**Short-term stabilization of organic matter in physically, chemically,
and biochemically protected pools in soils amended with municipal
wastes**

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Running title: Organic matter stabilization in soils amended with municipal wastes

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Abbreviations: MC, soil amended with municipal solid waste compost; MSW,
municipal solid waste; OM, organic matter; SC, soil amended with sewage sludge
compost; SS, sewage sludge; TS, soil amended with thermally dried sewage sludge;
UN, unameded control soil.

Keywords: Municipal solid waste compost; Physical fractionation; Sewage sludge; Soil
aggregates; Soil organic matter.

1   **Abstract**

2

3   Increased understanding of the stabilization mechanisms of the organic matter (OM)
4   entering the soil system through the application of organic amendments is of paramount
5   agronomic and environmental importance. Here we examined the organic C and total N
6   distribution in OM pools (i.e., free, intra-macroaggregate, intra-microaggregate,
7   mineral-associated, dissolved, and humic acid fractions) characterized by different
8   protection mechanisms in soils annually amended with different organic substrates of
9   municipal origin (i.e., municipal solid waste compost, sewage sludge compost, and
10   thermally dried sewage sludge) for three years. With respect to the unamended control
11   soil, the application of the three organic materials, especially thermally dried sewage
12   sludge, increased soil organic C and total N contents. The organic inputs accumulated
13   mostly in the physically unprotected free OM pool, as relatively fresh, low humified
14   materials, and secondarily in the intra-macroaggregate fraction, as partially decomposed
15   OM weakly protected by physical mechanisms. Our results suggest that mechanisms
16   related to strong physical protection in microaggregates and chemical inaccessibility by
17   intimate association with minerals do not govern OM stabilization in the short term.

18   **1 Introduction**

19

20   Preserving soil organic matter (OM) is crucial to maintain soil functioning in
21   agricultural ecosystems. Soil OM not only plays a central role in soil processes that
22   govern crop production and supports the growth of a variety of animals and
23   microorganisms, but also preserves water quality and influences atmospheric
24   concentrations of greenhouse gases at a global scale [1-3].

25   Soil amendment with treated municipal wastes, such as municipal solid waste
26   (MSW) compost and sewage sludge (SS), is widely backed as a means to improve soil
27   fertility and replenish, preserve, or increase soil OM content [4, 5]. Decomposition of
28   OM inputs into the soil is determined by biological and environmental conditions [2]
29   and a combination of biochemical, physical, and chemical mechanisms of protection
30   within the soil matrix. These mechanisms refer to inherent biochemical recalcitrance
31   and formation of structures more resistant to degradation (e.g., humic substances and
32   black carbon), physical protection in macro and microaggregates, and chemical
33   association to minerals [6-8].

34   A large number of studies in the literature on the stabilization of organic materials
35   in soils have focused on the biochemical protection mechanisms, examining quantitative
36   and qualitative changes in humified OM pools (especially in humic acid fractions) using
37   chemical fractionation methods and characterization techniques [9-11]. These studies
38   indicate that (a) organic amendments generally increase soil humified OM pools, as
39   well as non-humic pools, to an extent that depends on the quantity and quality of the
40   organic input; (b) organic amendments, especially non-composted “fresh” materials,
41   determine significant changes in the molecular composition and functionalities of the
42   isolated humic fractions in the short term; and (c) the composition and functional

43 properties of humic pools in amended soils do not differ significantly from those of
44 native soil humic pools in the long term [9-11].

45 Other studies have tried to capture the role of the formation of primary and
46 secondary organomineral complexes in the stabilization of organic inputs in the soil
47 matrix using physical fractionation procedures [12-15]. In particular, Courtier-Murias et
48 al. [16] recently fractionated soil OM pools from long-term (44-year-old) amended field
49 plots by means of a physical procedure that is believed to be well linked to the current
50 concepts of soil OM preservation mechanisms and advantageous for the molecular
51 characterization of the isolated fractions [17, 18]. Most of the increases in soil OM
52 stocks observed in the amended soils with respect to the unamended control were
53 explained by the OM pool intimately associated with soil minerals, which was found to
54 be predominately of microbial origin, followed by the intra-microaggregate OM
55 fraction, which were dominated by plant structures at relatively advanced stages of
56 decomposition. The findings of this study were strongly consistent with the theory that
57 microbes and microbial residues play a central role in soil OM stabilization and
58 sequestration, inasmuch as it is not the intact or partially degraded plant materials,
59 fragments, and tissues that are preserved in soil in the long term, but the microbial
60 structures and microbial derived by-products resulting from OM transformation and
61 degradation processes [16, 18-23].

