Structural changes in humic substances after long-term fertilization of a calcareous soil with pig slurries

Diana E. Jiménez-de-Santiago | Gonzalo Almendros | Àngela D. Bosch-Serra

Abstract

Pig slurries are widely used on calcareous soils in European rainfed systems. Here we assess their impact on the amount of soil organic carbon (SOC) and on the composition of humic-type substances (HTS). Seven doses of slurry (five from fattening pigs and two from sows) ranging from 1.0 to 4.8 Mg ha\(^{-1}\) yr\(^{-1}\) of organic matter were evaluated after a period of 12 years and compared with mineral fertilizer treatment. At the end of the last annual cropping season (September), SOC was quantified, and HTS were isolated by alkaline extraction followed by acid precipitation, and studied by visible spectroscopy (800–400 nm) and Fourier-transformed infrared spectroscopy (4000–400 cm\(^{-1}\)). Following the trend in the slurry organic matter applied rates, SOC increased from 9.5 g C kg\(^{-1}\) (mineral treatment) to 13.8 g C kg\(^{-1}\). This SOC increase was equivalent to c. 25.4% of the slurry organic carbon applied. The incorporation of aliphatic structures, mainly polyalkyl, from slurries into the HTS tends to modify the composition of the soil organic matter (SOM), which is reflected in a decrease in the intensity of FT-IR peaks related to aromatic structures. Despite the trend of significant increase in SOC with fattening slurries, mainly from the organic matter rate of 1.6 Mg ha\(^{-1}\) yr\(^{-1}\) (c. 185 kg N ha\(^{-1}\)), the composition of the HTS showed an important aliphatic enhancement. The FTIR results showed that using exclusively the relative intensities of specific peaks (alkyl, carboxyl, aromatic and amide groups) as variables for the discriminant analysis, it is possible to identify HA between different groups of soils treated with progressive levels of slurry. Although the new aliphatic components could be considered important to improve soil physical quality, after the incorporation of additional SOM, the spectroscopic characteristics of HTS in soils treated with slurries suggested a weak effect in long-term C sequestration, as the newly incorporated OC forms are not qualitatively similar to the presumably stable native SOM. These potential changes in SOC and SOM composition at field level are constrained by the maximum allowed N rates from organic origin in some agricultural systems.
Pig slurry, the main by-product of intensively farmed pigs, is a matter of major concern because of the intensification of livestock farming (European Commission, 2022). A traditional way of disposing of this by-product is to use it as a fertilizer. This practice is widely recognized as an effective strategy to improve crop production (Bosch-Serra et al., 2015; Domingo-Olível et al., 2016) and to increase and/or restore the soil organic matter (SOM) (Senesi et al., 2007; Zhang et al., 2017). Pig slurries show a wide range of variation in composition (Yagüe, Bosch-Serra, & Boixadra, 2012). In general, they are low in dry matter (>90% water) and ammonium-nitrogen is the predominant N-form (Antezana et al., 2016; Sánchez & González, 2005). However, the use of pig slurry in agriculture promotes changes in soil quality and fertility over time (Domingo-Olível et al., 2016; Mateo-Marín et al., 2021; Piccolo et al., 1992; Yanardağ et al., 2020).

The organic matter in pig slurry differs greatly from native soil organic matter (SOM), particularly with respect to its humic-type substances (HTS) (Chen et al., 2002; Plaza et al., 2002; Senesi et al., 1996). The three main components of pig manure are lipids, proteins/peptides and carbohydrates (Mao et al., 2008), the dominant groups being alkyl (62.7%), followed by carbohydrates (12.6%) and carboxyl/amide (11.0%), with low OCH₃ content indicating negligible amounts of lignin (Cao et al., 2011). In general, continuous inputs of organic fertilizers lead to SOM with comparatively low maturity or resistance to biological degradation in comparison with the pre-existing HTS in soil (Dorado et al., 2003), whereas in the course of its progressive transformation in the soil, the added organic matter can be transformed into substances similar to native humic substances (Senesi et al., 1996, 2007). Both native organic matter and organic matter provided as an organic amendment have a strong positive impact on soil-crop productivity, as SOM enhances soil water holding capacity and nutrient availability (Oldfield et al., 2018), and improves soil aggregate strength (Shepherd et al., 2006); this is also true when pig slurries are applied (Bosch-Serra et al., 2017), which is particularly interesting in semiarid rainfed Mediterranean systems.

