oxidative kinetics due to low temperature (e.g., De Feudis et al., 2019; Cardelli et al., 2019) allowing at higher
446 altitude greater accumulation of less transformed OM in mountain soils than in plain ones. No general trend
447 has been instead detected in amorphous forms, that according to Fitzpatrick (1993) are the end-product of
448 organic residues transformation.

449 Independently from sites, other processes than physical occlusion might however contribute to the higher
450 accumulation of organic matter in fine than in coarse macroaggregates. Processes related to interaction of
451 OM with minerals/metals can in fact contribute to OM stabilization (Conant et al., 2011).

452 Our data showed that OM accumulation in fine macroaggregates was due to the organic amorphous forms,
453 being organs missing in fine macroaggregates. Additionally, SEM-EDS microanalysis showed that that red and
454 black amorphous forms had the highest Al:C, Fe:C and Ca:C molar ratios. These molar ratios are chemical
455 indicators of OM stabilization reflecting the interaction of OMFs with the soil mineral phase, and high molar

ratio values indicate stronger organo-mineral interactions (Brown et al., 2000). OM stabilization by mineral interactions was thus mainly attributable to red and black OMFs. This was in agreement with the fact that red and black amorphous organic forms are the end-product of organic residues transformation (Fitzpatrick, 1993), and that plant residues or particulate organic matter during decomposition become encrusted with mineral particles and microbial by-products in macroaggregates (Six et al., 2004). It is well-known that this interactions form the core of smaller aggregates within the larger ones, increasing soil OM stabilization in microaggregates (Six et al., 2004). Our data allowed to detect that OM stabilization by mineral interactions was not exclusive only of microaggregates, but efficiently occurred in fine macroaggregates. Finally, the increasing of OM in fine macroaggregates should be ascribe to a more efficient OM stabilization by both physical occlusion and organo-mineral interactions than in coarse macroaggregates.

4.2 The dynamics of macroaggregates and organic matter stabilization

The stabilization of organic matter in soil aggregate limits the oxidative processes, which generally drive the transformation of soil OM. They may be in turn influenced by the degree of exposure of the OM to pores interfacing with the gaseous and biotic phase (Geisseler et al., 2011). For this reason, an exposure index (EI) of OM was calculated from the aggregate thin sections, and the relationships between EI and both C/N and $\delta^{13}\text{C}$ values have been investigated. We interpreted the EI as an index of the potential physical exposure of organic matter to microbial activity (Young et al., 2008), while the C/N ratio and the $\delta^{13}\text{C}$ signature provide information the degree of transformation of organic matter. The C/N ratio is in fact an indicator of the whole organic matter pool turnover (Bronick and Lal, 2005), and a high value of C/N suggests the presence of OM with low transformed status. Isotopic carbon fractionation instead occurs during the process of organic decomposition, leading to enrichment in ^{13}C due to oxidation of ^{12}C by microorganisms (Feng, 2002). Consequently, lower values of $\delta^{13}\text{C}$ (more negative) correspond to less oxidized organic matter (Angers et al., 1997). In our study, the C/N ratio and the $\delta^{13}\text{C}$ signature of fine and coarse macroaggregates were significantly correlated ($r=-0.675$, $p<0.01$; Figure S3), confirming the data convergence related to the degree of OM transformation.

Our findings showed that high values of EI was associated to fine macroaggregates. This was quite unexpected, because our data suggested higher physical occlusion of OM in fine macroaggregates due to lower porosity and smaller pore size than in coarse macroaggregate. Because of the methodological procedure used for EI determination, the EI measures the proximity of OM to the pore surface and thus assesses if the OM is encapsulated in the soil matrix or exposed. The apparent discrepancy between great degree of OM exposure and high physical occlusion in fine macroaggregates, in our opinion should be explain through the origin of fine macroaggregate themselves. Organic residues must initially be accessible (i.e., exposed) to microorganisms in order to form fine macroaggregates in coarse ones, in agreement with Six et al. (2004) who described how the transformations of the OM represent the driving processes for the formation of fine aggregates into coarse ones. Our hypothesis was thus that at least a part of the OM in fine macroaggregates inherited its localisation from its initial accessibility in coarse macroaggregates, and that *i)* its initial degradation allows the formation of fine macroaggregates causing OM encapsulation and *ii)* consequently favouring OM stabilization. This seemed to be supported by positive correlation between EI and C/N ratio, and the negative ones between EI and $\delta^{13}\text{C}$. They in fact indicated that high values of EI, typically associated with fine macroaggregates, was related to OM form with lower degree of transformation. Additionally, EI was positively linked to GMea. Thus, the apparent accessibility of OM defined by the EI did not compromise the C-cycle functionally related to enzyme activities related to C-cycle and thus the soil functionality was preserved (Wang et al., 2015; Wang et al., 2017).

5. CONCLUSIONS

This study offers a picture of the processes that are active within macroaggregates (4-1 and 1-0.25 mm) and which influence the transformation and stabilization of the OM as a function of its physical location. Our *in situ* investigation has allowed us to detect that:

- Coarse macroaggregates (4-1 mm) tended to be more porous and contained lower percentages of OMFs and organic C than and fine macroaggregates (1-0.25 mm).

• Fine macroaggregates accumulated OMFs characterized by a greater interaction with the mineral soil fraction, greater degree of exposure to the pores surface, lower degree of chemical transformation and higher maintenance of C-cycle functionality than in coarse macroaggregates.

• The fractions of the OM that have undergone the greatest chemical transformations (evaluated by C/N value and $\delta^{13}\text{C}$ signature) were in coarse macroaggregates and were not those that were more stable within the aggregates of the soil, in agreement with Schmidt et al. (2011).

The data of porosity, distribution of pores and organic forms determined by image analysis, confirmed that coarse and fine macroaggregates differed, and they were physically differentiated microhabitats for microorganisms. Specifically, fine macroaggregates had organic matter closer to the pores surface than in coarse macroaggregates probably due to the origin of fine macroaggregates themselves, whose genesis begins because of the decomposition of accessible particulate organic residues within coarse macroaggregates (Figure 6). In fine macroaggregates, the interaction between OM and metals and/or mineral phase and the physical occlusion of OM lead to its stabilisation (Figure 6).

Physical occlusion and interaction with minerals thus appeared as two complementary mechanisms enhancing OM stabilization in fine macroaggregates.

~~The OM stabilization in fine macroaggregates involved both physical occlusion and organo-~~
~~metals/mineral phase interactions processes. Both~~ these processes are often related to
microaggregates (<0.25 mm) rather than macroaggregates (>0.25 mm) and further researches need on
the study of their relative importance in fine macroaggregates. However, o- Our findings ~~thus~~ seem to
provide a new insight for studying the potentiality of OM stabilization and C sequestration in soil
macroaggregates.

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662

663

664 **Figure captions**

665 Figure 1. Pore size distribution in a) coarse and b) fine macroaggregates. In c) the ANOVA ~~analysis~~-results
666 are reported. M-OW and M-AA: 16-yrs old oak wood and 5-yrs old alfalfa in mountain area, respectively; P-
667 NF and P-FF: non-fertilized and fertilized walnut grove in plain area.

668

669 Figure 2. Al:C, Fe:C and Ca:C molar ratio of the different organic forms in coarse and fine macroaggregates.
670 Different capital letters represent the significant diffences among organic forms. Different lower letters
671 represent the significant difference between coarse and fine macroaggregates within the same organic
672 forms.

673 Figure 3. Box-plots of exposure index (EI, in mm⁻¹) values in the coarse and fine macroaggregates. ~~Different~~
674 ~~letters refer to significant differences (p<0.05). The box represents the interquartile range, the line represents~~
675 ~~the median value, the symbol represents the mean value, error bars represent the full range of data. Different~~
676 ~~letters refer to significant differences (p<0.05)~~

677 ~~119~~

678 Figure 4. Relationships between EI values measured in aggregate thin sections and chemical properties
679 measured on ground aggregates (C/N ratio and $\delta^{13}\text{C}$). The coarse (filled symbols) and fine macroaggregate
680 (open symbols) classes are display for each plot. The error bars indicate the standard deviation.

681 Figure 5. Relationships between EI values measured in aggregate thin sections and geometric mean of
682 assayed enzyme activities (GMea). The coarse (filled symbols) and fine macroaggregate (open symbols)
683 classes are display for each plot. The error bars indicate the standard deviation.

