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

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

--Manuscript Draft--

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Corresponding Author:	Gloria Falsone Dipartimento di Scienze e Tecnologie Agro-alimentari, Alma Mater Studiorum Università di Bologna Bologna, Italy
First Author:	Guidi Patrizia
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 macroggregates 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 d 13 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:

Gloria Falsone, Università di Bologna, DISTAL, via Fanin 40, I-40127 Bologna, Italy. Tel +39-0512096229, Email gloria.falsone@unibo.it

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 been 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, 13C 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 been 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 combing 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 13C, 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 13 C 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 $kg^{-1}_{aggreagte}$ (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 stablisation: 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 lengh 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 micrорgamisms 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 µm2.

- 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.

Title

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

Authors

Guidi Patrizia (1), Falsone Gloria (1), Wilson Clare (2), Cavani Luciano (1), Ciavatta Claudio (1), Marzadori Claudio (1)

Author Affiliations

(1) Dipartimento di Scienze e Tecnologie Agro-alimentari, Alma Mater Studiorum Università di Bologna, 40127 Bologna (I)

(2) Biological and Environmental Science, University of Stirling, FK9 4LA Stirling (UK)

Corresponding author

Gloria Falsone, email gloria.falsone@unibo.it

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 μm) 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 macroggregates 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:~~/~~N ratio (r=0.66), negatively to δ^{13}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~~ T~~t~~herefore~~ 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.

The ~~S~~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.

KEYWORDS

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

1. INTRODUCTION

The largest amount of organic C in terrestrial ecosystems is in the soil and it is three time the amount of C in the atmosphere and four time 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 OM~~OM~~ (Schmidt et al., 2011).

Several authors consider that, for a mechanistic understanding and modelling of soil ~~S~~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 (Kö~~o~~gel-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 ~~S~~OM 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, and therefore the aggregation process itself, must be considered as an important mechanism for stabilization of organic C~~ stabilization~~.

The physical protective capacity of aggregates to soil OM is related to the spatial separation of substrate and microorganisms, as well as to reduced ~~the decline of~~ microbial activity due to ~~reduced~~ a lower diffusion of gasses ~~oxygen~~ into and within aggregates (Mikutta et al., 2006; Six et al., 2002). Furthermore, organo-mineral associations acting in soil ~~S~~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.

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

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

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

(Ananyeva et al., 2013). The exposure of the OM to the pores surface can influence the contact with the gaseous and biotic phase of the soil, two fundamental factors in the transformation OM processes. We suggest thus that the localization of the OM within the aggregates is an aspect that needs to be taken into account and ~~requires~~ demands in-depth investigation~~to be more widely studied~~.

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

For this, optical micro-morphological investigations, coupled SEM-EDS technique, of thin sections of macroaggregates of different size (4-1 mm coarse macroaggregates, 1-0.25 mm fine macroaggregates) were carried out to study *in situ* OM properties. ~~We investigated four soils from sites that differed in key drivers of OM persistence, such as climate and management (Wiesmeier et al., 2019).~~ In order to increase our knowledge on soil OM persistence, the current research examined these microfeatures in soils characterized by different site conditions in two 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 ~~different~~ 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 ~~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 Eutrudepts (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 ~~plot~~site, 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 1S~~2~~.

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). ~~B~~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 m 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, the~~in this study,~~ area of interest in each thin section in this study~~-~~corresponded to the intr~~a -~~aggregate area. In coarse macroaggregate thin sections, from 9 to 16 single aggregates were analysed for each site~~s~~, while ~~for~~ in fine macroaggregate thin sections from 23 to 41 aggregates were investigated. Measurements on aggregates close to the edge of the thin sections or having inside/near artificial bubbles were avoided. Optical observations have been carried out using a polorised microscope Olimpus BX50.

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

2.3.1. Total porosity and pore size distribution

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

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

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

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

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

The images obtained by organic components analysis was exported and stacked upon the binary pore image thereby forming a map showing the distribution of organic matter in relation to soil pores. It was thus possible to identify the surface of organic matter in contact to the pores and to measure the length of contact perimeter between the two features. Then, the total length of the contact perimeter (in mm) was normalized by the total area of organic form (in mm^2), in order to obtain a measure of the proportion of the organic matter surface in contact to the pore. For each sample, an index, called exposure index (EI; mm^{-1}) was calculated. The EI gives information about the organic matter-pores contact, and therefore on the potential physical exposure of organic matter to the microbial activity (Young et al., 2008).

2.4. SEM-EDS analysis on the aggregate thin sections

Polished thin sections were analysed using an environmental scanning electron microscope (SEM) and elemental data were collected by energy-dispersive spectroscopy (EDS) detector using ZEISS SEM systems (EVO MA15) linked to an Oxford Instruments INCA X-max detector with an 80-mm^2 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 organic features at least on 100 points for each thin section.~~ 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).

2.5. ~~Chemical and biochemical a~~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, δ^{13}C signature and enzyme activities related to carbon cycle have been measured.