Humic substances, from a structural point of view, are the most characteristic components of SOM (Tan, 2014). Depending on their solubility in acid or basic media, humic substances are operationally divided into humic acids (HA) or fulvic acids (FA). Both are the end results of the humification process. The microbial and chemical reworking of raw organic matter in the soil stabilizes organic substances against biodegradation (Kögel-Knabner, 2002; Senesi et al., 1996). Moreover, humic substances act as a pool of N in continuous release forms for plant and microorganisms, as HA can contain between 2 to 6% nitrogen (Schnitzer, 1985).

Short- and medium-term research on HTS present in dryland agricultural environments has been published (Brunetti et al., 2007; Madrid et al., 2004; Plaza et al., 2002). However, long-term compositional studies on HTS are still scarce (Ferrari et al., 2011; Francioso et al., 2000; Zhang et al., 2017), even though they are needed to contribute with field data in the framework of ‘A Soil Deal for Europe’, towards healthy soils (European Commission, 2020). In particular, a series of investigations in recent years have focused their interest on characterizing the changes in the molecular composition of humic substances accumulated in the soil after the application of slurry (Benedet et al., 2020; Furtado e Silva, Do Amaral Sobrinho, et al., 2022; Furtado e Silva, García, et al., 2022; Sacomori et al., 2021; Yagüe & Lobo, 2020).

After twelve cropping seasons using pig slurry as fertilizer in a calcareous soil, the aim of this work was to evaluate the impact of such management practices on the total content of SOC and on the molecular composition of the HAs. We think that it is of special interest to evaluate if the repeated applications of slurry over time can lead to a simultaneous increase in the quality and quantity of SOM, or if the accumulated humic substances still retain characteristics pointing to low evolution or maturity. From the point of view of the assessment of the SOM quality, it is of significant interest to establish to what extent the application of the slurry has led to the formation of substances with a composition similar to the soil HAs, that is, characterized by their aromaticity, condensation and content of reactive oxygen-containing functional groups that make them susceptible to forming organomineral complexes that are resistant to biodegradation. Because the humification/biodegradation balance is a characteristic of the different soils depending on the environmental conditions, in this study, special attention is paid to the effect of progressive application doses, which would allow establishing the best agrobiological response provided by the slurry.
2 | MATERIALS AND METHODS

2.1 | Experimental location and design

A long-term field experiment was established in 2002 in Oliola, Lleida province, NE Spain (41°52′29″N, 1°09′13″E, 440 m a.s.l.). The climate is semiarid Mediterranean with an annual precipitation of 450 mm. Evapotranspiration is higher than precipitation for most of the year, especially in summer, where high average temperatures (>20°C) are recorded. Annual reference evapotranspiration (ET_0, FAO Penman–Monteith equation) averages 1100 mm.

The soil was classified as Typic Xerofluvent (Soil Survey Staff, 2014), non-saline and calcareous. The upper layer (0–0.30 m) has a silty loam texture (131 g kg⁻¹ sand, 609 g kg⁻¹ silt, and 260 g kg⁻¹ clay); pH 8.2 (1:2.5; soil: distilled water), oxidizable organic carbon content (Yeomans & Bremner, 1988) of 11.67 g kg⁻¹, bulk density 1650 kg m⁻³ and calcium carbonate content 300 g kg⁻¹. Illite and chlorite are the dominant minerals in the granulometric clay fraction, which includes traces of talc and 1:1 clays such as kaolinite. Cation exchange capacity was 11.1 cmol₉ kg⁻¹. The water holding capacity was 17% (w/w) at field capacity and 7% (w/w) at the permanent wilting point.