684 Figure 6. Conceptual scheme of the dynamics of macroaggregates and organic matter stabilization. Into
685 brackets the microfeatures used in this study and suggesting each step of the scheme

686

HIGHLIGHTS

- Soil macroaggregates (4-1 and 1-0.25 mm) thin sections have been investigated
- Optical microscopy and SEM-EDS allowed the *in situ* analysis of OM in macroaggregates
- Both physical occlusion and mineral interactions stabilized OM in macroaggregates
- The highest OM stabilization by both mechanisms was in fine macroaggregates
- In fine macroaggregate, both OM accumulation and functionality maintenance occurred

1 **Title**

2 **New insights into organic carbon stabilization in soil macroaggregates: an *in situ* study**

3

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15

16 **ABSTRACT**

17 The purpose of this study was to investigate the *in situ* characterization of organic matter (OM) within soil
18 macroaggregates, and to assess the relationships between OM characteristics and macroaggregate size
19 indicating different OM stabilization mechanisms. Optical micro-morphological investigations, coupled to
20 SEM-EDS technique, were carried out on thin sections of 4-1 and 1-0.25 mm soil aggregates (coarse and fine
21 macroaggregates, respectively) of four different sites in which soil structure were not disturbed by tillage.
22 Fine macroaggregates were significantly less porous (3.70-6.71% of total porosity) and had higher presence
23 of the finest pore class (<50 μm) compared to coarse macroaggregates (5.93-9.08% of total porosity),
24 independently from sites. The percentage of organic matter forms (OMFs) identified by optical investigation
25 was significant higher in fine (13.54-17.75%) than in coarse (4.19-8.27%) macroaggregates. In particular, fine
26 macroaggregates were richer in red and black amorphous organic forms, which were characterized by the
27 highest values of Al:C, Fe:C and Ca:C molar ratios. These findings suggested thus an accumulation of OM in
28 fine macroaggregates than in coarse macroaggregates occurred. It was probably due to a more efficient OM
29 stabilization in fine than in coarse macroaggregates related to both physical occlusion (lower porosity and
30 smaller pore size) and organo-minerals interaction (higher presence of OMFs characterized by the highest
31 Al:C, Fe:C and Ca:C ratios),
32 The OM exposure index (EI), a measurement of the OM surface exposed to pores and thus potentially
33 available for microbial activity, was unexpectedly higher in fine than in coarse macroaggregates (EI: 0.48-0.79
34 and 0.25-0.58 mm^{-1} in fine and coarse macroaggregates, respectively). However, the accessibility of OM
35 defined by the EI seemed to facilitate neither the oxidative transformation nor the damage of enzyme
36 activities, being the EI positively related to C:N ratio ($r=0.66$), negatively to $\delta^{13}\text{C}$ values ($r=-0.74$) and positive
37 to the geometric mean of the five assayed enzyme activities related to C-cycle ($r=0.79$). Therefore, even
38 more potentially exposed, in fine macroaggregates the OM was not accessible to microorganisms due to the
39 effective physical occlusion, and thus both accumulation of few transformed OM and maintenance of
40 functionality related to C-cycle occurred.

41 The OM stabilization in macroaggregates thus involved both physical occlusion and organo-metals/mineral
42 phase interactions processes. Both these processes are often related to microaggregates rather than
43 macroaggregates. Our findings thus seem to provide a new insight for studying the potentiality of OM
44 stabilization and C sequestration in soil macroaggregates.

45

46 **KEYWORDS**

47 Macroaggregate size; aggregate thin sections; optical microscopy; SEM-EDS; physical occlusion; organo-
48 mineral interactions.

49

1. INTRODUCTION

The largest amount of organic C in terrestrial ecosystems is in the soil and it is three times the amount of C in the atmosphere and four times that in the biota (Janzen, 2004). The persistence of this high amount of organic matter (OM) in soil depends on many factors including land use, edaphic factors and climate (Smith et al., 2008), and can be altered by human activities, which can indeed have contrasting effects (Lal, 2004a; Lal, 2004b; Lal et al., 2015; Bai et al., 2018; Baude et al., 2019). One of the objectives of the current soil science research is to model, in a reliable way, the flow of C from, within, and to the soil in order to allow the assessment of the different soil properties and management practices applied. To date, one of the main difficulties in reaching this goal is given by the lack of sufficiently detailed knowledge on the processes that govern the persistence of the soil OM (Schmidt et al., 2011).

Several authors consider that, for a mechanistic understanding and modelling of soil OM decomposition and stabilization, it is crucial to improve knowledge on processes such as occlusion of organic matter within aggregates and sorption of organics onto mineral surfaces (Kögel-Knabner et al., 2008;). Conant et al. (2011) proposed a conceptual model defining the resistance of soil OM to decomposition as being due to its chemical structure and its physicochemical protection. The former referred to the de-polymerization process, the latter to adsorption/desorption on mineral surface and aggregate turnover. Recently, Wiesmeier et al. (2019) stressed the role of physical protection within aggregates for soil OM persistence, stating that physical protection, and therefore the aggregation process itself, must be considered as an important mechanism for stabilization of organic C.

The physical protective capacity of aggregates to soil OM is related to the spatial separation of substrate and microorganisms, as well as to reduced microbial activity due to a lower diffusion of gases into and within aggregates (Mikutta et al., 2006; Six et al., 2002). Furthermore, organo-mineral associations acting in soil OM protection can be considered as structural units of soil aggregates and nanoparticulate fractions of the smaller aggregates themselves (Totsche et al., 2017), and are, therefore, strictly related to the aggregate formation process.

75 Six et al. (2000) postulated that SOM stabilization is based on microaggregate (<0.25 mm) formation within
76 macroaggregates (>0.25 mm), with C in microaggregates stabilized and sequestered for the long-term.
77 Macroaggregates would instead provide minimal C physical occlusion (Six et al., 2004). The efficiency of
78 macro- and microaggregates in soil OM stabilization is due to the different mechanisms that generate
79 aggregates of different size, as extensively described (e.g., Six et al., 2004). However, the role of
80 macroaggregates is essential in soil OM stabilization; macroaggregates being important environment where
81 both organic C is preferentially accumulated and microaggregate formation occurs (Six et al., 2000; Gioacchini
82 et al., 2016).

83 In addition to the aggregate size, the extent of the C transformation and stabilization in aggregates can be
84 influenced by the network of the intraggregate pores, and by the OM exposure to the pore surface (Ananyeva
85 et al., 2013). The exposure of the OM to the pores surface can influence the contact with the gaseous and
86 biotic phase of the soil, two fundamental factors in the transformation OM processes. We suggest thus that
87 the localization of the OM within the aggregates is an aspect that needs to be taken into account and
88 demands in-depth investigation.

89 Optical micro-morphological investigations of soil aggregate thin sections allows researchers to localize soil
90 OM in an undisturbed physical space within aggregates and, coupling them with SEM-EDS analysis, to
91 investigate *in situ* characteristics of OM. Considering that C preferentially accumulates in macroaggregates,
92 and that the processes leading to the long-term soil OM stabilization begins within macroaggregates (i.e., the
93 microaggregates formation begins within macroaggregates), we believe that a study of OM properties within
94 macroaggregates can provide new insights into the understanding of the processes of organic carbon
95 preservation into soil aggregates.

96 For this, optical micro-morphological investigations, coupled SEM-EDS technique, of thin sections of
97 macroaggregates of different size (4-1 mm coarse macroaggregates, 1-0.25 mm fine macroaggregates) were
98 carried out to study *in situ* OM properties. In order to increase our knowledge on soil OM persistence, the
99 current research examined these microfeatures in soils characterized by different site conditions in two
100 mountain and plain areas in the Northern Italy.

Specifically, this study focused on (i) the *in situ* characterization of soil organic matter within coarse and fine macroaggregates from soil in different site conditions, and (ii) the existence of relation between OM characteristics and macroaggregate size suggesting specific OM stabilization processes. We investigated four soils from sites that differed in key drivers of OM persistence, such as climate, soil properties and management (Wiesmeier et al., 2019) because we would test if the hypothesized relationships between OM characteristics and macroaggregate size were similar among different sites (i.e., sites which differed in climate, soil properties and management) and thus if a certain size-effect exists transgressing the environmental key properties.

2. MATERIALS AND METHODS

2.1. The study area

In this study we investigated both mountain and plain areas of different altitudes in the Emilia Romagna region (Northern Italy). The mountain area was located at Monzuno in the Appennine mountain, while the plain area was at Cadriano in the Po Valley (Table 1). The soils in the mountain area formed on limestone-marl and pelitic-sandstone stratifications, while those in the plain area develop on conoids, i.e. sedimentary bodies consisting of a clastic sediment accumulation. Both soils are ascribed to Inceptisols (Soil Survey Staff, 2014) as evinced from the Regional Soil Survey Service database (Regione Emilia Romagna, 2018). The climate of the mountain area is characterized by mean annual temperature of 11.6°C and mean annual precipitation of 967 mm, while in plain area by 12.9°C and 645 mm, respectively.

In both areas we selected two sites on the basis of soil management (Table 1), avoiding agricultural sites subject to annual tillage operations that would strongly affect soil aggregation (Bronick and Lal, 2005). In the mountain area, we thus selected a 16-yrs old oak wood (M-OW) and a 5-yrs old alfalfa (M-AA.). In the plain area we investigated an experimental walnut grove of the cv. Lara in place since 2001 selecting one fertilized area (P-FF) receiving 90 kg urea ha⁻¹ y⁻¹ as granular urea and one non-fertilized control area without urea distribution (P-NF). In each site, two different plots have been selected and pits wide about 0.3 m were dug.

126 From each pit, the 0-20 cm soil layer corresponding to A horizon was collected. The main physico-chemical
127 properties of the fine earth of 0-20 cm topsoil were reported in Table 1S.

128 All soil samples were air dried at room temperature and sieved in order to separate two different
129 macroaggregate (>0.25 mm; Six et al. 2000; Tisdall and Oades, 1982) size classes: coarse macroaggregates
130 (4-1 mm) and fine macroaggregates (1-0.25 mm).

131

132 *2.2. Soil aggregate thin sections preparation*

133 The aggregates in both macroaggregate classes have been gently mixed and at least 25 single aggregates and
134 50 single aggregates have been randomly kept for the preparation of thin section of the coarse and fine
135 macroaggregate, respectively. The method for preparing thin section was based on Takeda (1988) and
136 Tippkötter et al. (1986). Blocks of aggregates were obtained by impregnation of aggregates samples with
137 polyester resin. The blocks have been then cut along a diameter plane, shaven out and glued to the slide. The
138 slices were thinned to a standard thickness of 30-40 μm , using the Logitech precision lapping machine. The
139 slices were further reduced to few μm and hand-polished by rubbing the slices on paper coated using the
140 birefringence colours of the minerals as indicated in the Michel-Levy paper which reports the birefringence
141 colours of individual minerals according to their thickness. Finally, aggregates thin sections (28 x 48 mm) were
142 polished using diamond paste. The slides were not cover-slipped since the organic microfeatures in these
143 thin-sections were to be analysed for their elemental composition by scanning electron microscope (SEM)
144 equipped with an EDS probe.