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

On each aggregate fraction separated by sieving, the total organic C (g C kg$^{-1}_{aggregate}$) and total N (g N kg$^{-1}_{aggregate}$) 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- Finnigam, DE). The values were then expressed as $\delta^{13}C$, 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~~n~~ 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:

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

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

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

3. RESULTS ~~AND DISCUSSION~~

3.1. Porosity

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

The total detectable porosity, measured in the intra~~-a~~ggregates space, ranged from 5.93 to 9.08% and from 3.70 to 6.71% in coarse and fine macroaggregates, respectively (Table 2). Porosity varied significantly based on size factor ($p<0.001$), and the fine macroaggregates were less porous than the coarse ones (Table 2~~3~~). Within each aggregate class, among sites no differences in the ~~management factor did not significantly affect~~ intr~~a-~~aggregate porosity were found.~~.~~

Figure 1 (a-b) shows the pore size distribution (PSD) in the different aggregates. The PSD was significantly influenced by the size factor ($p <0.01$; Figure 1-c). The pores >200 μm were only present in coarse macroaggregates, while in fine macroaggregates pore <50 μm predominated. Fine macroaggregates, therefore, were significantly less porous and showing finer porosity compared to coarse macroaggregates. Between ~~management~~ sites~~factor~~, no differences in the PSD were found (Figure 1-c).

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

The concentration of organic C in aggregates, measured on ground samples, varied from 6.6 and 49.6 g kg^{-1} and from 7.8 and 52.4 g kg^{-1} in coarse and fine macroaggregates, respectively (Table 2~~3~~). The organic C content of fine and coarse macroaggregates was therefore characterized by a high variability, but ~~mainly attributable to different soil managements. However, despite the high variability made the size factor not significant,~~ it was possible to observe a tendency for which the accumulation of C significantly increased ~~decreased~~ passing from the coarse to the fine macroaggregates within each site (p always <0.01; Table 2).

~~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 type of investigation was performed through optical investigation of aggregate thin sections which allowed us the *in situ* identification and quantification of organic matter forms (OMFs).~~ The percentage of OMFs, measured on aggregate thin sections, ~~–~~ varied from 4.19 to 8.27 and from 13.54 to 17.75% in coarse and fine macroaggregates, respectively (Table 2~~3~~), confirming that accumulation of organic matter was higher in fine than in coarse macroagrregates (p<0.001). ~~.~~ The percentage of OMFs on macroaggregates thin section and organic C concentration measured by dry combustion on ground aggregates showed thus a significant similar trend (r=0.567, p<0.05). ~~The two methods used to quantify the OM content in the aggregates were thus correlated, supporting the use of optical micro-morphological parameter as an indicator of the OM present in the aggregates.~~

~~Table 3 shows that the amount of OMFs was higher in fine macroaggregates, with significant effects of size factor (p<0.001). In our opinion, the lowest porosity and smallest pore size in fine macroaggregates (Table 3) could allow a higher persistence of OM in finer than in coarse macroaggregates. The effect of pores network on soil OM stabilization has been observed by several authors. Kravchenko and Guber (2017) reported experimental evidences indicating pores of 30-90 μm in size as drivers in processes of organic carbon decomposition. Ananyeva et al. (2013) showed that abundance of 40-70 μm pores was negatively correlated with levels of organic carbon in macroaggregates, suggesting that aggregates with great amount of such pores poorly protected organic matter. Yang et al. (2019) suggest that total porosity has a significant role, 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, t~~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 δ^{13}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 δ^{13}C (more negative) correspond to less oxidized organic matter (Angers et al., 1997). The C/N ratio and the δ^{13}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.~~

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~~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 δ^{13}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 δ^{13}C values. Specifically, EI values was positively correlated to the C/N ratio and negatively to the δ^{13}C values. ~~Both observed relationships indicated that high~~

~~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 3), 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 ~~Interesting~~ significantly positively correlated also to ~~to note the significant positive correlation between EI and~~ GM*ea* (Figure 5).

~~This would suggest that the highest OM stabilization occurring in fine macroaggragates 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 quantification of organic matter forms (OMFs). The quantification of OM was also performed on ground samples by the well-standardized dry combustion method, which provides the quantification of the whole organic C in disturbed samples without any distinction among different forms. The quantification of OM content in the aggregates obtained by the two methods (i.e., the content of OC in ground aggregates and the presence of OMFs detected on aggregate thin section) showed similar trend and the data was significantly related, further supporting the use of optical micro-morphological parameter as an indicator of the presence of OM in the aggregates.

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

Yang et al. (2019) suggest that total porosity has a significant role, 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 conversely the greatest percentage of pores <50 μm in fine macroaggregates, according to cited authors, could have contributed to organic matter storage within them. Therefore, in our study the physical occlusion would be more efficient in fine macroaggregates than in coarse ones.

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

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

Our data showed that OM accumulation in fine macroaggregates was due to the organic amorphous forms, being organs missing in fine macroaggregates. Additionally, SEM-EDS microanalysis showed that that red and black amorphous forms had the highest Al:C, Fe:C and Ca:C molar ratios. These molar ratios are chemical 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 $\delta^{13}C$ 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 $\delta^{13}C$ 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 $\delta^{13}C$ (more negative) correspond to less oxidized organic matter (Angers et al., 1997). In our study, the C/N ratio and the $\delta^{13}C$ 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 macroggregates. 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 δ^{13}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 δ^{13}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. B~~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~~. O~~ur 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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Figure captions

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

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

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

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

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

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

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 macroggregates
- 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

Title

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

Authors

Guidi Patrizia (1), Falsone Gloria (1), Wilson Clare (2), Cavani Luciano (1), Ciavatta Claudio (1), Marzadori Claudio (1)

Author Affiliations

(1) Dipartimento di Scienze e Tecnologie Agro-alimentari, Alma Mater Studiorum Università di Bologna, 40127 Bologna (I)

(2) Biological and Environmental Science, University of Stirling, FK9 4LA Stirling (UK)

Corresponding author

Gloria Falsone, email gloria.falsone@unibo.it

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 μm) 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 macroggregates 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 δ^{13}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.