A rainfed winter cereal system with barley (Hordeum vulgare L.) rotated with wheat (Triticum aestivum L.) was maintained since the beginning of the field experiment, except for the 2007–2008 and 2013–2014 cropping seasons when the field was maintained under fallow conditions. Fertilizer treatments were distributed in three blocks (spatial replications). At the end of the 2015–2016 cropping season (September 2016), eight treatments were chosen for this study. The treatments studied were designed based on traditional fertilization practices used in the geographical region under study, where pig farms with fattening pigs predominate, followed by sows. It ensures that both the doses of products and the subsequent agronomic treatments will be as realistic as possible from the point of view of obtain generalizable results, and in line with EU and related national regulations (Generalitat de Catalunya, 2019). Furthermore, additional higher doses were included to intensify potential soil changes (SOM evolution). Before each application, the slurry was analysed for its total OM and N contents to calculate the exact application rates under comparable conditions in all treatments. The total annual OM rate applied with pig slurries (Table 1) were calculated based on the composition of slurries from two sources: fattening pigs (F) at 20, 30, 50, 70 and 90 Mg ha⁻¹ yr⁻¹ or from sows (S) at 60 and 90 Mg ha⁻¹ yr⁻¹. A mineral N fertilized plot (M000) received ammonium nitrate (120 kg N ha⁻¹ yr⁻¹), P (40 kg P ha⁻¹ yr⁻¹) and K (56 kg K ha⁻¹ yr⁻¹) as a control treatment. All slurry doses (Table 1) were applied at cereal tillering stage (early February) except for the 30, 50, 70 and 90 Mg ha⁻¹ where an equivalent fraction of 30 Mg ha⁻¹ was applied in October, just before sowing. Slurry rates started from c. 60% of the maximum N allowed rate from organic origin (190 kg N ha⁻¹) in rainfed non-nitrate vulnerable areas (Generalitat de Catalunya, 2019) and increased upon it up to 3 times. Over the years, slurries came from two nearby farms.

### Table 1: Fertilization treatments applied during 12 cropping seasons and annual averages of organic matter, total-N, organic-N and ammonium-N applied (± standard deviation). Accumulated biomass grain yield is included.

<table>
<thead>
<tr>
<th>Initial¹</th>
<th>Slurry rate²</th>
<th>Organic matter³</th>
<th>Total-N</th>
<th>Organic-N</th>
<th>Ammonium-N</th>
<th>Grain yield⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Mg ha⁻¹ yr⁻¹)</td>
<td>(kg ha⁻¹ yr⁻¹)</td>
<td></td>
<td></td>
<td></td>
<td>(Mg ha⁻¹)</td>
</tr>
<tr>
<td>M000</td>
<td>0–0</td>
<td>0 (±0)</td>
<td>120 (±0)</td>
<td>0 (±0)</td>
<td>60 (±0)</td>
<td>45.04</td>
</tr>
<tr>
<td>F096</td>
<td>0–20</td>
<td>959 (±355)</td>
<td>124 (±33)</td>
<td>43 (±22)</td>
<td>81 (±19)</td>
<td>44.30</td>
</tr>
<tr>
<td>S114</td>
<td>0–60</td>
<td>1136 (±1097)</td>
<td>114 (±59)</td>
<td>44 (±36)</td>
<td>71 (±30)</td>
<td>47.43</td>
</tr>
<tr>
<td>F161</td>
<td>30–0</td>
<td>1611 (±530)</td>
<td>185 (±40)</td>
<td>60 (±13)</td>
<td>126 (±34)</td>
<td>47.35</td>
</tr>
<tr>
<td>S211</td>
<td>0–90</td>
<td>2107 (±1421)</td>
<td>190 (±88)</td>
<td>74 (±50)</td>
<td>116 (±49)</td>
<td>50.34</td>
</tr>
<tr>
<td>F256</td>
<td>30–20</td>
<td>2563 (±741)</td>
<td>308 (±63)</td>
<td>101 (±29)</td>
<td>206 (±42)</td>
<td>53.66</td>
</tr>
<tr>
<td>F322</td>
<td>30–40</td>
<td>3224 (±1048)</td>
<td>403 (±72)</td>
<td>130 (±37)</td>
<td>271 (±47)</td>
<td>53.21</td>
</tr>
<tr>
<td>F480</td>
<td>30–60</td>
<td>4802 (±1249)</td>
<td>586 (±116)</td>
<td>192 (±61)</td>
<td>393 (±76)</td>
<td>48.71</td>
</tr>
</tbody>
</table>

