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1 **Distribution and thermal stability of physically and chemically protected organic**
2 **matter fractions in soils across different ecosystems**

3

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18

66 (Kästner 2000), thus suggesting that the molecular imprint of SOM by molecules and
67 fragments derived from microbial biomass is probably much more important than
68 previously considered (Miltner et al. 2012). When microorganisms degrade plant
69 residues, they use low-molecular-weight compounds from the plant biomass to build their
70 own biomass, and the majority of this material is then incorporated into non-living SOM
71 (Kindler et al. 2006; Miltner et al. 2012, and ref. therein). Cotrufo et al. (2013)
72 hypothesized that labile plant constituents are the main source of microbial by-products,
73 and these in turn are the main precursors of SOM stabilized by the soil matrix, through
74 the formation of physically or chemically realized organo-mineral associations (Vogel et
75 al., 2014). For example, McGill and Paul (1976) and Turchenek and Oades (1979)
76 observed that microbial cell wall materials were a major component of SOM accumulated
77 in the coarse clay fraction, while Ladd et al. (1996, and ref. therein) reported that clay-
78 size fractions were enriched of organic nitrogen (N) of microbial origin. Moreover,
79 particle size fractionation after ultrasonic dispersion and subsequent dating showed young
80 radiocarbon ages of the fine clay-associated organic C, indicating that this organic C was
81 more active than that associated with the coarse clay (Anderson and Paul, 1984).

82 Several other studies have suggested that thermally labile, microbial derived
83 materials and easily metabolizable compounds (*e.g.*, carbohydrates and amino acids) are
84 dominant in the mineral-associated organic matter (MAOM) pool, whereas the LF free
85 (LF_free) or occluded within macro- (LF_M) and especially micro-aggregates (LF_m)
86 are believed to be mainly of plant origin and characterized by an increasing degree of
87 persistence (Kleber et al., 2011; Hatton et al., 2012; Keiluweit et al., 2012; Plaza et al.,
88 2013, 2016). Given its chemical nature, the MAOM pool is also the richest in N among
89 SOM pools, *i.e.*, with the lowest C/N (Christensen, 2001), pointing to the need of a better
90 understanding of N distribution in the soil. In fact, despite the importance of N

91 sequestration to ecosystem functioning, to date there has not been a comprehensive
92 examination of the implications of SOM stabilization in ecologically meaningful fractions
93 for long-term N sequestration in different soils and ecosystems (Bingham and Cotrufo,
94 2016).

95 Soil C stock and sink capacity were recently described as “ecosystem properties”
96 (Schmidt et al., 2011), sharply affected by several factors including land use (*e.g.*,
97 undisturbed *versus* agricultural soils subjected to aggressive human imprints) (Amundson
98 et al., 2015). Besides climate, ~~v~~Vegetation and parent material are main drivers of soil
99 formation (Jenny, 1941), and their role in SOM stocks and persistence has been
100 investigated in different ecosystems, spanning from spruce and deciduous forests to
101 grasses and arable soils (Guggenberger et al., 1994, 1995; Leifeld and Kögel-Knabner,
102 2005; Simpson et al., 2007; Clemente et al., 2011; Wieismeyer et al., 2012; Deneff et al.,
103 2013; Catoni et al., 2016). Despite all these research efforts, an accurate assessment of
104 soil organic C and total N distribution across pools with different mechanisms of physical
105 and chemical protection is still needed to improve our knowledge of the response of soil
106 properties, including C stocks, to land management and/or changes in land use and cover
107 (Guo and Gifford, 2002; Wieismeyer et al., 2012; Wei et al., 2017). This would help better
108 understand if SOM persistence, and therefore the distribution among fractions, is an
109 ecosystem property rather than the main common denominator across different soils and
110 ecosystems. In addition, technosols, *i.e.* man-made soils, are widely distributed
111 throughout the world as they are found where human activity has led to the construction
112 of artificial soils, sealing of natural soil, or extraction of material normally not affected
113 by surface processes (*e.g.*, mines, dumps, oil spills, earth movements, coal fly ash
114 deposits) (IUSS Working Group WRB, 2006). Many technosols, in particular those in
115 dumps, are originated covering man-made substrate with a layer of natural soil in order

116 to permit revegetation. Although technosols have been recently included in soil
117 classifications, often associated with other soil groups in a complex pattern, here they are
118 considered as a “modern form” of soil in anthropogenic ecosystems. Only few attempts
119 to understand SOM stabilization dynamics in technosols are reported in literature
120 (Chaudhuri et al.; 2013; Rodríguez-Vila et al.; 2016; Moreno-Barriga et al.; 2017),
121 pointing to the need of including them when contrasting different ecosystems, for a
122 generalizable understanding of the characteristic of SOM pools protected by different
123 mechanisms of stabilization.