145

146 *2.3. Optical micromorphology observations, image analysis of pores and organic components in the* 147 *aggregate thin sections*

148 Conventional descriptions of thin sections were made at 40X following the guidelines of Stoops (2003) and
149 Fitzpatrick (1980). To achieve our research aim, the area of interest in each thin section in this
150 study corresponded to the intraggregate area. In coarse macroaggregate thin sections, from 9 to 16 single
151 aggregates were analysed for each site, while in fine macroaggregate thin sections from 23 to 41 aggregates

152 were investigated. Measurements on aggregates close to the edge of the thin sections or having inside/near
153 artificial bubbles were avoided. Optical observations have been carried out using a polarised microscope
154 Olympus BX50.

155 For image analysis of intra-aggregates porosity and organic matter, high-resolution images were captured at
156 40x using a digital camera, and connected to a computer equipped with an images frame grabber. Captured
157 images were then available for computerised analysis carried out by AnalySIS v 510 (Olympus Soft Imaging
158 Solutions GmbH) image analysis software. Image analysis provides quantitative information from the
159 scanned image.

160

161 2.3.1. *Total porosity and pore size distribution*

162 To measure pores, multiple images of the same representative aggregates were taken under both plane (PPL;
163 Figure S1-a) and crossed polarized light (XPL) at 0.5 and 15° (Falsone et al., 2014). This was necessary to
164 distinguish between pores and quartz, since both were translucent under PPL. These images were additively
165 combined and the result inverted. The inverted images were multiplicatively layered with a natural light
166 image to produce a composite binary image in which minerals were readily distinguished from voids, with
167 minerals and soil matrix represented by black pixels and pores by white pixels (Figure S1-b). To exclude any
168 electronic noise and difficulties in removing quartz, the minimum size for detecting pores was set at 100 μm^2 .
169 The pores were classified according to four different size classes (Zhou et al., 2012; Pagliai et al., 2004): <50,
170 50–100, 100–200, >200 μm , on the basis of their equivalent diameters. The total surface of pores and the
171 surface of each pore classes were measured. The percentage of total porosity (total porosity %, i.e., total
172 surface of pores/surface of investigated area) and pore size distribution (% of <50, 50–100, 100–200, >200
173 μm ; i.e., surface of each pore class/total surface of pores) were thus calculated.

174

175 2.3.2. *Total surface of organic matter forms (OMFs) and their distribution*

176 Under PPL and XPL conditions, the organic forms were identified and categorized as being either organ or
177 amorphous in form (Babel, 1975; Figure S2). Once classified according to their form, organic components

178 have been further described according to the extent of their decomposition following the classification
179 proposed by Fitzpatrick (1993) where amorphous forms were strongly decomposed organic fragments, and
180 were further described by their colour, with change in colour from yellow to red and black indicating greater
181 decomposition due to oxidative and microbial processes (Bullock et al., 1985; Figure 2). A manual delimitation
182 of each organic component has been provided using image analysis software within PPL images (Figure S1-
183 c). Images were thus segmented selecting for organic fragments, and the total area of organic fragments and
184 the area of each class of organic features was measured.

185 The percentage of total surface of organic forms (organic matter forms %, i.e., surface of organic forms/
186 surface of investigated area) was calculated. The distribution of different organic components recognised (%
187 of organs and amorphous forms, classified according to their decomposition degree and colour, respectively)
188 was also calculated (i.e., surface of each organic form/surface of investigated area).

189

190 2.3.3. *Organic matter-pores contact: the exposure index (EI)*

191 The images obtained by organic components analysis was exported and stacked upon the binary pore image
192 thereby forming a map showing the distribution of organic matter in relation to soil pores. It was thus possible
193 to identify the surface of organic matter in contact to the pores and to measure the length of contact
194 perimeter between the two features. Then, the total length of the contact perimeter (in mm) was normalized
195 by the total area of organic form (in mm²), in order to obtain a measure of the proportion of the organic
196 matter surface in contact to the pore. For each sample, an index, called exposure index (EI; mm⁻¹) was
197 calculated. The EI gives information about the organic matter-pores contact, and therefore on the potential
198 physical exposure of organic matter to the microbial activity (Young et al., 2008).

199

200 2.4. *SEM-EDS analysis on the aggregate thin sections*

201 Polished thin sections were analysed using an environmental scanning electron microscope (SEM) and
202 elemental data were collected by energy-dispersive spectroscopy (EDS) detector using ZEISS SEM systems
203 (EVO MA15) linked to an Oxford Instruments INCA X-max detector with an 80-mm² SDD. For this work, the

instrument setup was: low vacuum conditions (>30 kPa), accelerating voltage of 5-20 keV, process time of 5.0, working distance of 8.5 mm, spot-size between 500-560. EDS analysis was performed at high magnifications (500-1000x). The microanalysis was carried out for the detected organic features (Figure S2) in coarse and fine macroaggregates. About 50 points were scored for each organic feature. Data was normalized to 100%, giving a semi-quantitative measure of elemental concentrations. Thus elemental molar ratios are discussed in this work rather than absolute concentrations. Additionally, using elemental molar ratios any C resin effect has been avoided. The ratios are thus being interpreted relative to one another rather than being presented as actual soil ratios.

In this work we took into account the Al:C, Fe:C and Ca:C molar ratios as indicators of the degree of organic-metals/minerals interactions (Falsone et al., 2014).

214

2.5. Aggregate properties measured on aggregate fractions separated by sieving

In order to check the relationships between the features measured *in situ* on aggregate thin sections and chemical and biochemical aggregate properties, the organic carbon, total nitrogen, $\delta^{13}\text{C}$ signature and enzyme activities related to carbon cycle have been measured.

219

2.5.1. Organic C, total N and $\delta^{13}\text{C}$

On each aggregate fraction separated by sieving, the total organic C ($\text{g C kg}^{-1}_{\text{aggregate}}$) and total N ($\text{g N kg}^{-1}_{\text{aggregate}}$) concentration were determined on finely ground aggregate subsamples (ground to <0.5 mm) by dry combustion (CHNS-O Elemental Analyser 1110, Thermo Scientific GmbH, Dreieich, DE). The relative abundance of C stable isotopes was determined by continuous flow- isotope ratio mass spectrometry (CF-IRMS) using an isotopic mass spectrometer Delta V advantage (Thermo- Finnigan, DE). The values were then expressed as $\delta^{13}\text{C}$, as deviation in parts per thousand compared to the universal reference standard.

227

2.5.2. The geometric mean of enzyme activities (GMea)

229 The geometric mean of the assayed enzyme activities (GMea) was used as a comprehensive index of soil
 230 quality in order to compare enzyme activities in coarse and fine aggregates (Liu et al., 2013). For each
 231 aggregates class the geometric mean of the assayed enzyme activities (GMea) was calculated as:

$$232 \quad GMea = \sqrt[5]{\beta - GLU \cdot \alpha - GLU \cdot N - AG \cdot \beta - XYL \cdot \beta - CEL} \quad (1)$$

233

234 where β -GLU, α -GLU, N-AG, β -XYL and β -CEL were β -glucosidase, α -glucosidase, N-acetyl β -glucosaminidase,
 235 β -xylosidase, β -cellobiosidase, respectively. These enzyme activities were chosen on their relevance for C
 236 cycle (Liu et al., 2013, Qin et al., 2010). The activity of these five extracellular hydrolytic enzymes was
 237 determined using MUF conjugates at final concentrations (Microplate fluorometer infinite200, TECAN,
 238 Männedorf, CH) ensuring substrate saturating conditions in according to Giacometti et al. (2014).

239

240 2.6. Statistical analysis

241 Differences in the micromorphological features (porosity, organic forms, EI) between coarse and fine
 242 macroaggregates (size factor) were checked by the one-way ANOVA.

243 Differences in the molar ratio (Al:C, Fe:C and Ca:C), determined from EDS analysis, among organic forms and
 244 between coarse and fine macroaggregates in thin section were tested by the one-way ANOVA.

245 The assumption of ANOVA was tested by Shapiro-Wilks test for normality and data distribution and Levene
 246 test for homogeneity of variances.

247 The relationships between EI micromorphological property and both the chemical and biochemical
 248 properties measured on the aggregates were evaluated using the Pearson's correlation coefficient.

249 The threshold used for significance in all statistical tests was set at 0.05. All data treatments were carried out
 250 using R *agricolae* package (R core team, 2019).

251

252 3. RESULTS

253 3.1. Porosity

254 The total detectable porosity, measured in the intraggregates space, ranged from 5.93 to 9.08% and from
255 3.70 to 6.71% in coarse and fine macroaggregates, respectively (Table 2). Porosity varied significantly based
256 on size factor ($p < 0.001$), and the fine macroaggregates were less porous than the coarse ones (Table 2).
257 Within each aggregate class, among sites no differences in the intraggregate porosity were found.
258 Figure 1 (a-b) shows the pore size distribution (PSD) in the different aggregates. The PSD was significantly
259 influenced by the size factor ($p < 0.01$; Figure 1-c). The pores $> 200 \mu\text{m}$ were only present in coarse
260 macroaggregates, while in fine macroaggregates pore $< 50 \mu\text{m}$ predominated. Fine macroaggregates,
261 therefore, were significantly less porous and showing finer porosity compared to coarse macroaggregates.
262 Between sites, no differences in the PSD were found (Figure 1-c).