¹Treatments are arranged according to the amount of organic matter applied. The letter of each reference indicates the fertilizer origin: mineral fertilizer (M), slurry from fattening pigs (F) or slurry from sows (S). The numbers indicate the average rate of organic matter applied annually (kg 10⁻¹ ha⁻¹).

²The experiment was maintained from 2002 to 2016; during the 2007–2008 and 2013–2014 cropping seasons the field was under fallow.

³The first number indicates the amount of slurry applied at sowing and the second the amount applied at cereal tillering as topdressing.

⁴A polynomial quadratic equation (γ = −1.7095x² + 10.841x + 36.112; R² = 0.87) was found between the mean value of organic matter applied with slurries (x, Mg ha⁻¹) and the accumulated biomass grain yield (y, Mg ha⁻¹).
Slurry was applied over the soil surface by the splash-plate machine method. Tractor speed was adjusted and applied doses were supervised by differences in tank weight after each application. At pre-sowing, the same day after application of the slurry, it was buried with an offset disc harrow (0–0.15 m), but it was left on the soil surface at cereal tillering.

2.2 | Soil and slurry sampling

Pig slurry samples were collected on 10 February 2015. The solid slurry fraction was separated by centrifugation. Subsequently, it was freeze-dried, ground with an agate mortar, suspended for 24 h in water (1:20, ground slurry: distilled water), centrifuged prior the 0.45μm filtration and freeze-dried.

A composite soil sample in each treatment (1 kg) was collected on 20 September 2016, prior to sowing. It consisted of a mixture of five subsamples obtained from a soil depth of 0–0.1 m. The samples were dried at room temperature and passed through a 2-mm sieve. A total of 24 soil samples were processed.

2.3 | Organic matter fractionation and quantification

Humic acids from soil samples were isolated following Duchaufour and Jacquin (1975) and Velthorst et al. (1999). Each extraction was repeated threefold in series of 50 g soil per treatment to obtain at least 0.5 g HAs. Firstly, the soil sample was suspended in 2 M H₃PO₄ (100 mL), which also leads to carbonate removal. The supernatant (free FA) was filtered to separate the floating free organic matter fraction (FOM) from the soil heavy centrifugation residue. The FOM was washed with distilled water until pH = 7. Both the FOM and the free FA extract were kept for further analysis.

Then, the rest of the initial soil samples followed a series of 10 sequential extractions following a standard procedure (Dabin, 1971). Samples were treated twice with 0.1 M Na₂HPO₄ (150 mL each time) and eight times with 0.1 M NaOH (150 mL each time). At the end of the extractions, the volume of the recovered solution was determined for further analysis of the total humic extract (THE) and HA using of 25 and 50 mL aliquots of each extract respectively. The preparative isolation and purification of the HAs from the remaining extract was carried out by precipitating the THE at pH = 1 with 6 M HCl for 24 h to promote HA precipitation, then the suspension was decanted. The supernatant solution (soluble FA) was discarded. The HA precipitate was re-dissolved with 0.5 M NaOH and centrifuged at 3622 g for 5 min to sediment clay minerals, which were discarded. The clay-free sodium humate solution was reprecipitated with 6 M HCl until pH = 1, centrifuged at 3622 g for 5 min and decanted. The purified HA in the gel state was recovered with distilled water and transferred into cellophane dialysis bags (Visking® dialysis tubing, molecular weight cut-off 18,000, equivalent to pore diameter of about 25 Å) for 3–4 days in order to remove soluble mineral salts. The distilled water was replaced every day until no reaction of chloride with silver nitrate was observed. Finally, the resulting HA suspension was transferred to Petri dishes and dried at 40 °C. Isolated HAs were stored in glass vials for further analyses.