62 Long-term studies are needed to assess the sustainability of agricultural systems and
63 C sequestration following soil management change scenarios; nevertheless, stabilization
64 and mineralization processes of OM inputs may significantly affect soil fertility, quality,
65 and functioning in the short term. In this context, the objective of this work was to test
66 whether OM from treated municipal organic wastes of different quality entering the soil
67 are stabilized in a relatively short term by the same aforementioned mechanisms

68 observed in the long term for other organic materials (i.e., chemical association of
69 microbes and microbial by-products with mineral surfaces and physical protection by
70 entrapment within small microaggregates). For this purpose, we examined the organic C
71 and N distribution in OM pools characterized by different protection mechanisms in
72 soils amended with MSW compost, SS compost, and thermally dried SS for three years.
73 We used a physical fractionation method to isolate free, intra-macroaggregate, intra-
74 microaggregate, mineral-associated, and dissolved OM pools, and a classic chemical
75 fractionation approach to isolate humic acids.

76

77

78 **2 Materials and methods**

79

80 **2.1 Field experiment and sampling**

81

82 The field experiment was established in 2008 at Arganda del Rey, Madrid, Spain
83 (40°19' N, 3°29' W, 535 m above sea level). The climate of this site is Mediterranean.
84 The average annual rainfall is 436 mm and average annual temperature of 14 °C. The
85 soil is classified as a Xerofluvent and has a clay loam texture [24]. Before initiation of
86 the experiment, the site had been under intensive crop production.

87 The experimental plots (7.5 m by 3 m) were laid out in a randomized complete
88 block design with four treatments and four replicates. The treatments consisted of a
89 control with no amendment and either MSW compost, SS compost, or thermally dried
90 SS applied at a rate of 30 t ha year⁻¹ for three consecutive years; this rate of organic
91 amendment is traditional in the study area. Hereafter, UN is used for unamended soil,
92 MC for soil amended with MSW compost, SC for soil amended with SS compost, and

93 TS for soil amended with thermally dried SS. The MSW compost and the SS compost
94 were produced using a conventional windrow composting system at waste treatment
95 facilities located in Toledo and Madrid, respectively. The thermally-dried SS was
96 produced by heating SS with a stream of hot air at 380-450 °C at a wastewater treatment
97 plant located in Madrid. The three amendments were hand broadcast in late October and
98 immediately incorporated into the upper 15 cm of soil with a rotary tiller. The cropping
99 system was a three year rotation of winter wheat (*Triticum aestivum* L.), sunflower
100 (*Helianthus annuus* L.), and winter barley (*Hordeum vulgare* L.).

101 Samples of MSW compost, SS compost, and thermally dried SS were taken at the
102 time of application and ground with a ball mill for organic C and total N determination.
103 Soil samples representative of the plow layer (0-15-cm depth) were collected randomly
104 from each plot in late June 2011, just after barley harvest. The soil samples were gently
105 crushed and passed through a 2-mm sieve for physical fractionation. An aliquot of each
106 sieved sample was ground with a ball mill for chemical fractionation (humic acid
107 extraction) and organic C and total N analyses.

108

109 2.2 Physical fractionation

110

111 Physical fractionation of soil OM was conducted according to the procedure of
112 Plaza et al. [17] with modifications as described in detail by Plaza et al. [18]. This
113 procedure is mainly based on the densimetric method of Golchin et al. [25] and Sohi et
114 al. [26] for the fractionation of free and occluded OM and the procedure of Six et al.
115 [27, 28] for the breakup of macroaggregates preserving microaggregates. In brief, free
116 OM was separated by density flotation in a NaI solution and subsequent suction and
117 filtration. Macroaggregates in the remaining soil were broken up by using the

118 microaggregate isolator device developed by Six et al. [27, 28] for liberating intra-
119 macroaggregate OM, which was then recovered by a second density separation. Finally,
120 intra-microaggregate OM was isolated from mineral-associated OM by a third density
121 separation after ultrasonic disruption. Here NaI was used for density separations instead
122 of sodium polytungstate as reported in Plaza et al. [17, 18], because sodium
123 polytungstate may contain some N that inevitably remains in the heavy fraction after the
124 last separation and hampers the examination of N distribution. The recovered free, intra-
125 macroaggregate, intra-microaggregate, and mineral-associated OM fractions were oven-
126 dried at 70 °C, weighed, and ground with a ball mill.