KEYWORDS

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

1. INTRODUCTION

The largest amount of organic C in terrestrial ecosystems is in the soil and it is three time the amount of C in the atmosphere and four time 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 gasses 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.

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

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

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

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

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

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: coarse macroaggregates (4-1 mm) and fine macroaggregates (1-0.25 mm).

2.2. Soil aggregate thin sections preparation

The aggregates in both 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.

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, the area of interest in each thin section in this studycorresponded to the intraggregate area. In coarse macroaggregate thin sections, from 9 to 16 single aggregates were analysed for each site, while in fine macroaggregate thin sections from 23 to 41 aggregates were investigated. Measurements on aggregates close to the edge of the thin sections or having inside/near artificial bubbles were avoided. Optical observations have been carried out using a polorised microscope Olimpus BX50.

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

2.3.1. *Total porosity and pore size distribution*

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

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

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

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

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

The images obtained by organic components analysis was exported and stacked upon the binary pore image thereby forming a map showing the distribution of organic matter in relation to soil pores. It was thus possible to identify the surface of organic matter in contact to the pores and to measure the length of contact perimeter between the two features. Then, the total length of the contact perimeter (in mm) was normalized by the total area of organic form (in mm^2), in order to obtain a measure of the proportion of the organic matter surface in contact to the pore. For each sample, an index, called exposure index (EI; mm^{-1}) was calculated. The EI gives information about the organic matter-pores contact, and therefore on the potential physical exposure of organic matter to the microbial activity (Young et al., 2008).

2.4. SEM-EDS analysis on the aggregate thin sections

Polished thin sections were analysed using an environmental scanning electron microscope (SEM) and elemental data were collected by energy-dispersive spectroscopy (EDS) detector using ZEISS SEM systems (EVO MA15) linked to an Oxford Instruments INCA X-max detector with an 80-mm^2 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).

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, $\delta^{13}C$ signature and enzyme activities related to carbon cycle have been measured.

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

On each aggregate fraction separated by sieving, the total organic C (g C $kg^{-1}_{aggregate}$) and total N (g N $kg^{-1}_{aggregate}$) 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- Finnigam, DE). The values were then expressed as $\delta^{13}C$, 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:

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

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 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 macroaggregaes (size factor) were checked by the one-way ANOVA.

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.

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

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

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

3. RESULTS

3.1. Porosity

The total detectable porosity, measured in the intraggregates space, ranged from 5.93 to 9.08% and from 3.70 to 6.71% in coarse and fine macroaggregates, respectively (Table 2). Porosity varied significantly based on size factor ($p<0.001$), and the fine macroaggregates were less porous than the coarse ones (Table 2). Within each aggregate class, among sites no differences in the intraggregate porosity were found.

Figure 1 (a-b) shows the pore size distribution (PSD) in the different aggregates. The PSD was significantly influenced by the size factor ($p <0.01$; Figure 1-c). The pores >200 μm were only present in coarse macroaggregates, while in fine macroaggregates pore <50 μm predominated. Fine macroaggregates, therefore, were significantly less porous and showing finer porosity compared to coarse macroaggregates. Between sites, no differences in the PSD were found (Figure 1-c).

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

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

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

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 ($p<0.001$).

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.

3.4. Exposure Index (EI)

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 $\delta^{13}C$ values. Specifically, EI values was positively correlated to the C/N ratio and negatively to the $\delta^{13}C$ values. The EI was significantly positively correlated also to GM*ea* (Figure 5).

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

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

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

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

Our data showed that OM accumulation in fine macroaggregates was due to the organic amorphous forms, being organs missing in fine macroaggregates. Additionally, SEM-EDS microanalysis showed that that red and black amorphous forms had the highest Al:C, Fe:C and Ca:C molar ratios. These molar ratios are chemical 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 $\delta^{13}C$ 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 $\delta^{13}C$ 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 $\delta^{13}C$ (more negative) correspond to less oxidized organic matter (Angers et al., 1997). In our study, the C/N ratio and the $\delta^{13}C$ 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 macroggregates. 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 δ^{13}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 $\delta^{13}C$ 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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Figure captions