Soil organic carbon from the soil sample and the carbon content in the different fractions (FOM, free FA, THE, and HA) was quantified by dichromate oxidation and subsequent titration with ferrous ammonium sulphate (Walkley & Black, 1934; Yeomans & Bremner, 1988). Fulvic acids were calculated by difference between THE and HA.

2.4 | Spectroscopic analyses

In this study, FTIR spectroscopy was considered especially suitable in the case of HA extracted from soils treated with residues at a low degree of transformation and maturity. By applying resolution enhancement techniques, FTIR spectra show well-resolved peak patterns suitable to describe macromolecular substances at different degrees of alteration. This is the case of lignin and other constituents such as acids, esters and proteins, which can be easily distinguished from alkyl compounds and proteins. The same is not the case with other techniques that yield quantitative values more directly, for example, 13C-NMR spectra, with several overlapping signals produced by different structures (e.g. alkyl, carboxyl and amide). This does not allow specifying their proportions with precision, nor clearly differentiating the humified material from the biomass present in residues such as slurry. On the other hand, the rich peak pattern of the resolution-enhanced FTIR spectra provides a greater number of descriptors that are directly, for example, 13C-NMR spectra, with several overlapping signals produced by different structures (e.g. alkyl, carboxyl and amide). This does not allow specifying their proportions with precision, nor clearly differentiating the humified material from the biomass present in residues such as slurry. On the other hand, the rich peak pattern of the resolution-enhanced FTIR spectra provides a greater number of descriptors that are suitable for chemometric studies on the effect of sludge addition on HA composition.

In HA samples, spectroscopic determinations were carried out in the visible range from solutions adjusted to an equal concentration in carbon (Ghosh & Schnitzer, 1979). The HAs were dissolved into 0.01 M NaOH at a concentration equivalent to 0.2 mg C mL⁻¹. Spectra in the visible range (400–800 nm) were obtained with a Hewlett Packard 8452A diode array...
satisfactory and the E4/E6 ratios were calculated.

In soil and slurry samples, Fourier-transformed infrared (FT-IR) spectra in the 4000–400 cm\(^{-1}\) wavelength range were acquired with a spectrophotometer, Bruker IFS28 (Karlsruhe, Germany). Baseline was corrected. Pellets were prepared with 2 mg of HA and 200 mg KBr. In addition, and for resolution enhancement, a digital treatment was used based on subtracting the original spectrum from a positive multiple of its second derivative (Almendros et al., 1992). Finally, the spectra were full-scale normalized.

Absorption bands were assigned according to different authors (Fengel & Wegener, 1983; Hernández, 2009; Kononova, 1982; MacCarthy & Rice, 1985). The identification of the lignin band pattern was carried out following Fengel and Wegener (1983) and Hernández et al. (2010). The determination of the intensities of the main spectral peaks (normalized values of peak-valley distances), was carried out in the second derivative of the spectra (Fernández-Getino et al., 2010).

2.5 | Statistical analysis

The ANOVA analysis was performed for SOC and SOM fractions (FOM, free FA, FA, HA), for the HA/SOC, HA/THE and HA/FA ratios and for optical densities (E4, E6 and E4/E6) according to a randomized block design. If significant, differences between mean values were identified with Duncan Multiple Range Test at \(\alpha = 0.05\). Analyses were performed with the statistical package SAS version 9.4 (SAS Institute, 2014).