263

264 3.2. Organic C concentration and organic matter forms (OMFs)

265 The concentration of organic C in aggregates, measured on ground samples, varied from 6.6 and 49.6 g kg⁻¹
266 and from 7.8 and 52.4 g kg⁻¹ in coarse and fine macroaggregates, respectively (Table 2). The organic C content
267 of fine and coarse macroaggregates was therefore characterized by a high variability, but it was possible to
268 observe a tendency for which the accumulation of C significantly increased passing from the coarse to the
269 fine macroaggregates within each site (p always < 0.01 ; Table 2). The percentage of OMFs, measured on
270 aggregate thin sections, varied from 4.19 to 8.27 and from 13.54 to 17.75% in coarse and fine
271 macroaggregates, respectively (Table 2), confirming that accumulation of organic matter was higher in fine
272 than in coarse macroaggregates ($p < 0.001$). The percentage of OMFs on macroaggregates thin section and
273 organic C concentration measured by dry combustion on ground aggregates showed thus a significant similar
274 trend ($r = 0.567$, $p < 0.05$).

275

276 3.3. OMFs distribution and Al:C, Fe:C and Ca:C molar ratios

277 The OMFs detected on thin sections were differently distributed between aggregates (Table 3): the organs
278 have been detected only in coarse macroaggregates of mountain sites, while the organic amorphous forms

279 have been found both in coarse and fine macro-aggregates. The lack of organs in the plain area evidenced
280 difference according sites, which was confirmed also for amorphous forms ($p<0.05$). However, independently
281 from the sites, according to the size factor the fine macroaggregates were clearly the richest in red and black
282 organic amorphous forms ($p<0.001$).

283 Coupling the optical analysis to the SEM-EDS technique, it was possible to perform a semi-quantitative
284 measurement of the element concentrations for each identified OMFs class. In particular, Al:C, Fe:C and Ca:C
285 molar ratios of each OMFs class in coarse and fine macro-aggregates were determined (Figure 2). The
286 morphologically recognised OMFs showed different values of molar ratios. In particular, red and black
287 amorphous forms were characterized by the highest values of Al:C and Fe:C ($p<0.05$) and black amorphous
288 forms had also the highest values of Ca:C molar ratios ($p<0.05$). This occurred both in coarse and fine
289 macroaggregates.

290 3.4. Exposure Index (EI)

291 The EI values (Figure 3) varied from 0.25 to 0.58 mm⁻¹ in coarse macroaggregates and from 0.48 to 0.79 mm⁻¹
292 ¹ in fine macroaggregates, being significantly higher in fine macroaggregates ($p<0.05$). Figure 4 showed the
293 relationships between EI values and both C/N ratio and $\delta^{13}\text{C}$ values. Specifically, EI values was positively
294 correlated to the C/N ratio and negatively to the $\delta^{13}\text{C}$ values. The EI was significantly positively correlated
295 also to GMea (Figure 5).

296

297 4. DISCUSSION

298 4.1 The effect of macroaggregate size on OM characteristics

299 One of the objectives of our work was to characterize soil organic matter (OM) fractions according to their
300 specific physical location within the fine and coarse macroaggregates (*in situ*). Technically this was performed
301 through optical investigation of aggregate thin sections which allowed us the *in situ* identification and
302 quantification of organic matter forms (OMFs). The quantification of OM was also performed on ground
303 samples by the well-standardized dry combustion method, which provides the quantification of the whole

304 organic C in disturbed samples without any distinction among different forms. The quantification of OM
305 content in the aggregates obtained by the two methods (i.e., the content of OC in ground aggregates and the
306 presence of OMFs detected on aggregate thin section) showed similar trend and the data was significantly
307 related, further supporting the use of optical micro-morphological parameter as an indicator of the presence
308 of OM in the aggregates.

309 The *in situ* quantification of OMFs showed that fine macroaggregates were richer in organic matter than
310 coarse ones. Organic C accumulation in small aggregates is often reported (Tisdall and Oades, 1982; Six et al.,
311 2000; Six et al., 2004), and in general this C-enrichment refers to microaggregates (i.e, aggregate <0.25 mm).
312 Our findings thus showed that C accumulation can occur also in small macroaggregates of 1-to-0.25 mm size
313 class. Additionally, our data showed that OC accumulation in fine macroaggregates was coupled to a decrease
314 in porosity. In fact, the effect of macroaggregate size was also observed in the aggregate porosity, with the
315 lowest porosity and the smallest pore size in the fine macroaggregate class. In our opinion, thus, the lowest
316 porosity and smallest pore size in fine macroaggregates could enhance the persistence of OM. The effect of
317 pores network on soil OM stabilization has been in fact observed by several authors. Toosi et al. (2017)
318 demonstrated by their long-term experiment that, in natural succession system, the abundance of specific
319 size classes of pores affected OM decomposition and thus its chemistry in macroaggregates. Kravchenko and
320 Guber (2017) reported experimental evidences indicating pores of 30-90 μm in size as drivers in processes of
321 organic carbon decomposition. Ananyeva et al. (2013) showed that abundance of 40-70 μm pores was
322 negatively correlated with levels of organic carbon in macroaggregates, suggesting that aggregates with great
323 amount of such pores poorly protected organic matter. Quigley et al. (2018) agreed that pores of 40–90 μm
324 size range are associated with quick organic C decomposition, while pores <40 μm tend to be associated with
325 C protection. Yang et al. (2019) suggest that total porosity has a significant role, increasing soil aggregate
326 organic carbon respiration. With regard to our data, we suggest that the highest total porosity could have
327 favored a greater degradation of organic matter in coarse macroaggregates, and that conversely the greatest
328 percentage of pores <50 μm in fine macroaggregates, according to cited authors, could have contributed to

329 organic matter storage within them. Therefore, in our study the physical occlusion would be more efficient
330 in fine macroaggregates than in coarse ones.

331 The effect of size classes of macroaggregates on physical occlusion seemed to transgress that of the site
332 conditions, being both porosity and OMFs amount similar within fine and coarse macroaggregates. This
333 finding was quite unexpected, because site conditions (climate, parent material, soil texture, soil OM, etc.)
334 are considered as key factors in the aggregation process (e.g., Saker et al., 2018; Bronick and Lal, 2005).
335 However, in our study a certain site effect has been detected on organic C content of ground aggregates.
336 Thus, even if clearly the aggregate size strongly affected the physical occlusion of OM, we can not completely
337 excluded a specific site effect on OM stabilization. The OMFs distribution in fact differed among sites: in the
338 aggregates from the soils located in the plain areas organs completely lacked while they were present in the
339 coarse macroaggregates of soils in the mountain areas. Fitzpatrick (1993) suggested that organs consist of
340 plant residues containing cells and represent thus less transformed soil OM. The presence of less transformed
341 soil OM in mountain areas was indeed in agreement with the well-known slowing down of organic matter
342 oxidative kinetics due to low temperature (e.g., De Feudis et al., 2019; Cardelli et al., 2019) allowing at higher
343 altitude greater accumulation of less transformed OM in mountain soils than in plain ones. No general trend
344 has been instead detected in amorphous forms, that according to Fitzpatrick (1993) are the end-product of
345 organic residues transformation.

346 Independently from sites, other processes than physical occlusion might however contribute to the higher
347 accumulation of organic matter in fine than in coarse macroaggregates. Processes related to interaction of
348 OM with minerals/metals can in fact contribute to OM stabilization (Conant et al., 2011).

349 Our data showed that OM accumulation in fine macroaggregates was due to the organic amorphous forms,
350 being organs missing in fine macroaggregates. Additionally, SEM-EDS microanalysis showed that that red and
351 black amorphous forms had the highest Al:C, Fe:C and Ca:C molar ratios. These molar ratios are chemical
352 indicators of OM stabilization reflecting the interaction of OMFs with the soil mineral phase, and high molar
353 ratio values indicate stronger organo-mineral interactions (Brown et al., 2000). OM stabilization by mineral
354 interactions was thus mainly attributable to red and black OMFs. This was in agreement with the fact that

red and black amorphous organic forms are the end-product of organic residues transformation (Fitzpatrick, 1993), and that plant residues or particulate organic matter during decomposition become encrusted with mineral particles and microbial by-products in macroaggregates (Six et al., 2004). It is well-known that this interactions form the core of smaller aggregates within the larger ones, increasing soil OM stabilization in microaggregates (Six et al., 2004). Our data allowed to detect that OM stabilization by mineral interactions was not exclusive only of microaggregates, but efficiently occurred in fine macroaggregates. Finally, the increasing of OM in fine macroaggregates should be ascribe to a more efficient OM stabilization by both physical occlusion and organo-mineral interactions than in coarse macroaggregates.

4.2 The dynamics of macroaggregates and organic matter stabilization

The stabilization of organic matter in soil aggregate limits the oxidative processes, which generally drive the transformation of soil OM. They may be in turn influenced by the degree of exposure of the OM to pores interfacing with the gaseous and biotic phase (Geisseler et al., 2011). For this reason, an exposure index (EI) of OM was calculated from the aggregate thin sections, and the relationships between EI and both C/N and $\delta^{13}\text{C}$ values have been investigated. We interpreted the EI as an index of the potential physical exposure of organic matter to microbial activity (Young et al., 2008), while the C/N ratio and the $\delta^{13}\text{C}$ signature provide information the degree of transformation of organic matter. The C/N ratio is in fact an indicator of the whole organic matter pool turnover (Bronick and Lal, 2005), and a high value of C/N suggests the presence of OM with low transformed status. Isotopic carbon fractionation instead occurs during the process of organic decomposition, leading to enrichment in ^{13}C due to oxidation of ^{12}C by microorganisms (Feng, 2002). Consequently, lower values of $\delta^{13}\text{C}$ (more negative) correspond to less oxidized organic matter (Angers et al., 1997). In our study, the C/N ratio and the $\delta^{13}\text{C}$ signature of fine and coarse macroaggregates were significantly correlated ($r=-0.675$, $p<0.01$; Figure S3), confirming the data convergence related to the degree of OM transformation.