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

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

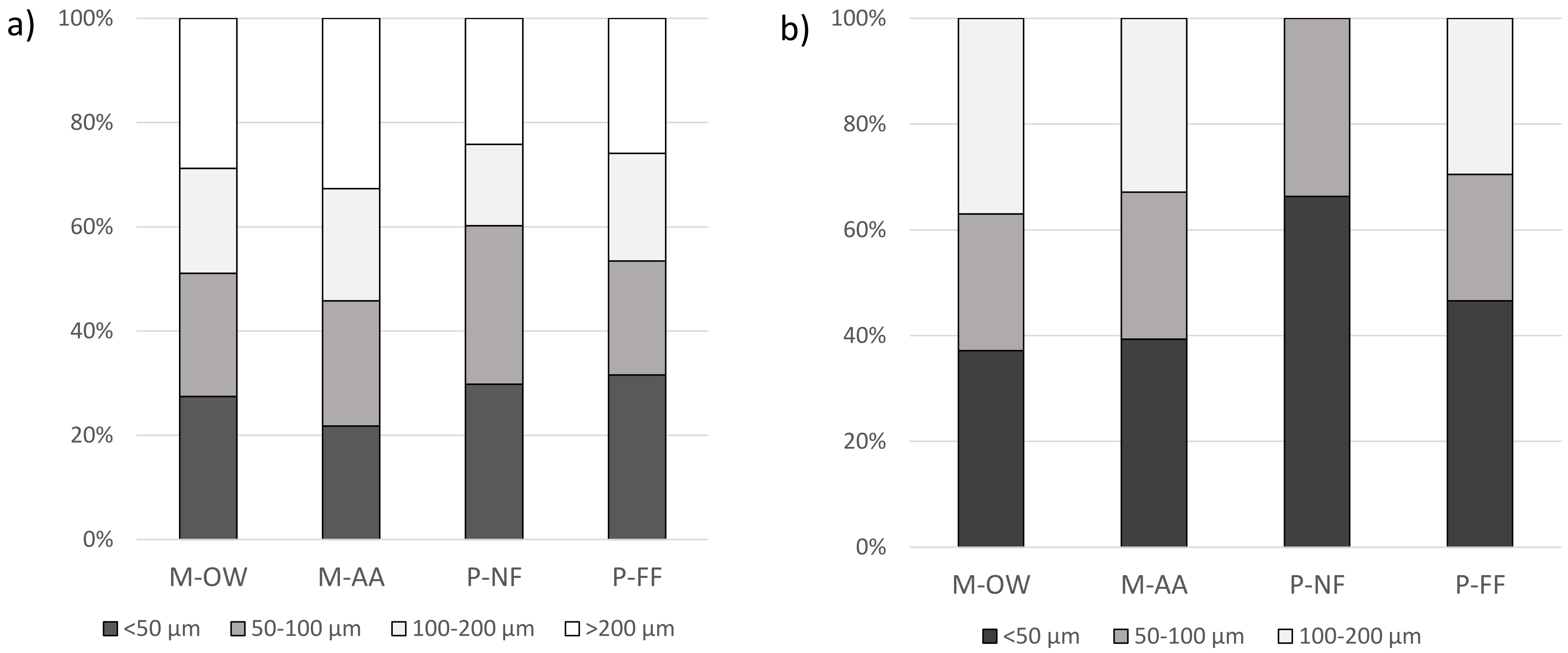
Figure 3. Box-plots of exposure index (EI, in mm^{-1}) values in the coarse and fine macroaggregates. The box represents the interquartile range, the line represents the median value, the symbol represents the mean value, error bars represent the full range of data. Different letters refer to significant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

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

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

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

Figure 1

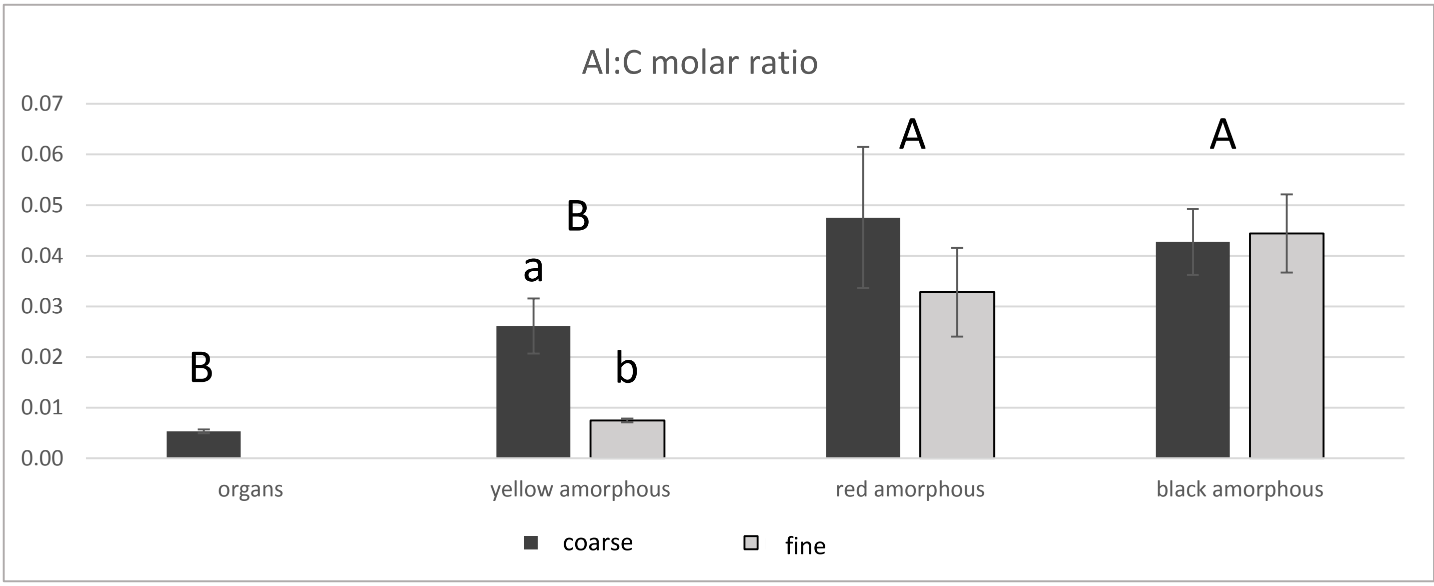


c)