124 In this study, we measured: (1) the organic C and total N contents of bulk soils under
125 five different ecosystems, including coniferous and broadleaved forests, grasslands,
126 technosols in anthropogenic systems and agroecosystems; and (2) their distribution in
127 ecologically meaningful SOM fractions, namely: a light fraction ($LF < 1.85 \text{ g cm}^{-3}$), found
128 free or occluded in macro- (2000-250 μm), and micro-aggregates ($>250 \mu\text{m}$), thus
129 stabilized by either inherent recalcitrance and/or an increasing strength of physical
130 protection, and the heavy ($> 1.85 \text{ g cm}^{-3}$) MAOM fraction, mostly stabilized by chemical
131 association to minerals. The main objectives of this work were to: (a) elucidate SOM
132 distribution among primary fractions characterized by different mechanisms of
133 protection, in a variety of soils from different ecosystems; and (b) investigate if the
134 thermal fingerprint of SOM fractions (possibly mirroring their “inherent recalcitrance”)
135 is an ecosystem property or can be generalized across different soils and ecosystems.
136 Finally, including technosols in this study would improve our understanding of the kind
137 and rate of stabilization mechanisms taking place during the initial phases of pedogenetic
138 processes (Zaccone et al.; 2014), being technosols young soils that develop in technogenic
139 man-made parent materials.

140

141

142 **Materials and methods**

143 *Study sites and soil sampling*

144 Five ecosystem types, known to have contrasting climatic and edaphic conditions, were
145 considered in this study. To achieve a broad range of soils, we collected samples from 4
146 to 5 different sites for each of the five ecosystem types (~~Table 1~~). Five coniferous forests
147 (CF), five broadleaved forests (BF), five grassland soils (GL) and five technosols (TS)
148 were sampled between May and June 2016 in different sites of the Marche Region (Italy).
149 The coniferous forests consisted mainly of *Pinus nigra* woodlands occurring between 350
150 and 1400 m a.s.l., the broadleaved ~~forests-woodlands~~ in stands of different species (e.g.,
151 *Fagus sylvatica*, ~~Orno~~-*Ostrya carpinifolia*etum associations with *Quercus pubescens*)
152 growing between 320 and 1300 m a.s.l., the grassland soils in meadows and pastures
153 characterized by meso- to xerophilous species (e.g., *Bromus erectus*) and *Medicago sativa*
154 and developing between 890 and 1700 m a.s.l.. The technosols included two open-pit
155 mines, filled and then reclaimed with *Helianthus annuus* (since 2010) and with *Triticum*
156 (since 2000), respectively, two dumps, reclaimed in 2000 and in 2011, respectively, and
157 a site of disposal of compost and other non-hazardous waste materials (all TS sites <300
158 m a.s.l.). Mean annual temperature of sampling sites ranged from 7.3 and 15.0 °C,
159 whereas mean annual precipitation ranged between 810 and 1570 mm. Four agricultural
160 soils (AG) cropped with barley and collected in Arganda del Rey (Madrid, Spain) were
161 also included in this study; ~~t~~. These soils were located at 530 m a.s.l. and characterized
162 by a mean annual precipitation of about 440 mm and a mean annual temperature of 14 °C
163 (Plaza et al., 2016). More details about the sampling sites are reported in Table 1.

164 At each location, 3 to 10 soil samples were collected randomly from several points,
165 depending on site surface and homogeneity separated 50 to 100 cm apart, using a soil

166 auger and mixed to obtain a composite sample. The depth of sampling varied from 10 to
167 25 cm, and corresponded to the top soil horizon (Table 1).

168

169 *Bulk soil characterization*

170 Prior to analysis, soil samples were gently crushed and passed through a 2-mm sieve. Soil
171 reaction (pH units) was measured on suspensions of 1:2.5 sample:water and sample:KCl
172 (1M) (Thomas 1996), whereas electrical conductivity (EC) was measured on water
173 extracts obtained at a sample-to-water ratio of 1:5 (RoadesSparks, 1996). All soils were
174 also analyzed for texture by the pipette method, whereas C and N elemental composition
175 was determined as described in section below. Soil properties averaged by ecosystem type
176 are reported in Table 2.