Our findings showed that high values of EI was associated to fine macroaggregates. This was quite unexpected, because our data suggested higher physical occlusion of OM in fine macroaggregates due to lower porosity

381 and smaller pore size than in coarse macroaggregate. Because of the methodological procedure used for EI
382 determination, the EI measures the proximity of OM to the pore surface and thus assesses if the OM is
383 encapsulated in the soil matrix or exposed. The apparent discrepancy between great degree of OM exposure
384 and high physical occlusion in fine macroaggregates, in our opinion should be explain through the origin of
385 fine macroaggregate themselves. Organic residues must initially be accessible (i.e., exposed) to
386 microorganisms in order to form fine macroaggregates in coarse ones, in agreement with Six et al. (2004)
387 who described how the transformations of the OM represent the driving processes for the formation of fine
388 aggregates into coarse ones. Our hypothesis was thus that at least a part of the OM in fine macroaggregates
389 inherited its localisation from its initial accessibility in coarse macroaggregates, and that *i)* its initial
390 degradation allows the formation of fine macroaggregates causing OM encapsulation and *ii)* consequently
391 favouring OM stabilization. This seemed to be supported by positive correlation between EI and C/N ratio,
392 and the negative ones between EI and $\delta^{13}\text{C}$. They in fact indicated that high values of EI, typically associated
393 with fine macroaggregates, was related to OM form with lower degree of transformation. Additionally, EI
394 was positively linked to GMea. Thus, the apparent accessibility of OM defined by the EI did not compromise
395 the C-cycle functionally related to enzyme activities related to C-cycle and thus the soil functionality was
396 preserved (Wang et al., 2015; Wang et al., 2017).

397

398 5. CONCLUSIONS

399 This study offers a picture of the processes that are active within macroaggregates (4-1 and 1-0.25 mm) and
400 which influence the transformation and stabilization of the OM as a function of its physical location. Our *in*
401 *situ* investigation has allowed us to detect that:

- 402 • Coarse macroaggregates (4-1 mm) tended to be more porous and contained lower percentages of
403 OMFs and organic C than and fine macroaggregates (1-0.25 mm).
- 404 • Fine macroaggregates accumulated OMFs characterized by a greater interaction with the mineral soil
405 fraction, greater degree of exposure to the pores surface, lower degree of chemical transformation
406 and higher maintenance of C-cycle functionality than in coarse macroaggregates.

- The fractions of the OM that have undergone the greatest chemical transformations (evaluated by C/N value and $\delta^{13}\text{C}$ signature) were in coarse macroaggregates and were not those that were more stable within the aggregates of the soil, in agreement with Schmidt et al. (2011).

The data of porosity, distribution of pores and organic forms determined by image analysis, confirmed that coarse and fine macroaggregates differed, and they were physically differentiated microhabitats for microorganisms. Specifically, fine macroaggregates had organic matter closer to the pores surface than in coarse macroaggregates probably due to the origin of fine macroaggregates themselves, whose genesis begins because of the decomposition of accessible particulate organic residues within coarse macroaggregates (Figure 6). In fine macroaggregates, the interaction between OM and metals and/or mineral phase and the physical occlusion of OM lead to its stabilisation (Figure 6).

Physical occlusion and interaction with minerals thus appeared as two complementary mechanisms enhancing OM stabilization in fine macroaggregates. Both these processes are often related to microaggregates (<0.25 mm) rather than macroaggregates (>0.25 mm) and further researches need on the study of their relative importance in fine macroaggregates. However, our findings seem to provide a new insight for studying the potentiality of OM stabilization and C sequestration in soil macroaggregates.

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555

556 **Figure captions**

557 Figure 1. Pore size distribution in a) coarse and b) fine macroaggregates. In c) the ANOVA results are
558 reported. M-OW and M-AA: 16-yrs old oak wood and 5-yrs old alfalfa in mountain area, respectively; P-NF
559 and P-FF: non-fertilized and fertilized walnut grove in plain area.

560 Figure 2. Al:C, Fe:C and Ca:C molar ratio of the different organic forms in coarse and fine macroaggregates.
561 Different capital letters represent the significant differences among organic forms. Different lower letters
562 represent the significant difference between coarse and fine macroaggregates within the same organic
563 forms.

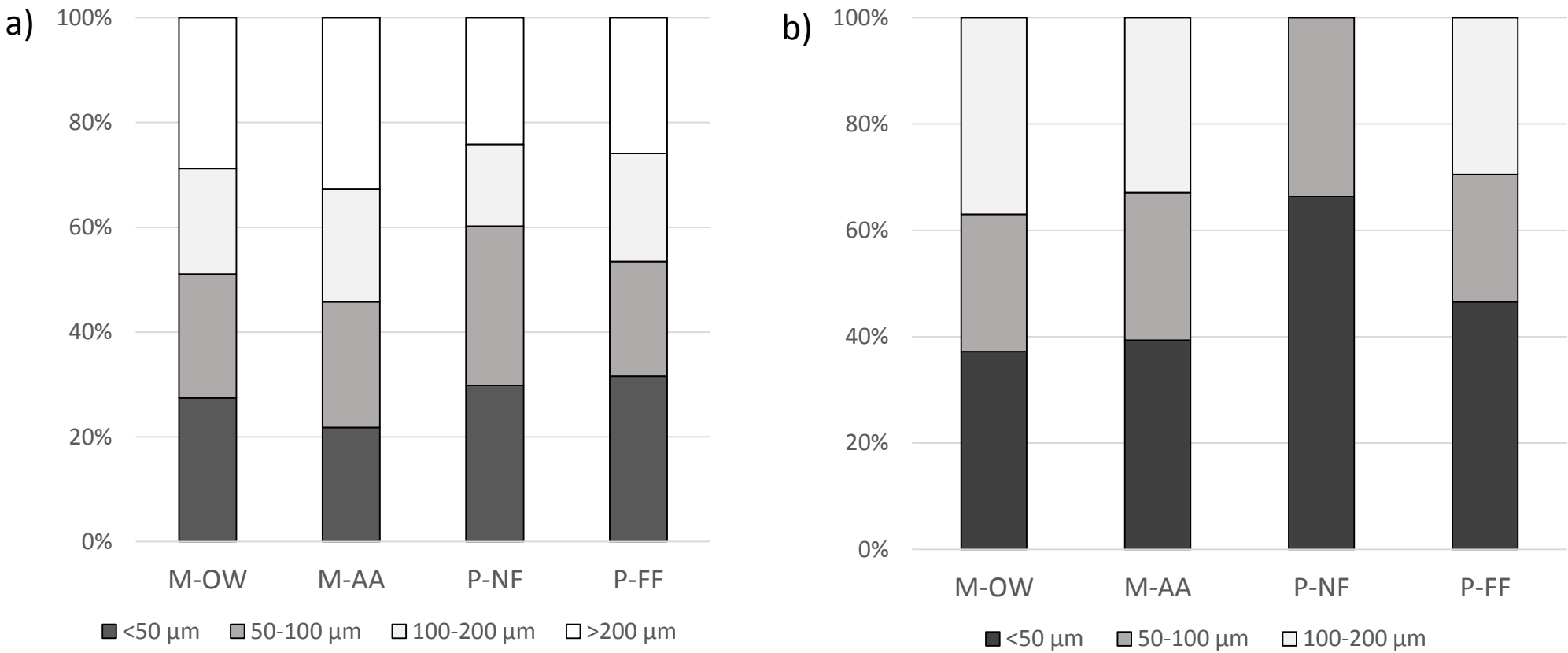
564 Figure 3. Box-plots of exposure index (EI, in mm^{-1}) values in the coarse and fine macroaggregates. The box
565 represents the interquartile range, the line represents the median value, the symbol represents the mean
566 value, error bars represent the full range of data. Different letters refer to significant differences ($p < 0.05$)

567 Figure 4. Relationships between EI values measured in aggregate thin sections and chemical properties
568 measured on ground aggregates (C/N ratio and $\delta^{13}\text{C}$). The coarse (filled symbols) and fine macroaggregate
569 (open symbols) classes are display for each plot. The error bars indicate the standard deviation.

570 Figure 5. Relationships between EI values measured in aggregate thin sections and geometric mean of
571 assayed enzyme activities (GMea). The coarse (filled symbols) and fine macroaggregate (open symbols)
572 classes are display for each plot. The error bars indicate the standard deviation.

573 Figure 6. Conceptual scheme of the dynamics of macroaggregates and organic matter stabilization. Into
574 brackets the microfeatures used in this study and suggesting each step of the scheme