		<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


Al:C molar ratio
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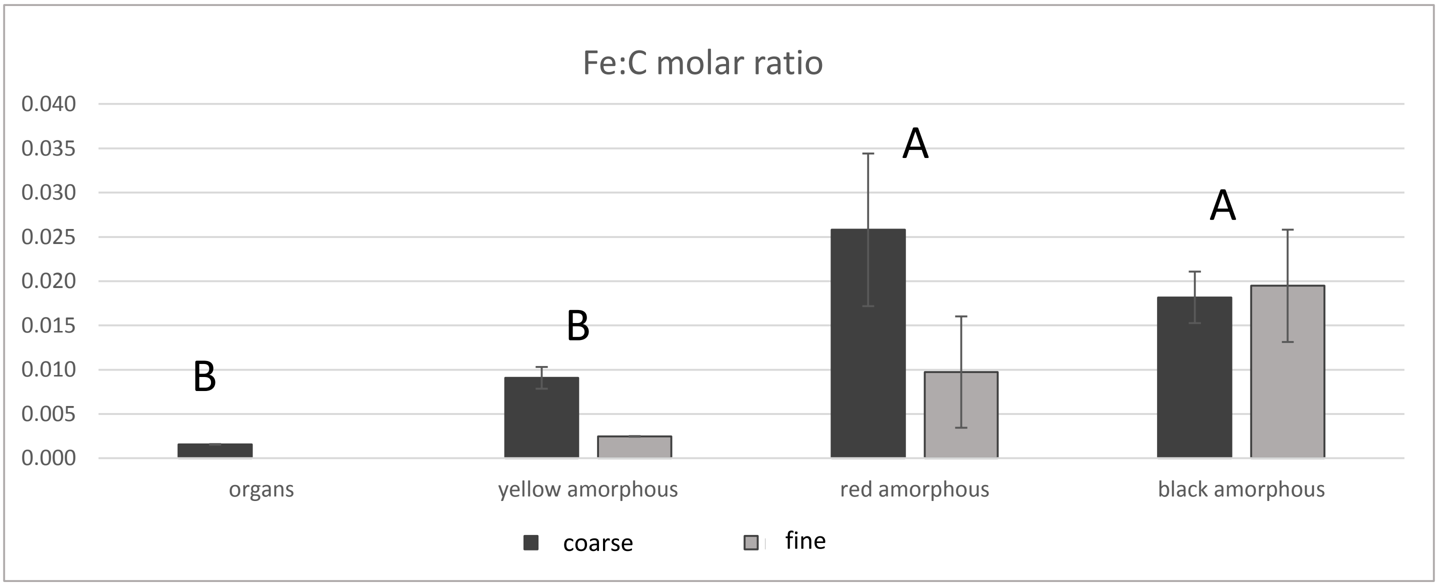
0.06
0.05
0.04
0.03
0.02
0.01
0.00
B
a
B
b
A
A
organs
yellow amorphous
red amorphous
black amorphous
coarse
fine


Fe:C molar ratio
0.040
0.035
0.030
0.025
0.020
0.015
0.010
0.005
0.000
B
B
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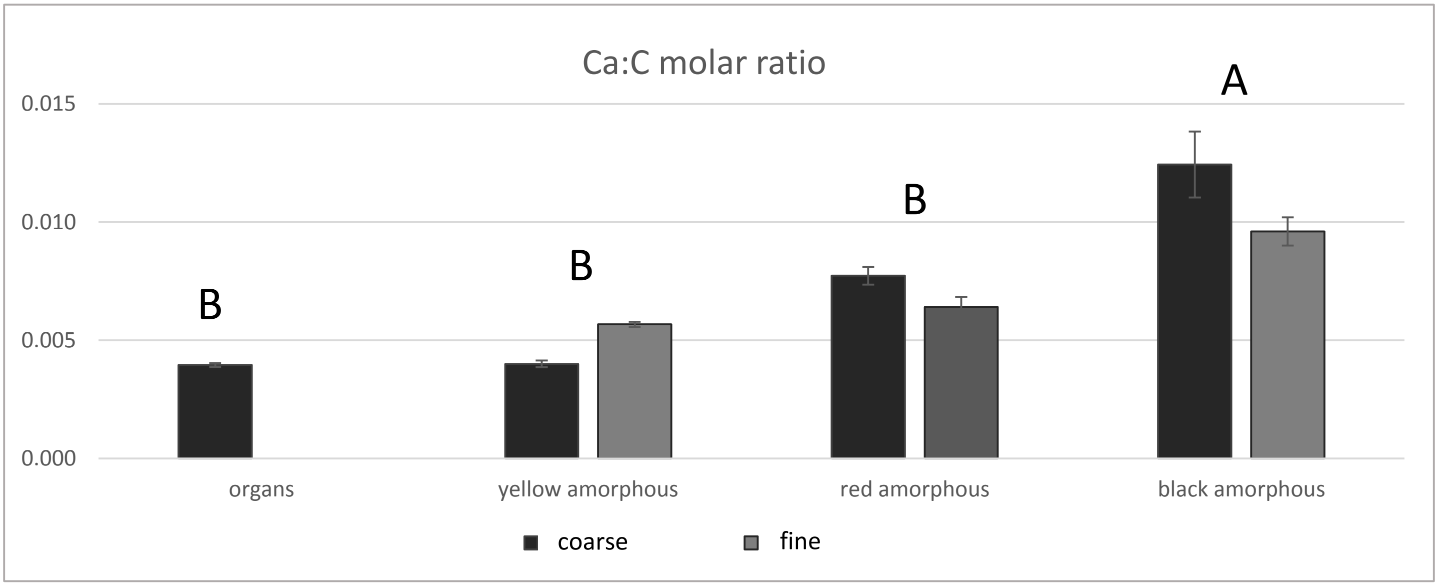
A
organs
yellow amorphous
red amorphous
black amorphous
coarse
fine