177

178 *Physical fractionation of SOM*

179 The physical fractionation of SOM was carried out using the method described by Plaza
180 et al. ~~(2012) with modifications detailed by Plaza et al. (2013)~~, with the aim to separate
181 the heavy MAOM fraction from the light particulate~~r~~ organic matter (LF) fraction, and
182 distinguish the latter into free LF and LF occluded in aggregates of different sizes.
183 Specifically, we separated four fractions: (a) free LF, located between aggregates
184 (unprotected C pool; LF_free); (b) LF occluded within macroaggregates (SOM pool
185 weakly protected by physical mechanisms; LF_M); (c) LF occluded within
186 microaggregates (SOM pool strongly protected by physical mechanisms; LF_m); and (d)
187 a heavy SOM pool associated with the minerals (highly persistent mostly chemically-
188 protected; MAOM). This method is based on the densimetric procedure by Golchin et al.
189 (1994) and Sohi et al. (2001) for the fractionation of free and occluded LF, and on the
190 method by Six et al. (2000, 2002) for the breakup of macroaggregates preserving

191 microaggregates. First, 148 g of NaI at a density of 1.85 g mL⁻¹ ~~were~~ was added to 10 g
192 of 2-mm sieved, air-dried soil in a 100-mL polycarbonate centrifuge tube. Free LF was
193 separated from the heavy fraction by density flotation in the NaI solution and subsequent
194 suction, filtration through a glass fiber filter and scrupulous washings with deionized
195 water. Macroaggregates remaining in the heavy fraction were broken up, using the
196 microaggregate isolator device designed by Six et al. (2000, 2002), into stable
197 microaggregates, MAOM and LF_M, which was collected after a second density
198 separation. Another density separation after an ultrasonic disruption at an energy input of
199 1500 J g⁻¹ allowed the LF_m isolation from the MAOM. The recovered LF_free, LF_M,
200 LF_m and MAOM fractions were oven-dried at 60°C, weighed and ground with a ball
201 mill for further analyses.

202

203 *Organic C and total N analysis*

204 Organic C and total N contents of both bulk soil samples and SOM fractions were
205 determined by dry combustion using a Thermo Flash 2000 NC Soil Analyzer. For organic
206 C determination, the whole soil samples and mineral-associated SOM fractions were
207 subject to acid fumigation before analysis to remove carbonates (Harris et al., 2001). All
208 analyses were carried out in triplicate.

209

210 *Thermogravimetric analysis*

211 Thermogravimetric analysis (TGA) was carried out on all SOM fractions using a Perkin
212 Elmer Thermogravimetric Analyzer TGA 4000. An aliquot (*ca.* 20 mg) of each sample
213 was placed in a ceramic crucible and heated from 30 to 850 °C at 20 °C min⁻¹ under an
214 oxidizing atmosphere of air at a flow rate of 30 mL min⁻¹. Two indexes of overall thermal
215 stability were calculated: T₅₀ (°C), representing the temperature at which 50% of the SOM

291 SOM in CF compared to the other ecosystems (Castellano et al., 2015). On the opposite,
292 AG soils generally show the lowest C/N ratios for all fractions, with the exception of
293 LF_free (Table 4).

294 For all ecosystems, the C/N ratio of the MAOM pool is significantly lower than those
295 of the other fractions (Table 4). A number of studies have reported a decrease in the C/N
296 ratio from coarse to fine fractions suggesting both an increase of the decomposition
297 degree of the SOM and a relative accumulation of microbial-derived materials (Kiem et
298 al., 2002; Chen et al., 2014). Thus, the low C/N ratio of MAOM fractions is probably
299 indicative of a depletion in lignin and plant-derived components and of an enrichment in
300 materials of prevailing microbial origin (Paul and Clark, 1989; Golchin et al., 1994;
301 Guggenberger et al., 1995; Kögel-Knabner et al., 2008; Cotrufo et al., 2013; Courtier-
302 Murias et al., 2013; Plaza et al., 2013, 2016). In a parallel work, Zaccone et al. (submitted)
303 reported the MAOM fraction to be characterized by high total DNA contents and very low
304 C/N ratios at the same time. According to the Microbial Efficiency Mineral Stabilization
305 (MEMS) framework, high quality N litter may lead to greater accumulation of microbial
306 products and concomitant formation of MAOM in soils with a high soil matrix capacity
307 (Cotrufo et al., 2013). Moreno-Barriga et al. (2017), studying the evolution of C and N
308 pools following the addition of different materials to mine tailings, concluded that easily-
309 available organic compounds triggered microbial growth during the first days of
310 incubation and enhanced the formation of aggregates. This phenomenon probably
311 occurred in TS, mirroring the early stage of pedogenic evolution, and suggested that
312 aggregate stability may increase following a decrease of labile C forms, being then either
313 used as fuel by microorganisms or stabilized by association with mineral particles. The
314 accumulation of organic C and total N in the MAOM fraction confirms that these pools
315 are less susceptible to further mineralization and offer a longer-term storage (Courtier-