575



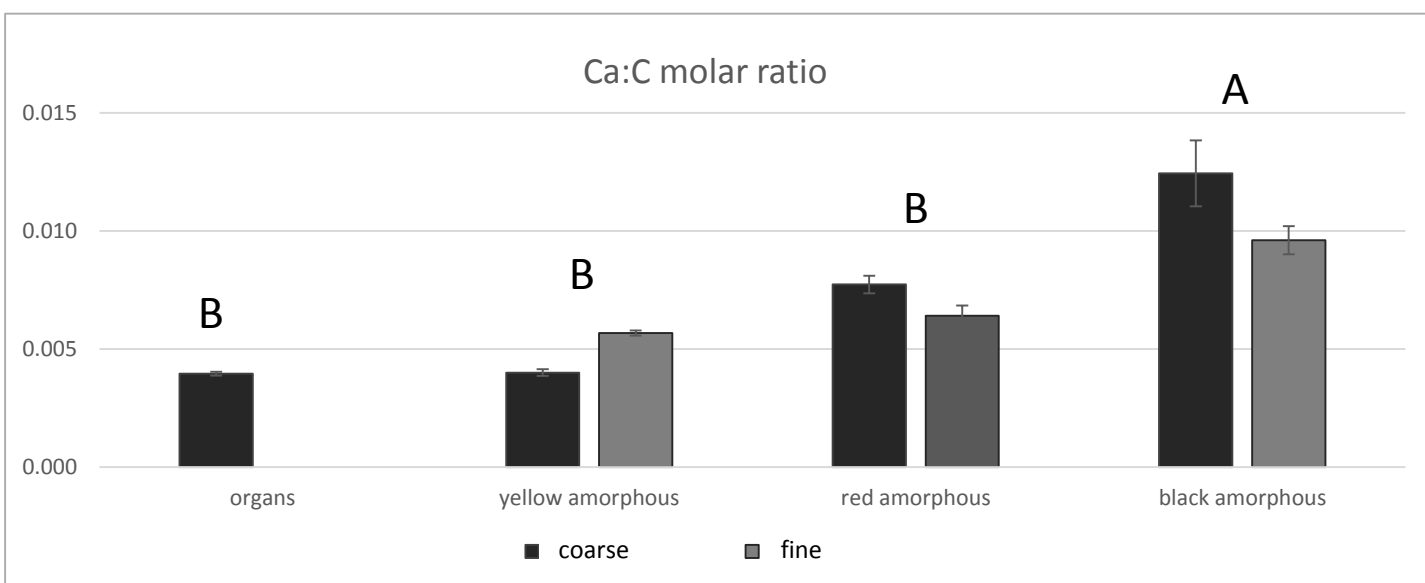
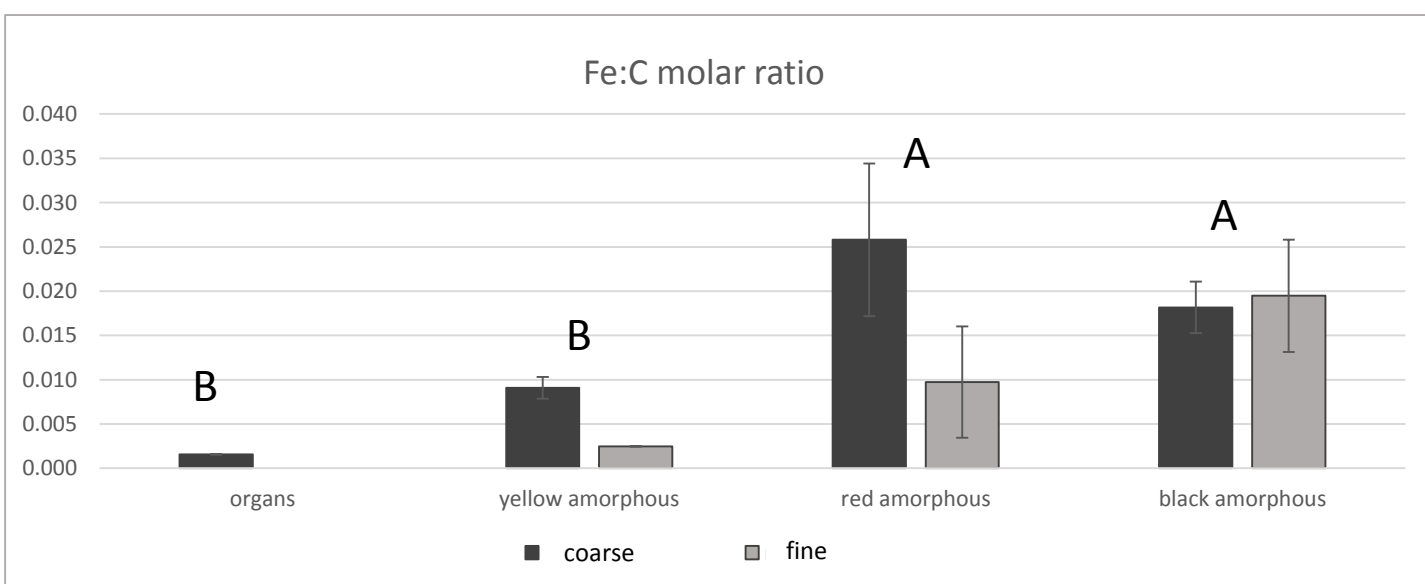
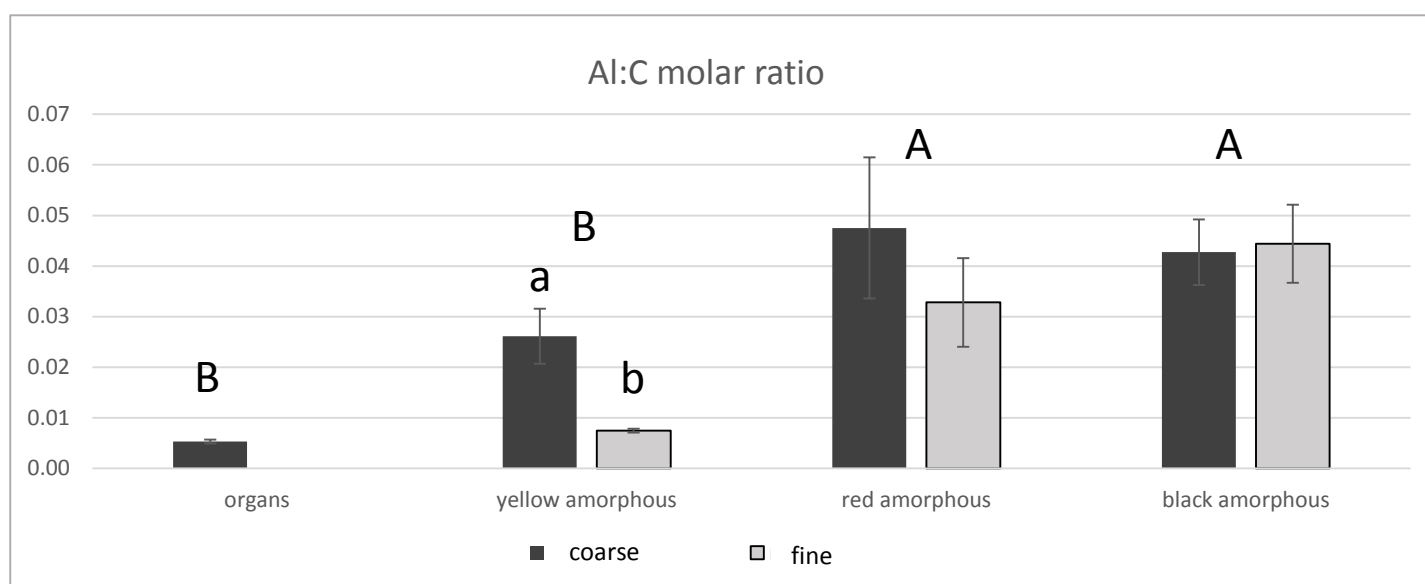
c)

		<50 μm (%)	50-100 μm (%)	100-200 μm (%)	>200 μm (%)
coarse vs fine macroaggregates		***	**	***	nd
within coarse macroaggregates	site	ns	ns	ns	ns
within fine macroaggregates	site	ns	ns	ns	nd