Ca:C molar ratio
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0.010
0.005
0.000
B
B
B
A
organs
yellow amorphous
red amorphous
black amorphous
coarse
fine

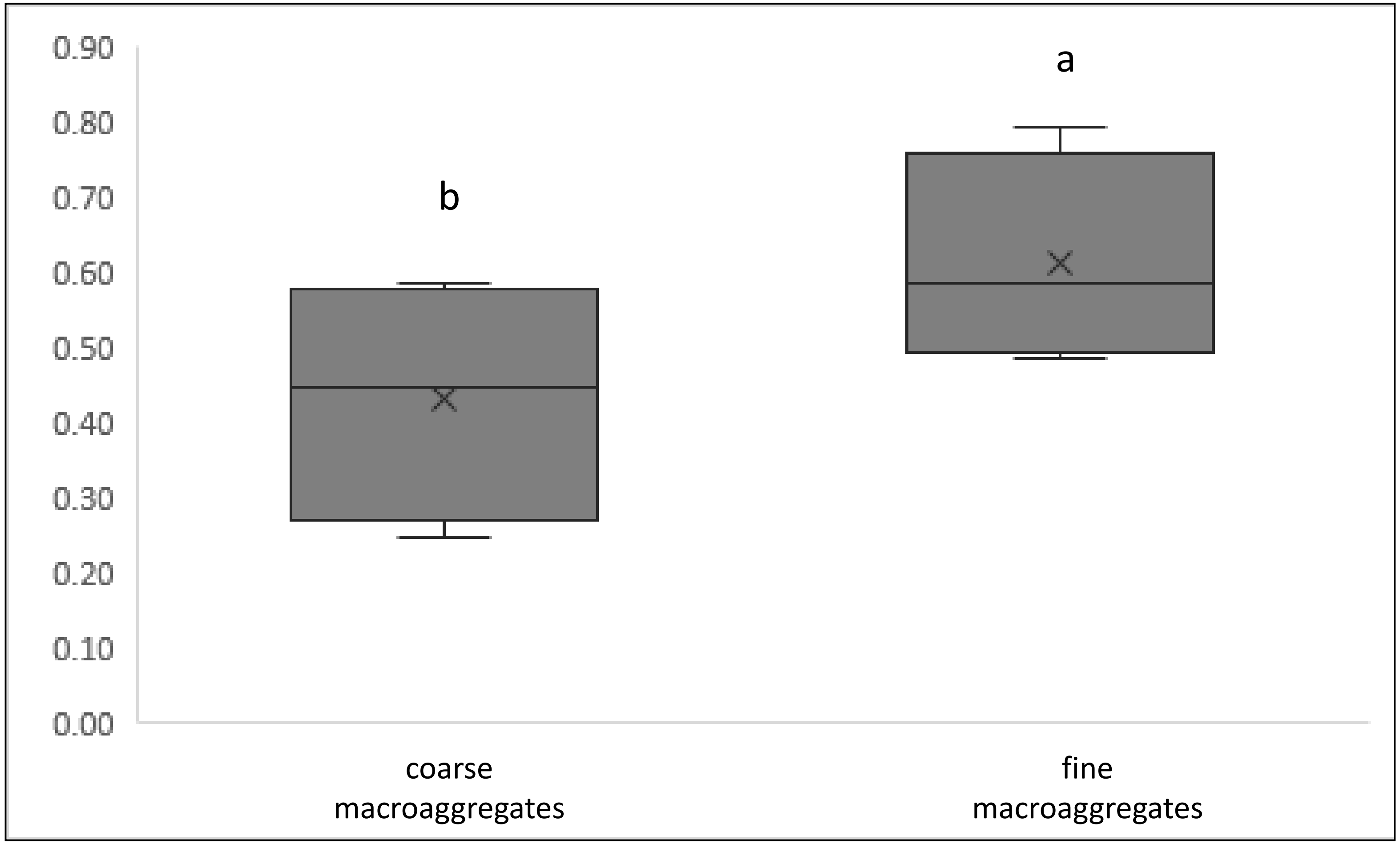

0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
b
a
coarse macroaggregates
fine macroaggregates