127

128 2.3 Chemical fractionation

129

130 Soil humic acids were extracted on the basis of their acid-base properties by means
131 of a classic chemical fractionation procedure [29]. Briefly, a solution of $\text{Na}_4\text{P}_2\text{O}_7$ and
132 NaOH was added to soil samples at a weight-to-volume ratio of 1 to 3, shaken
133 mechanically, and centrifuged. This procedure was repeated three times on the residues.
134 The alkaline extracts were combined, acidified with HCl, and centrifuged. The humic
135 acid precipitates were recovered with distilled water, dialyzed, and freeze-dried.

136

137 2.4 Organic C and total N determination

138

139 The organic C and total N contents of the organic amendments, whole soil samples,
140 and OM fractions were determined by dry combustion with a Thermo Flash 2000 NC
141 Soil Analyzer. The acid fumigation method described by Harris et al. [30] was used to
142 remove carbonates from the organic amendments, whole soil samples, and mineral-

143 associated OM fractions before the analysis. The results were expressed on a dry soil
144 weight basis. Dissolved organic C and N contents (dissolved OM pool) were estimated
145 by subtracting the free, intra-aggregate, and mineral-associated organic C and N
146 contents from the total organic C and N contents in the whole soil. Soil humic acid C
147 content was determined according to the method of Ciavatta et al. [31], which is based
148 on the same acid-base fractionation procedure described above for humic acid
149 extraction. To calculate the soil humic acid N content, the soil humic acid C content
150 determined by the method of Ciavatta et al. [31] was divided by the humic acid C/N
151 ratio determined by dry combustion on the freeze-dried humic acid samples.

152

153 2.5 Statistical analysis

154

155 Dependent variables (organic C contents, total N contents, and C/N ratios of whole
156 soil samples, and OM fractions) were subjected to one-way ANOVA to test whether the
157 effects of the experimental treatments were significant. The Bonferroni test at the 0.05
158 level was used for mean separation. These statistical calculations were performed with
159 the IBM SPSS 20 software.

160

161

162 3 Results and discussion

163

164 The main chemical properties of the MSW compost, SS compost, and thermally
165 dried SS applied to the soil are shown in Table 1. In agreement with data reported in
166 previous studies [32-36], the thermally dried SS has 15% more organic C and 58% more
167 N than the MSW compost, and 50% more organic C and 48% more N than the SS

168 compost. Compared to the values found for both kinds of SS, the C/N ratio of MSW
169 compost is higher and closer to the C/N ratio of the soil (Table 1).

170 With respect to the control treatment, the application of the three organic materials
171 significantly increases soil organic C and total N contents and tends to decrease soil
172 C/N ratio after 3 years (Table 2). Numerous studies in the literature report accrued
173 organic C and N contents in organically amended soils compared with unamended soils,
174 the magnitude of the effect depending on a variety of factors, such as the amendment
175 rate, soil type, and climate [13, 37-40]. Here the highest increases in organic C and total
176 N contents are observed in TS, followed by MC and SC, while the lowest C/N ratio was
177 observed in MC. The percent of organic C added with the amendment that is stored in
178 the soil decreases in the order $SC > TS > MC$, whereas the percent of added total N
179 stored in the soil decreases in the order $MC > SC > TS$. Previous results obtained by
180 laboratory incubation with measurement of respired C, chemical (elemental analysis)
181 and spectroscopic (infrared and nuclear magnetic resonance) methods, and thermal
182 analysis techniques (thermogravimetry and differential scanning calorimetry) indicated
183 that the OM in thermally dried SS is less stable than that in MSW and SS compost [34].
184 There are data in the literature showing that the amount of organic C stored in amended
185 soils is not dependent on the type of residue applied [37, 38]. However, other works
186 suggest that undecomposed organic materials (e.g., fresh plant residues) are less
187 efficient at building up soil OM than relatively more stable amendments (e.g., manures
188 and composts) [16, 41].