ns: not significant (p>0.05);*: p<0.05; **: p<0.01; ***: p<0.001

nd: not determined

Figure 2



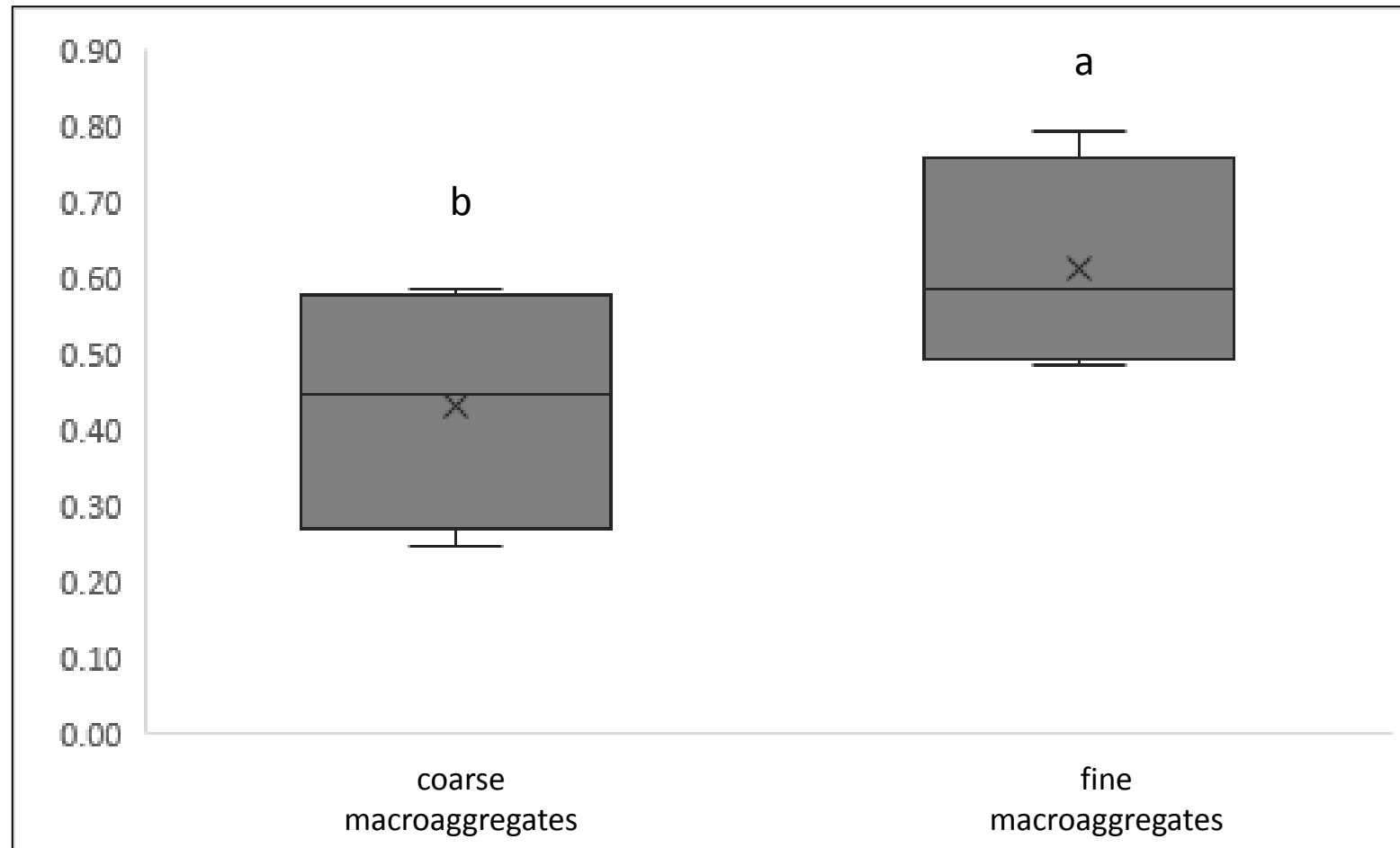


Figure 4

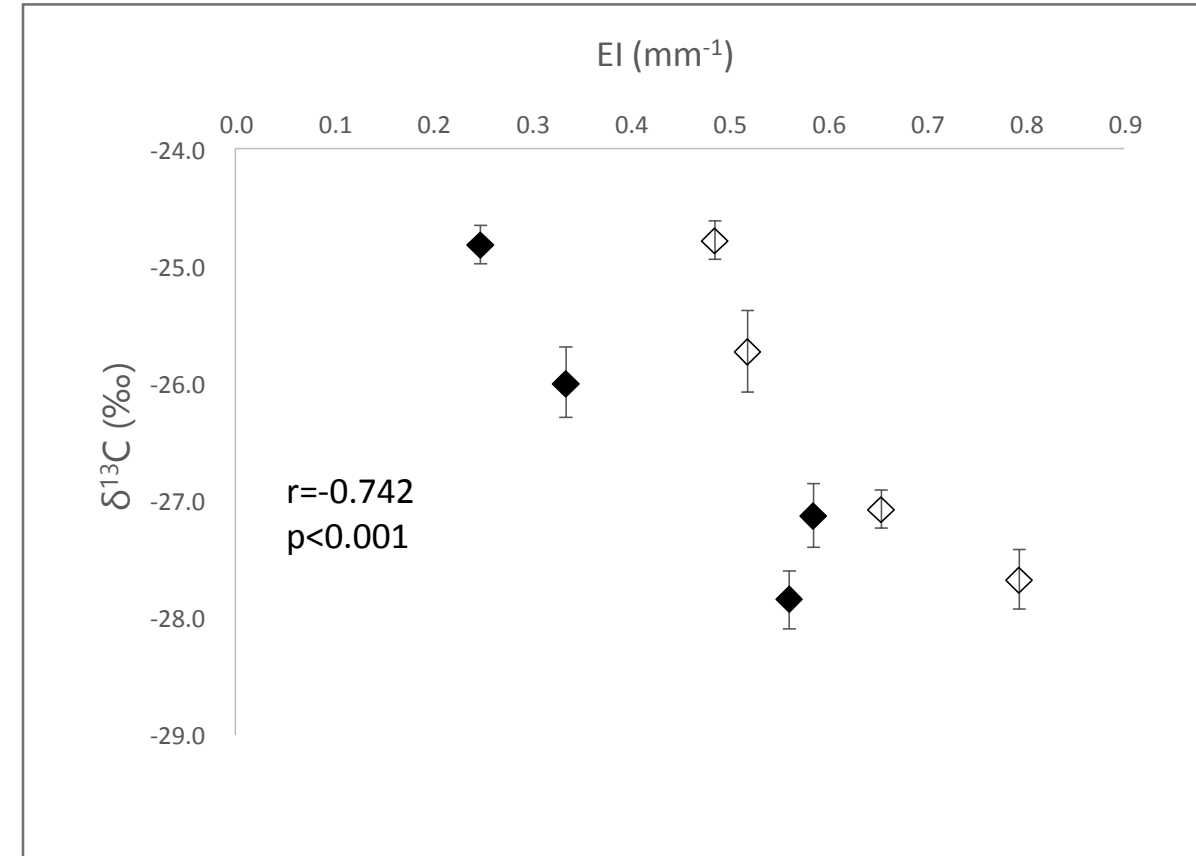
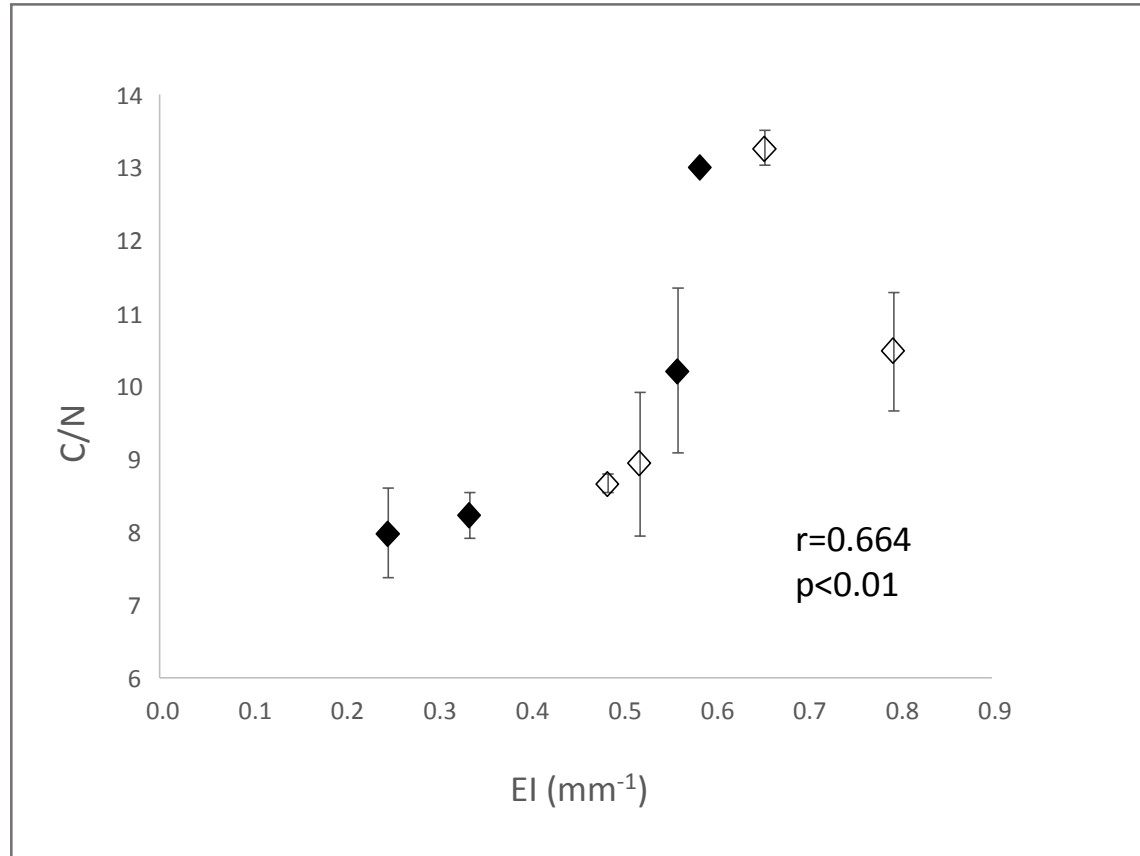
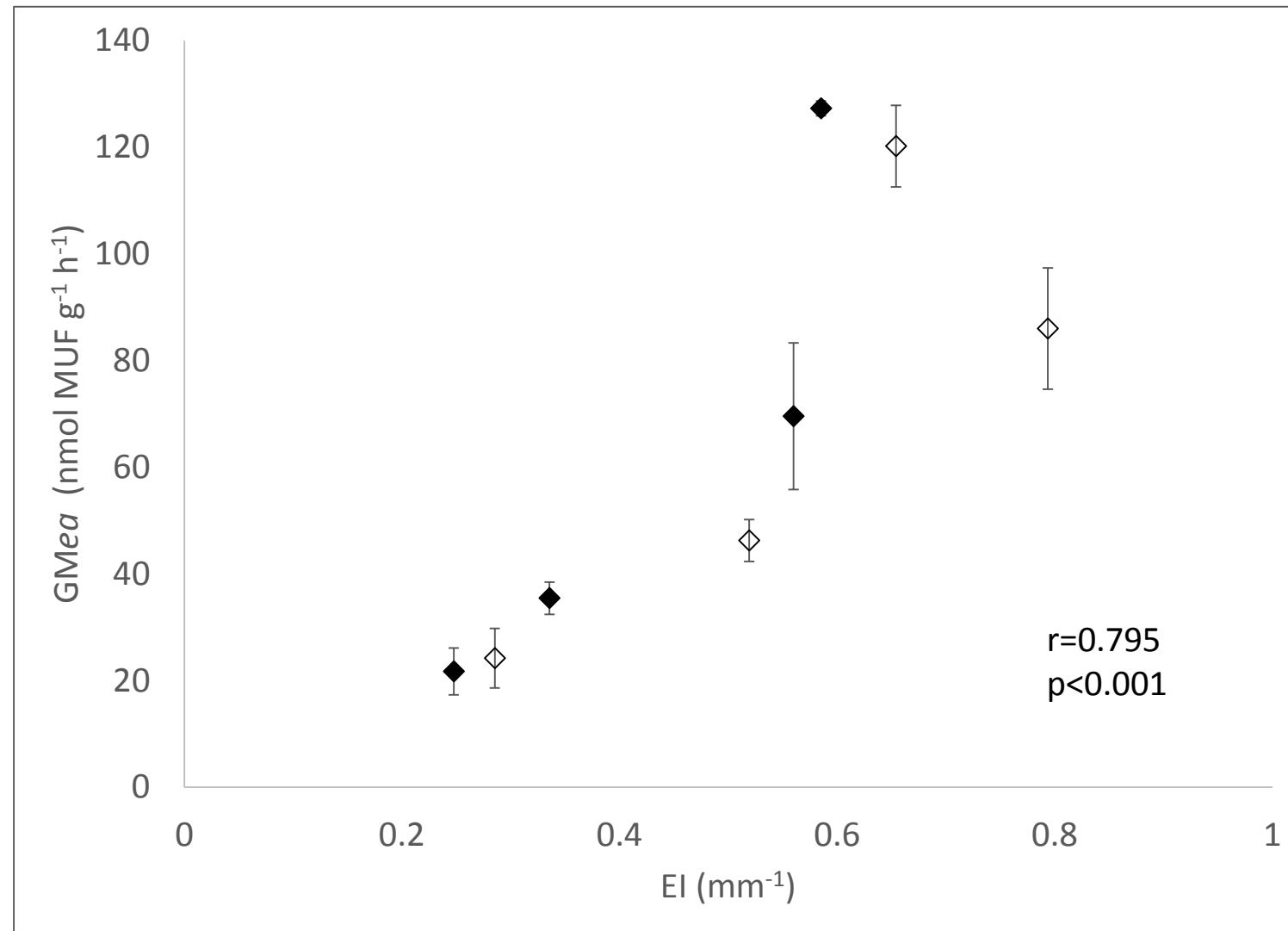