The multivariate treatment of the spectroscopic data was carried out by discriminant analysis, applied to distinguish data groups (treatments that differ in the slurry application rates) based on a set of observed quantitative variables (intensities of the main peaks of the FTIR spectra). This statistical procedure is based on the construction of discriminant functions that are linear combinations of the variables and that allow the differential aspects of the spectra belonging to different groups to be identified. Discriminant analysis seems adequate to analyse the results of spectroscopic data of HAs obtained in experiments under field conditions, generally with wide spatial variability, because (a) it forces the calculation of the appropriate discriminant functions to classify the observed cases in order to group them as well as possible, and (b) it allows evaluating to what extent each of the observations belongs to the different predefined groups, which is also a way of evaluating whether the treatments have really been reflected in the characteristics of each of the HAs (Statgraphics Technologies, 2022).

3 | RESULTS

3.1 | Changes in soil fertility after slurry application

Soil organic carbon, THE and its fractions: HA and FA, showed significant differences between treatments (Tables S1 and S2). Addition of fattening pig slurries at rates higher than 1.6 Mg ha\(^{-1}\) yr\(^{-1}\) (from F161 treatment onwards) increased SOC significantly (c. 46%) when compared with the mineral treatment (Figure 1). However, no significant SOC differences \((p > 0.05)\) in comparison with the M000 treatment were found when slurries came from sows. The proportion of HA increased by almost 75%, from 1.5 g C kg\(^{-1}\) soil in M000 to 2.6 g C kg\(^{-1}\) soil (F256, Figure 1). The HA accounted for 61% up to 80% of the THE. Significant differences \((p = 0.05)\) were also found in the HA/FA ratio, which means that only treatments S211 and F256 had higher ratios than the control (Table S1). The lowest contribution to SOC came from the FOM fraction, which averaged between 0.1 and 0.7 g C kg\(^{-1}\) soil, followed by free FA (Figure 1).

3.2 | Characteristics of HAs in soils treated with slurry

The HAs from soils treated with slurries at the highest rates (F322, F480) showed significantly decreased values of optical density at 465 nm compared with mineral fertilization (Table S3), following from F096 there was a smooth decline in E4 optical density as slurry rate increases. Calculated E4/E6 ratio values ranged from 4.9 up to 5.9 (Figure 2).

The resolution-enhanced FT-IR spectra greatly assist in the identification of the characteristic bands of HAs from the slurries (Figure 3a) and soil samples from the experimental field (Figure 3b). The spectra show a common pattern of well-defined bands, with differences in their intensities between sow and fattening slurries (Figure 3a) and between soil samples from different fertilization treatments (Figure 3b). In slurries, C–O–C polysaccharide band 1130 cm\(^{-1}\), the C–N amide II and III bands (1550 cm\(^{-1}\) and 1270 cm\(^{-1}\), respectively) and C=O stretching band (1720 cm\(^{-1}\)) were detected. In fertilized soils, there were three prominent peaks: the band for aliphatic C–H stretching (2920 cm\(^{-1}\)); the complex 1620 cm\(^{-1}\) band to which aromatic and unsaturated structures and conjugated carboxyl groups contribute; and the intense complex band at
1030 cm\(^{-1}\) for C–O stretching of polysaccharide-like substances as well as methoxyl groups. Other characteristic peaks were the carboxyl C=O stretching (1720 cm\(^{-1}\)), the CH\(_3\) symmetric bending from carboxylic acids (1380 cm\(^{-1}\)) and the amide II band at 1550 cm\(^{-1}\). The C–H asymmetric bending (1460 cm\(^{-1}\)) of aliphatic groups was identified as well as aromatic vibrations of lignin methoxyphenols (1420 cm\(^{-1}\)). Other well-resolved peaks present in the resolution-enhanced spectra coincided with the amide I structures (1650 cm\(^{-1}\)); aromatic C (1510 cm\(^{-1}\)) and syringyl (1330 cm\(^{-1}\)) and guaiacyl (1270 cm\(^{-1}\)) groups of lignin structures.