189 The free, intra-macroaggregate, intra-microaggregate, mineral-associated, and
190 dissolved organic C and total N contents in the unamended and amended soils are
191 shown in Figures 1 and 2, respectively. As found in other studies [16, 18, 25, 42], the
192 mineral-associated OM is the most abundant organic C pool, constituting 65% of the

193 total soil organic C in UN, 53% in SC, 52% in MC, and 44% in TS; similarly, the
194 mineral-associated OM fraction accounts for the greatest proportion of the total N in the
195 soils examined, with contributions of 68% in UN, 60% in SC, 54% in MC, and 48% in
196 TS.

197 Treatment effects were significant on free organic C and total N ($P < 0.001$), intra-
198 macroaggregate organic C and N ($P < 0.05$), and dissolved N ($P < 0.05$), and not
199 significant ($P > 0.05$) on dissolved organic C and intra-microaggregate and mineral-
200 associated organic C and total N. In particular, with respect to the unamended soil, the
201 application of thermally dried SS, MSW compost, and SS compost significantly
202 increases free and intra-macroaggregate organic C by 223 and 196%, 192 and 159%,
203 and 126 and 39%, respectively, and free total N by 419, 308, and 205%, respectively. In
204 the three amended soils, the free OM pool accounts for most of the increase in total
205 organic C content measured with respect to the unamended soil. In particular, this
206 fraction explains 69, 55, and 61% of the total organic C increase in MC, SC, and TS
207 soils, respectively. These results clearly differ from those of Courier-Murias et al. [16],
208 who reported that the mineral-associated OM fraction, followed by the intra-
209 microaggregate OM pool, explains most of the increase in total organic C content in
210 soils amended with manure and crop residues for 44 years.

211 The C/N ratio of the solid OM pools in the unamended soil decreases in the order
212 free OM > intra-macroaggregate OM > intra-microaggregate OM > mineral-associated
213 OM (Figure 3), passing from a value of 18, typical of plant residues, to a value of 9,
214 indicative of greater degree of OM decomposition and associated with an enrichment in
215 microbial derived materials [1, 19]. The C/N ratio of the free OM pool in the amended
216 soils, especially with thermally dried SS, is significantly smaller than that of the
217 unamended soil and relatively closer to the C/N ratios of the organic amendments

218 (Figure 3). In contrast, no significant differences among treatments are found in the
219 mineral-associated and dissolved OM pools, and only the application of MSW compost
220 slightly decrease the C/N ratio of the intra-aggregate fractions (Figure 3).

221 Humic acid C and N contents increase with the application of the organic
222 amendments, especially with the composted materials (Figures 1 and 2). The C/N ratios
223 of the humic acid fractions in the amended soils, especially in the soil treated with
224 thermally dried SS, are significantly smaller than that of the unamended soil (Figure 3).
225 The C/N ratios of humic acid-like fractions present in organic amendments are typically
226 smaller than those of native soil humic acids, indicative of lower humification degree
227 [9-11]. Thus, consistently with previous studies on similar systems [9-11; 42], our
228 results suggest a partial incorporation of simple and low humified components into the
229 humic acid pools of the amended soils.

230

231

232 **4 Concluding remarks**

233

234 Annual soil amendment for three consecutive years with organic materials derived
235 from different municipal organic wastes increased soil OM content. Regardless of their
236 chemical composition, the organic inputs accumulated mostly in the unprotected free
237 OM pool, as relatively fresh, low humified materials, and secondarily in the intra-
238 macroaggregate fraction, as partially decomposed OM weakly protected by physical
239 mechanisms. Most likely, stabilization mechanisms related to strong physical protection
240 in microaggregates and chemical inaccessibility by intimate association with minerals
241 would become more and more important in longer periods of time. This warrants further
242 investigation. Extensive research is also needed to unravel the links between short- and

243 long-term stabilization of municipal organic wastes in the soil matrix and their
244 composition, the treatments applied to stabilize them prior to application, and their
245 effects on soil fertility, quality, and functioning.

246   **Acknowledgements**

247

248   This work was supported by the Spanish Ministry of Science and Innovation
249   (research grant AGL2009-09124). José Manuel Fernández was the recipient of a
250   fellowship from the CSIC JAE-DOC (Junta de Ampliación de Estudios) program
251   cofinanced by the European Social Fund.