Figure 5



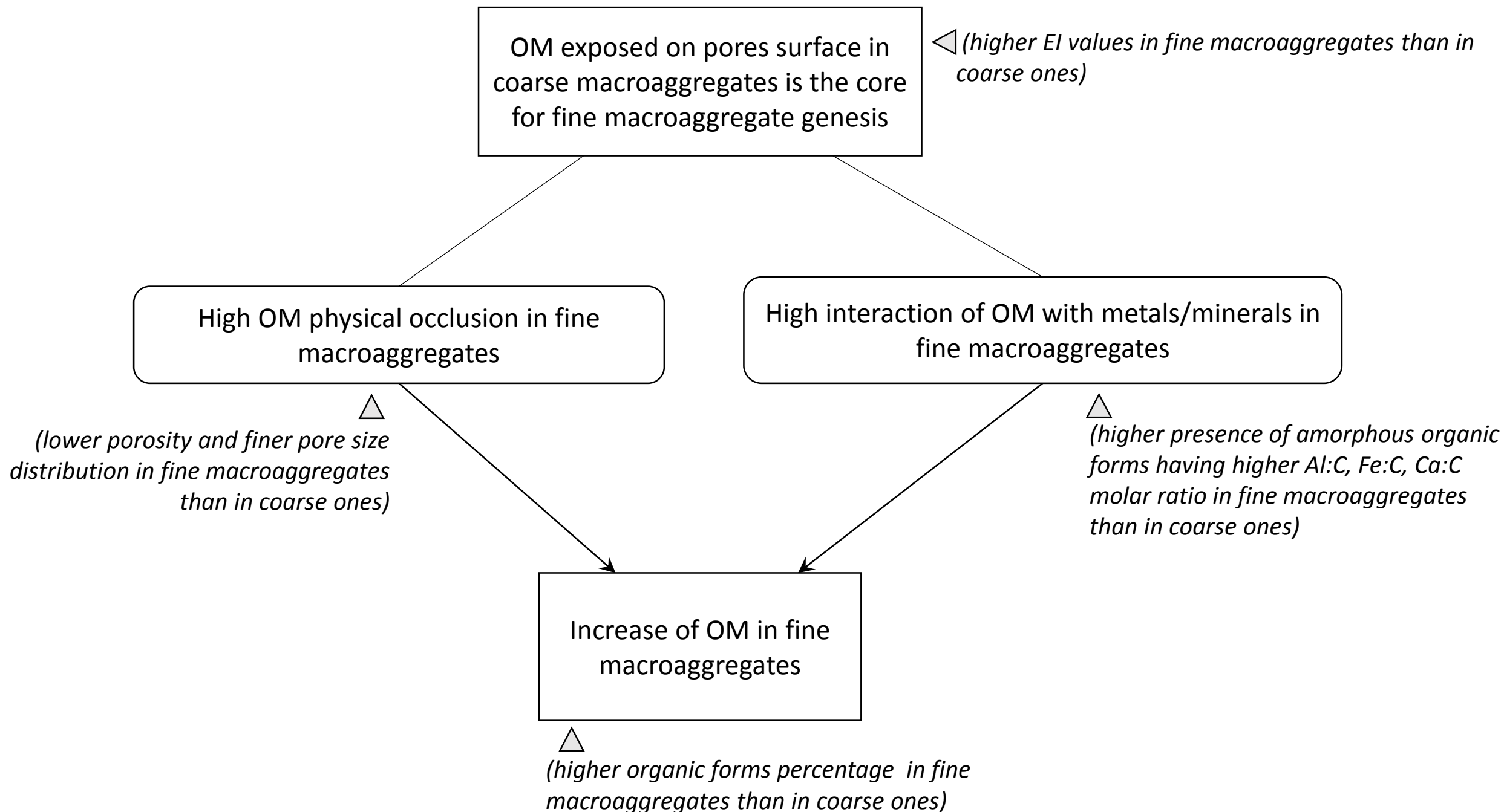


Table 1. General information of the investigated sites

Area	Site	Coordinates	Elevation (m a.s.l.)	Soil management and sampling information
Mountain	M-OW	44° 16'29''N 11°14'53''E	630	The oak wood was a 16-year-old wood exploited for firewood. At sampling time, the wood was at the end of its cutting cycle.
Mountain	M-AA	44°16'28''N 11°15'25''E	663	The alfalfa was a 5-year-old crop not-fertilizer. At sampling time, the alfalfa was at the end of its cropping cycle.
Plain	P-FF	44°32'18''N 11°23'07''E	34	Since 2001, 90 kg/ha/yr g of urea has been distributed for granular treatment subdividing in two doses (45 kg/ha/yr g in April/May and 45 kg/ha/yr in October). The soil was not tilled and covered by spontaneous grasses. The soil sampling was done along the plant rows.
Plain	P-NF	44°32'19''N 11°23'07''E	34	Since 2001, the site was not fertilized. The soil was not tilled and covered by spontaneous grasses. The soil sampling was done along the plant rows.

Table 2. Percentage of total porosity, organic carbon and presence of organic matter forms in coarse and fine macroaggregates. Numbers in the brackets represent the standard deviation values. In the bottom, the ANOVA results are reported

Macroaggregate class	Sites	Total porosity ^a (%)	Organic carbon ^b g kg ⁻¹ _{aggregate}	Organic matter forms ^a (%)
coarse	M-OW	5.96 (1.67)	49.6 (4.6)	8.27 (1.29)
	M-AA	9.08 (1.00)	10.9 (3.5)	6.51 (0.95)
	P-NF	5.93 (1.89)	6.6 (1.8)	4.19 (0.88)
	P-FF	8.19 (1.43)	7.9 (1.2)	5.58 (1.10)
fine	M-OW	4.42 (0.97)	52.4 (7.3)	17.16 (4.48)
	M-AA	6.71 (1.43)	12.1 (3.4)	17.75 (1.94)
	P-NF	3.70 (0.78)	7.8 (1.2)	14.39 (2.99)
	P-FF	6.53 (1.18)	9.3 (3.0)	13.54 (3.41)
coarse vs fine macroaggregates		***	ns	***
within coarse macroaggregates		ns	***	ns
within fine macroaggregates		ns	**	ns

^ameasured on macroaggregate thin sections; ^bmeasured on grounded macroaggregates.

M-OW and M-AA: 16-yr old oak wood and 5-yr old alfalfa in mountain area, respectively; P-NF and P-FF: non-fertilized and fertilized walnut grove in plain area.

ns: not significant (p>0.05); **:p<0.01; ***: p<0.001

Table 3. Organic matter forms distribution in coarse and fine macroaggregates. Numbers in the brackets represent the standard deviation values

macroaggregate	sites	Organs (%)	Yellow	Red	Black
class			amorphous	amorphous	amorphous
			forms (%)	forms (%)	forms (%)
coarse	M-OW	3.31 (0.9)	1.87 (0.2)	2.32 (0.6)	3.16 (0.7)
	M-AA	1.11 (0.4)	-	2.09 (0.9)	3.51 (0.3)
	P-NF	-	1.10 (0.4)	1.29 (0.3)	2.07 (0.4)
	P-FF	-	1.10 (0.5)	2.14 (0.5)	3.14 (0.7)
fine	M-OW	-	1.21 (0.4)	7.77 (0.8)	8.51 (1.2)
	M-AA	-	1.84 (0.8)	6.07 (0.7)	12.06 (1.2)
	P-NF	-	-	6.64 (0.7)	9.72 (1.5)
	P-FF	-	-	5.65 (0.8)	5.30 (0.9)
coarse vs fine		-	-	***	***
macroaggregates					
within coarse					
	site	-	-	*	*
macroaggregates					
within fine					
	site	-	-	***	***
macroaggregates					

M-OW and M-AA: 16-yr old oak wood and 5-yr old alfalfa in mountain area, respectively; P-NF and P-FF: non-fertilized and fertilized walnut grove in plain area.

ns: not significant ($p>0.05$); *: $p<0.05$; ***: $p<0.001$

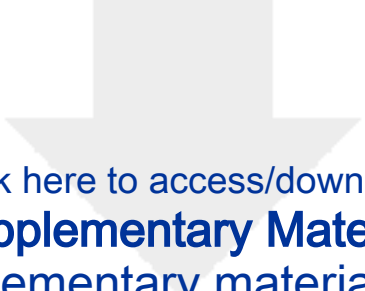
Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:



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