Figure 4

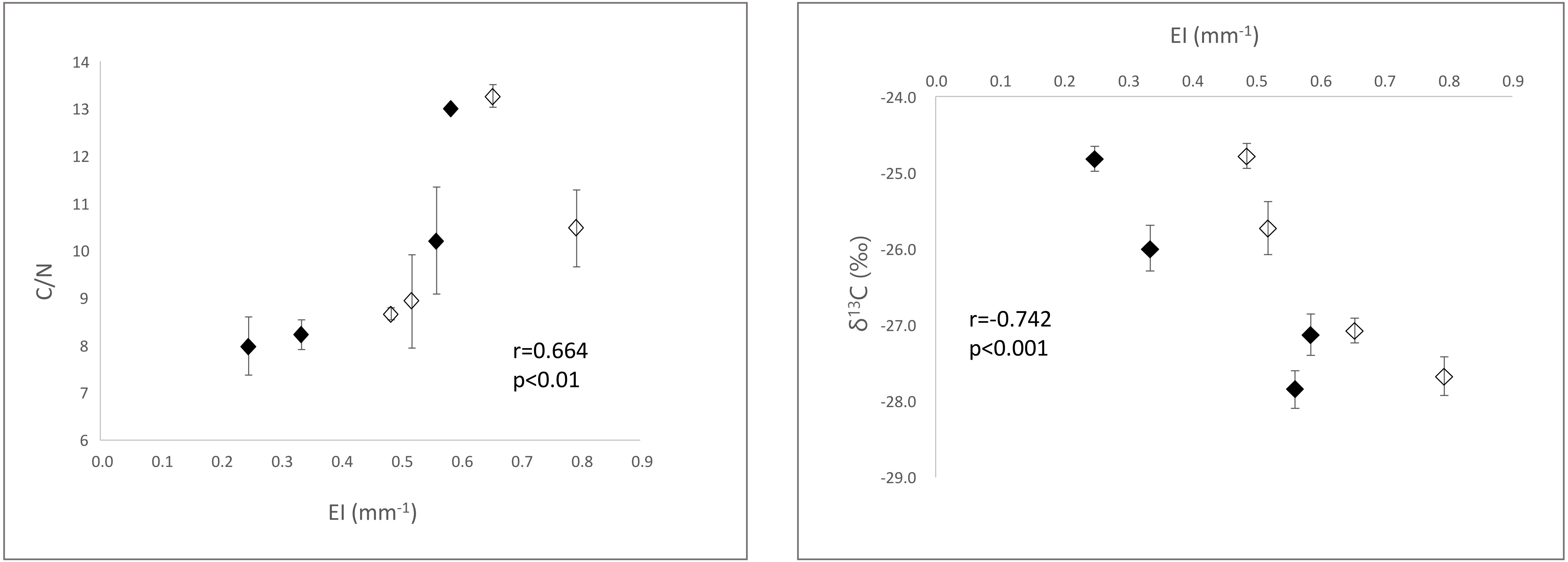

14
13
12
11
10
9
8
7
6
C/N
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
EI (mm-1)
r=0.664
p<0.01
EI (mm-1)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
-24.0
-25.0
-26.0
-27.0
-28.0
-29.0
δ13C (‰)
r=-0.742
p<0.001