252

253   *The authors have declared no conflict of interest.*

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FIGURE CAPTIONS

Figure 1. Free, intra-macroaggregate, intra-microaggregate, mineral-associated, dissolved, and humic acid C content in unamended soil (UN) and soil amended with either municipal solid waste compost (MC), sewage sludge compost (SC), or thermally dried sewage sludge (TS). Free, intra-macroaggregate, intra-microaggregate, mineral-associated, and dissolved organic matter (OM) pools were isolated by physical fractionation, whereas humic acids were isolated by chemical fractionation. Error bars indicate the standard error of the mean. Within the same OM pool, different letters indicate statistically significant differences according to the Bonferroni test at the 0.05 level.

Figure 2. Free, intra-macroaggregate, intra-microaggregate, mineral-associated, dissolved, and humic acid N content in unamended soil (UN) and soil amended with either municipal solid waste compost (MC), sewage sludge compost (SC), or thermally dried sewage sludge (TS). Free, intra-macroaggregate, intra-microaggregate, mineral-associated, and dissolved organic matter (OM) pools were isolated by physical fractionation, whereas humic acids were isolated by chemical fractionation. Error bars indicate the standard error of the mean. Within the same OM pool, different letters indicate statistically significant differences according to the Bonferroni test at the 0.05 level.

Figure 3. C/N ratio of free organic matter (OM), intra-macroaggregate OM, intra-microaggregate OM, mineral-associated OM, dissolved OM, and humic acid fraction in unamended soil (UN) and soil amended with either municipal solid waste compost (MC), sewage sludge compost (SC), or thermally dried sewage sludge (TS). Free, intra-macroaggregate, intra-microaggregate, mineral-associated, and dissolved OM pools were isolated by physical fractionation, whereas humic acids were isolated by chemical

fractionation. Error bars indicate the standard error of the mean. Within the same OM pool, different letters indicate statistically significant differences according to the Bonferroni test at the 0.05 level.

Table 1. Mean chemical properties ($\pm$ standard error) of the municipal solid waste (MSW) compost, sewage sludge (SS) compost, and thermally dried SS added annually for three years ($n = 3$) and the soil before starting the field experiment ($n = 4$, corresponding to composite samples collected from the 4 experimental blocks).

Property	MSW compost	SS compost	Thermally dried SS	Soil
pH	7.0 ± 0.4	7.1 ± 0.2	6.9 ± 0.3	8.5 ± 0.0
Electrical conductivity (dS m ⁻¹)	7.9 ± 2.8	7.6 ± 0.1	3.8 ± 0.9	0.14 ± 0.01
Organic C (g kg ⁻¹)	238 ± 55	140 ± 5	279 ± 6	10.3 ± 0.2
Total N (g kg ⁻¹)	18.4 ± 2.2	22.7 ± 1.1	43.9 ± 1.1	0.99 ± 0.03
C/N ratio	12.6 ± 1.7	6.2 ± 0.1	6.4 ± 0.1	10.5 ± 0.2

Table 2. Organic C contents, total N contents, and C/N ratios of unamended soil (UN) and soil annually amended with either municipal solid waste compost (MC), sewage sludge compost (SC), or thermally dried sewage sludge (TS) for three years, and percent of organic C and total N added with the amendment stored in soil at the end of the experiment.

	Organic C (g kg ⁻¹)	Organic C stored (%)	Total N (g kg ⁻¹)	Total N stored (%)	C/N ratio
UN	10.7 ± 0.5 b	-	1.14 ± 0.03 b	-	9.31 ± 0.21 a
MC	14.0 ± 0.7 a	34.2 ± 3.4 a	1.62 ± 0.08 ab	63.0 ± 7.1 a	8.65 ± 0.11 b
SC	13.4 ± 0.3 a	48.0 ± 13.1 a	1.52 ± 0.02 b	39.8 ± 5.2 b	8.86 ± 0.13 ab
TS	15.1 ± 0.6 a	38.7 ± 6.1 a	1.71 ± 0.07 a	31.6 ± 3.6 b	8.81 ± 0.03 ab

Within columns, means followed by the same letter are not significantly different according to the Bonferroni test at the 0.05 level.

Organic C and total N distribution in organic matter pools characterized by different protection mechanisms in soils amended with different organic materials of municipal origin was investigated. After three years of annual amendment, organic inputs accumulated mostly in the physically unprotected organic matter pool, as relatively fresh, low humified materials, and secondarily in the intra-macroaggregate fraction, as partially decomposed organic matter weakly protected by physical mechanisms. These results suggested that mechanisms related to strong physical protection in microaggregates and chemical inaccessibility by intimate association with minerals do not govern organic matter stabilization in the short term.