A series of significant correlations were found between the normalized intensities of some peaks from soil samples and the average applied organic matter rate (Figure 4). The alkyl C–H bending (1460 cm\(^{-1}\)), and the peak for C–O structures (1030 cm\(^{-1}\) for carbohydrate, methoxyl groups…) were positively related to the organic matter rate applied with slurries. The concomitant relative decrease in the intensity of unspecific aromatic structures peaking at 1620 cm\(^{-1}\) was also observed (Figure 4), including some prominent aromatic peaks of the lignin pattern (e.g. peaks at 1420 cm\(^{-1}\) and 1270 cm\(^{-1}\)).

### 3.3 Multivariate analysis of FTIR spectroscopic data of humic acids

The results of the discriminant analysis are shown in Figure 5, where the classification of the HA samples represented in the space defined by the two discriminant functions is shown.

The results of the analysis showed that 100% of the samples are correctly classified into the three predefined groups, corresponding to the control soils (exclusively mineral fertilization), and the groups corresponding to low annual manure application rates (>96 < 211 kg 10\(^{-1}\) ha\(^{-1}\)) and high (>256 < 480 kg 10\(^{-1}\) ha\(^{-1}\)). This is reflected in
the fact that totally disjoint sets appear that are separated mainly based on the first discriminant function, which presented a significance index of $p < .014$ (79.8% of the variance is explained).

The general comment regarding the classification obtained is the possibility of differentiating large groups of samples obtained from soils treated with different doses of slurry, exclusively using the intensities of the FTIR spectra bands as descriptors. The different intensity of the spectral bands in pre-established groups determines the values of the coefficients of the discriminant functions shown on the axes (Figure 5). In particular, the first discriminant axis allows a clear differentiation of the levels of the progressive amounts of organic matter application, while the second axis provides some additional information on the different doses.

4  |  DISCUSSION

4.1  |  Changes in soil carbon levels as a consequence of slurry application

Slurry application led to a positive trend in SOC content, which increased as applied rates increased (Figure 1). This trend was less marked when sow slurry had been applied, probably due to its greater impact on the increase in the relative proportion of the FOM (i.e. the light or labile particulate organic matter) as has been stated by Yagüe, Bosch-Serra, Antúnez, and Boixadera (2012). This light organic matter fraction is assumed to be an unprotected C pool, consisting of plant residues in various stages of microbial decomposition (Six et al., 2002). In our case, FOM includes decomposition products of sawdust used as
bedding after a sow’s farrowing process. This is not the case for fattening pigs as they are housed directly on slats.

The general SOC increase cannot be attributed to a higher stubble biomass input as grain yields follow a polynomial quadratic equation and yields tend to stabilize and even decrease after reaching a value of \( c = 50 \text{ Mg ha}^{-1} \) (Table 1). The fact that the increase in plant production is progressively less when the dose is increased, until it decreases slightly in the treatments with the highest dose of slurry, corresponds to an expected behaviour typical of the classical Law of Diminishing Returns (Mitscherlich, 1909). This suggests that it has been possible to determine the so-called 'technical optimum' for application of slurry to our soil, and that the experiment has covered the entire range of concentrations necessary to evaluate the effects of the dose of slurry on the composition of the HA.

In the F480 treatment, SOC rose up to 4.3 g C kg\(^{-1}\) soil\(^{-1}\) (Figure 1) which means an increment of 45% when compared with M000. Our results agree with findings reported from Yost et al. (2022) and Santos et al. (2022), but differ from those by other authors who reported small or non-significant increases in SOC (Domingo-Olivé et al., 2016; Ndayegamiye & Côté, 1989; Plaza et al., 2002). Such differences can be explained because in our case a wide range of rates were tested (Table 1). Besides, doses were maintained over 12 years, which makes it possible to consistently define the cumulative effect of fertilization on SOC.