316 Murias et al., 2013).

317 The low C/N of MAOM recorded for all the land uses is also consistent with the
318 adsorption of nitrogenous compounds (*e.g.*, amino acids, amino sugars, proteins, cell wall
319 constituents), possibly of microbial origin (Stevenson 1994; Ladd et al. 1996; Knicker;
320 2004; Sollins et al., 2006; Nannipieri and Paul, 2009), ~~possibly of microbial origin~~, onto
321 mineral surfaces.

322 In fact, clay strongly affects stabilization of organic N directly (Ladd et al. 1996;
323 Nielsen et al. 2006; Sollins et al. 2006) and through the formation of aggregate-protected
324 particulate and non-particulate organic matter (*e.g.*, Yoo and Wander 2008), although
325 most of these studies have been carried out in vitro using purified clay particles and
326 purified proteins (Nannipieri and Paul 2009). By means of a 14-month laboratory
327 incubation of a sandy loam soil amended with ¹⁵N-clover, DiCosty et al. (2003) showed
328 that both in whole soil and particle-size fractions, the clover-derived N was always 85–
329 90% amide. Simpson et al. (2007) estimated that 88%, 86% and 82% of organic N in the
330 soil humic fractions from grasses, mixed-aspen dominated forests and pine forests
331 respectively was of microbial origin, in the form of amide in peptides and proteins. On
332 the opposite, according to Rasmussen et al. (2005), the high C/N ratio found in LF_free
333 and occluded LF fractions suggests a relatively high occurrence of slightly-to-partially
334 decomposed plant materials in these fractions.

335

336 *Thermal stability of SOM pools*

337 Being the thermal stability of any material related to its chemistry and surface properties,
338 the thermal stability of SOM may serve as a proxy for biogeochemical stability and
339 provide insight about the energy of interaction between SOM and soil minerals (Plante et
340 al. 2009). At the same time, thermal analysis does not provide information at molecular

341 level, and is affected by several operational parameters, including arrangement and type
342 of sample holder, packing density and grain size of sample, heating rate, initial sample
343 mass, crucible material, and furnace atmosphere (Plante et al. 2009 and ref. therein).

344 Thermogravimetric (TG) curves of all SOM fractions generally show two main steps
345 of weight loss in the range of temperature 100-550 °C. The first, between 250 and -350
346 °C, is associated with more easily oxidizable compounds, including polysaccharides (*e.g.*,
347 cellulosic material) and aliphatic structures (Plante et al., 2009). The second, between
348 350- and 550 °C, due to the thermal degradation of recalcitrant, aromatic structures
349 including lignin and non-hydrolyzable compounds (Schnitzer and Hoffman, 1966; Lopez-
350 Capel et al., 2005) (Fig. 1). The ratio between these two weight losses, here defined as *H*,
351 as well as the T_{50} value, *i.e.* the temperature at which 50% of the SOM mass is lost, are
352 thus generally used to describe the SOM stability and/or recalcitrance (*e.g.*, Rovira and
353 Vallejo, 2000). In fact, within each fraction, the *H* and T_{50} values found are significantly
354 correlated to each other ($R=0.666-0.981$, $p\leq 0.001$, $n=24$), independently of the land use.

355 Differences in *H* and T_{50} among pools in CF, BF and GL soils are not statistically
356 significant, while TS and AG the LF_free and MAOM fractions showed lower values of
357 both thermal indexes compared to LF_M and LF_m (Table 4), being these latter fractions
358 the result of residual plant material depleted in labile compounds. Technosols generally
359 show the highest values of both *H* and T_{50} in all SOM fractions (Table 4). Moreover, TS
360 is the only soil group in which a significant correlation was found between C/N ratios and
361 both *H* ($R=0.463$, $p=0.040$, $n=20$) and T_{50} ($R=0.630$, $p=0.003$, $n=20$).