Figure 5

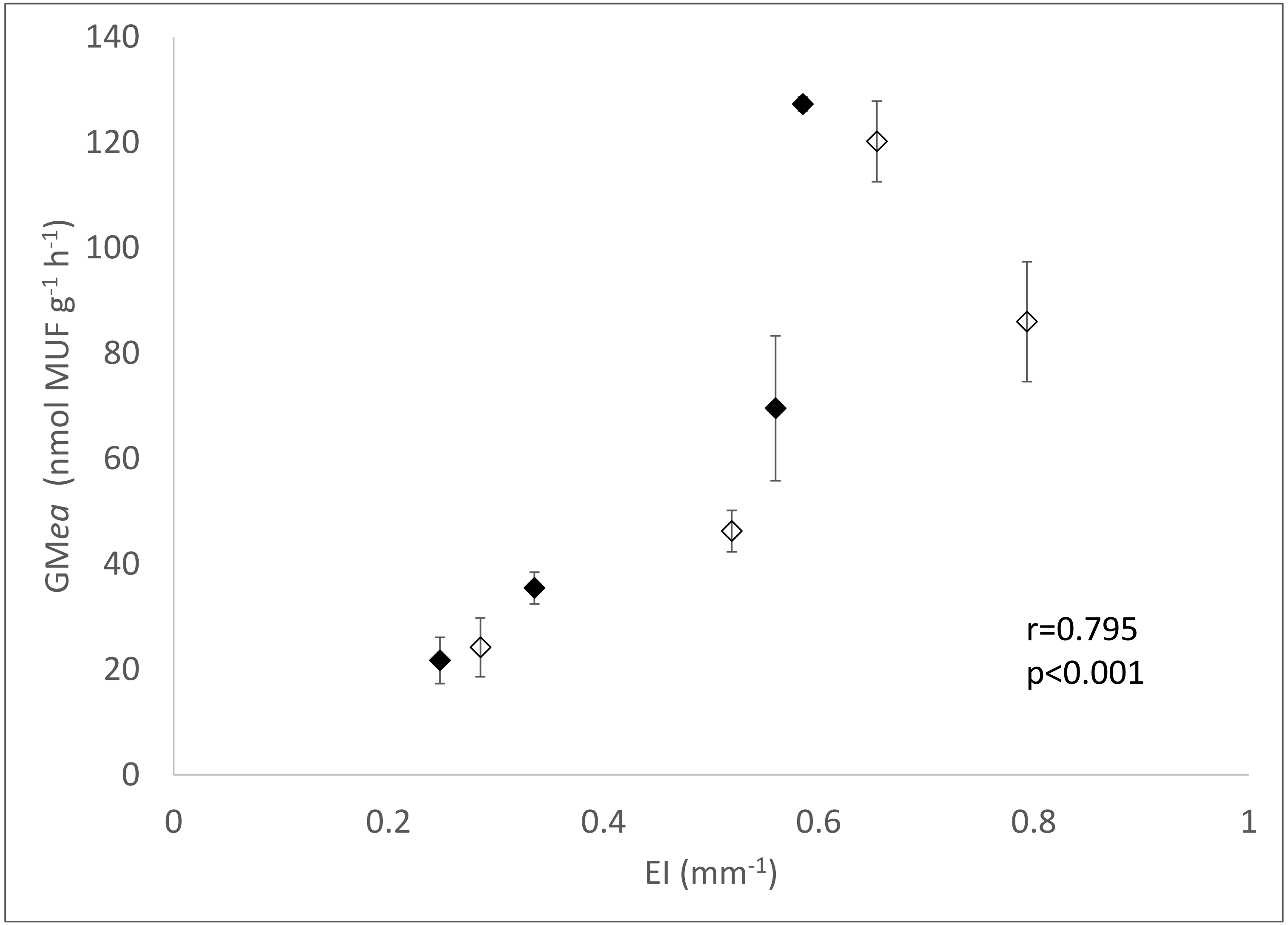
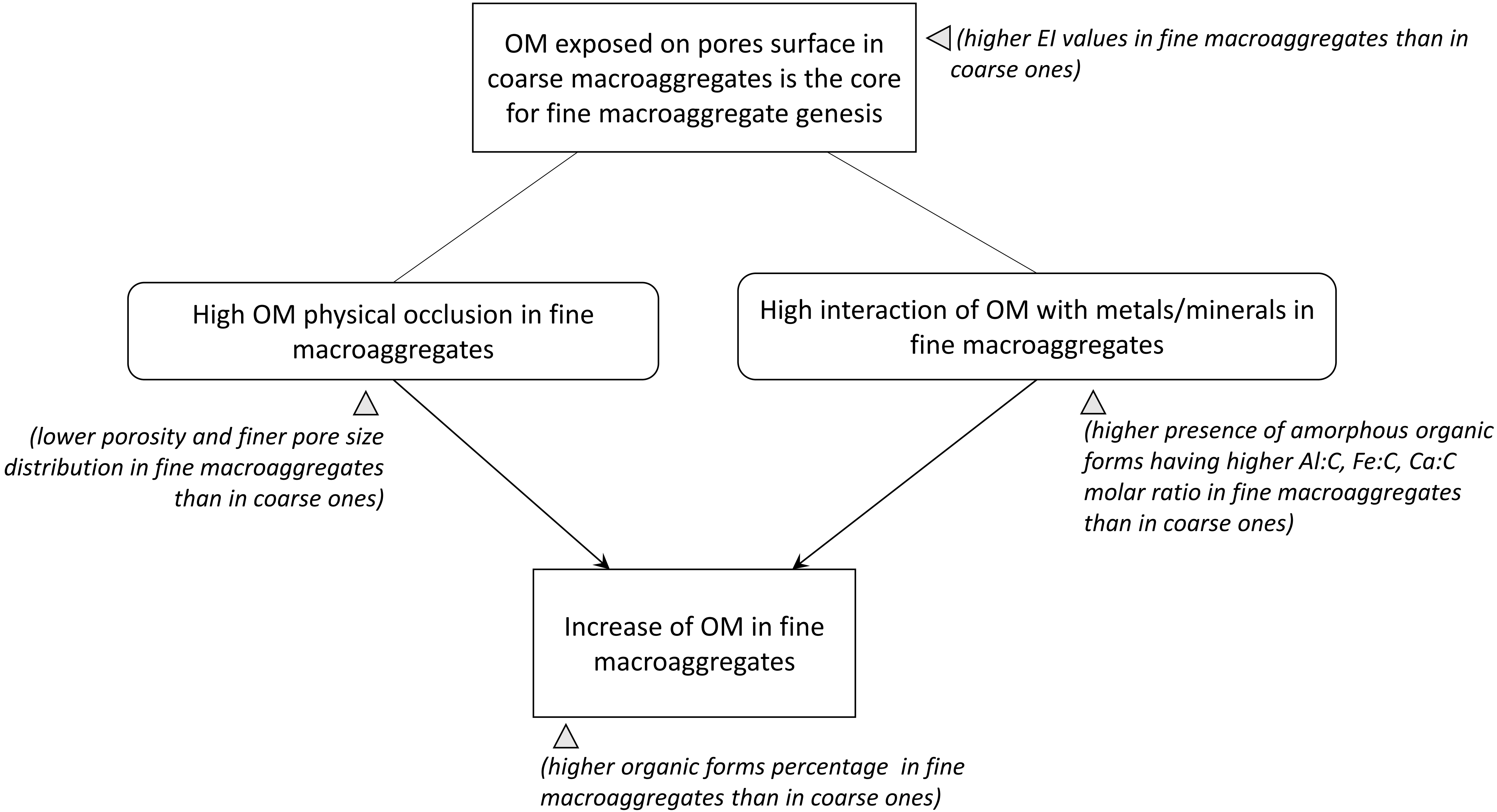

140
120
100
80
60
40
20
0
GMea (nmol MUF g-1 h-1)
0
0.2
0.4
0.6
0.8
1
EI (mm-1)
r=0.795
p<0.001

Figure 6



OM exposed on pores surface in coarse macroaggregates is the core for fine macroaggregate genesis

◁ *(higher EI values in fine macroaggregates than in coarse ones)*

High OM physical occlusion in fine macroaggregates

△ *(lower porosity and finer pore size distribution in fine macroaggregates than in coarse ones)*

High interaction of OM with metals/minerals in fine macroaggregates

△ *(higher presence of amorphous organic forms having higher Al:C, Fe:C, Ca:C molar ratio in fine macroaggregates than in coarse ones)*

Increase of OM in fine macroaggregates

△ *(higher organic forms percentage in fine macroaggregates than in coarse ones)*

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	site	ns	***	ns
within fine macroaggregates	site	ns	**	ns

[a]measured on macroaggregate thin sections; [b]measured on grounded macroaggregates.

M-OW and M-AA: 16-yrs old oak wood and 5-yrs 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 class	sites	Organs (%)	Yellow amorphous forms (%)	Red amorphous forms (%)	Black amorphous 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 macroaggregates	site	-	-	*	*
within fine macroaggregates	site	-	-	***	***

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

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

Conflict of Interest

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:

Supplementary Material - Table 1S