362 When the weight loss (%) between 150 and -250 °C is considered (Fig. 2), the
363 occurrence of more labile molecules in the MAOM fraction becomes evident, in
364 accordance with the low C/N ratios characterizing this pool. In fact, the percentage of
365 weight loss in the MAOM fraction is always higher than in the other fractions (Fig. 2).

366 The interaction of labile molecules (*e.g.*, sugars, amino acids, proteins) with mineral
367 surfaces has been reported to be a key mechanism for the long-term stabilization of
368 organic C (Lynch and Cotnoir, 1956; Baldock and Skjemstad, 2000; Kiem and Kögel-
369 Knabner, 2003), allowing low molecular weight metabolic compounds to be stored in
370 relatively “passive” pools for up to millennia (Paustian et al., 1992). For example,
371 Schulten and Leinweber (1999), analyzing the Ah horizon of a Gleysol, found pyrolysis-
372 field ionization mass spectra and thermograms of the light SOM fraction (< 2.0 g cm⁻³)
373 to be similar to those of primary plant materials while the corresponding heavy fraction
374 (> 2.0 g cm⁻³) characterized by larger abundances of carbohydrates, lignin decomposition
375 products, N-containing compounds and peptides.

376 Finally, the comparatively small variation in both *H* and *T*₅₀ values characterizing the
377 MAOM fraction (<16% and <3%, respectively) regardless of the ecosystem from each it
378 is derived (Table 4) are indicative of new molecules resulting from a common microbial
379 transformation process rather than highly degraded litter inputs. This finding is in
380 agreement with Kallenbach et al. (2016) who provided evidences about the influence of
381 substrate–microbe interactions on the synthesis of novel SOM constituents that become
382 stabilized by clay minerals.

383

384

385 **Conclusions**

386 We provide evidence that physical fractionation coupled with thermal analysis and C/N
387 ratios allow obtaining new insights about SOM sequestration in mineral soils across
388 different ecosystems. We found that MAOM, followed by the LF_{free} fraction, represents
389 the major pool of both organic C (44-64% of the whole SOC stock) and total N (58-70%
390 of the whole N stock) independently of ecosystem type. The lowest C/N ratios and the

391 highest percentage of weight loss recorded between 150-250 °C, both characterizing the
392 MAOM fraction, indicate that this pool is comparatively richer in labile compounds. At
393 the same time, the very small variability observed for these proxies (*H*, *T*₅₀ and *C/N*),
394 independently of land use and SOM inputs, is indicative of new molecules resulting from
395 a common microbial transformation process. Technosols store two-third of the whole
396 organic C and TN in the MAOM fraction, and show the highest values of both *H* and *T*₅₀
397 in all SOM fractions. Moreover, the significant correlation found between *C/N* ratio and
398 both *H* and *T*₅₀ exclusively in TS underlines how, in this young, man-made soils,
399 mechanisms of SOM sequestration probably mirror processes taking place during the first
400 phases of the pedogenetic processes. As a whole, different ecosystems may differ in terms
401 of absolute SOM content among fractions; however, our results suggest a common
402 mechanism of SOM dynamics, where plant input and their inherent recalcitrance control
403 LF quality and distribution, while microbial by-products, largely independent of
404 ecosystem type, govern SOM accumulation in the MAOM fraction. Further studies are
405 needed to better understand not only the mechanisms of SOM stabilization at the
406 molecular lever by state-of-the-art tools currently available, but also the implications of
407 the molecular mechanisms at larger scales and the ecological functions of the different
408 SOM pools in the environment.

409

410

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416

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648 **Figure caption**

649

650 **Figure 1.** Thermogravimetric curves of free light fraction (LF_free), intra-
651 macroaggregate light fraction (LF_M), intra-microaggregate light fraction (LF_m) and
652 mineral-associated organic matter fraction (MAOM) of a representative sample for each
653 soil group (coniferous forest soils, CF; broadleaved forest soils, BF; grassland soils, GL;
654 technosols, TS; agricultural soils, AG).

655

656 **Figure 2.** Percentage of weight loss (mean \pm st. dev.) occurring in the temperature range
657 150-250 °C for each SOM fraction and within each ecosystem type (coniferous forest
658 soils, CF; broadleaved forest soils, BF; grassland soils, GL; technosols, TS; agricultural
659 soils, AG).

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