Protected SOC, in close association with silt and the 1:1 clay particles might increase. According to Beare et al. (2014), if a log/log relationship between the specific surface area of the fine fraction (15 m\(^2\) g\(^{-1}\)) and its SOC content is found, this protected SOC fraction could theoretically rise up to \( c = 23 \text{ g C kg}^{-1}\). At this point, Telles Rodrigues et al. (2020) warned of the risk of possible enhancement of microbial oxidation of the SOM of the deep horizons because of the high doses of N applied with the slurries.

4.2 Changes in soil humic substances after slurry application

Despite the low organic matter rates applied, from 1 to 5 Mg ha\(^{-1}\) yr\(^{-1}\) (Table 1) for a period of 12 years, significant differences in the HA/FA ratio were observed (Table S2). The changes in the HA/FA ratio is because of the initial increment of HA (with a progressive slurry rate) that further stabilized, showing a smooth plateau, and the concomitant decrease of FA until the F256 rate from where FA arose (Figure 1). This result can be interpreted as an increase in the quality of humification parallel with the increase in C content stored in the soil.

Much of the HA in a calcareous soils is bound to Ca\(^{2+}\). In calcium saturated soils, the humic materials (to a lesser extent the FAs) have reduced mobility in the presence of an excess of divalent cations (Greenland, 1971).

4.3 Effect of slurry addition on the FT-IR spectroscopic patterns of humic acids

In our soil samples (Figure 3), the increase of alkyl-groups (mainly at 1460 cm\(^{-1}\)) with slurry organic matter doses can be related to the incorporation to the SOM of fatty acids and waxes from slurries (Mahieu et al., 1999). These substances might be responsible for soil hydrophobicity when pig slurries are applied (Jiménez-de-Santiago et al., 2019). Although their transitory effect suggests a limited effect on C soil storage because of transformation processes over time (oxidation), it is considered that alkyl-C is easily retained by clay components (Lorenz et al., 2009). Nevertheless, macroaggregation is promoted...
by the accumulation of methoxyl/N-alkyl C in microaggregates (Yu et al., 2015), followed by an increase of SOC content. Our results from previous studies suggest that this accumulation is responsible for the resistance of dry aggregates to slaking (Bosch-Serra et al., 2017).

The applied slurries are raw materials with a low degree of humification (Provenzano et al., 2014) and their faecal origin, where the importance of lignocellulosic residues (i.e. lignin) is low because of the nature of the pig feed. The spectra in the visible range corroborated these assumptions, since E4 (an indicator of the progress of the humification) tend to decline with slurry organic matter rates (Figure 2). Tinoco et al. (2015) established that significant positive correlations exist between E4 and aromatic compounds in HAs, while the correlation between E4 and yields of aliphatic and lignin-derived methoxyphenols is negative. An enrichment of the less stable compounds added with the slurries as structures of aliphatic character, mainly polyalkyl, would explain the corresponding relative decrease in the intensity of the aromatic structures, including the characteristic FT-IR lignin pattern. The resulting relative decrease in the aromatic peak intensities in long-term experiments has also been observed by other authors (Almendros et al., 1991; Dorado et al., 2003).

4.4 | Discriminant analysis of the changes in the intensities of the bands of the FTIR spectra of humic acids treated with different doses of slurry

The simple visual inspection of the samples classified in the space defined by the discriminant functions demonstrates the possibility of satisfactorily categorizing the HAs of samples treated with different levels of slurry based exclusively on the information provided by the intensities of the main bands of their FTIR spectra (Figure 5). The differences between the spectral patterns of the groups depending on the slurry levels can be easily described by observing the trends (Figure 4), which shows the correlations between the slurry dose and the intensity of the selected bands.

The progressive decrease with the dose of slurry in the intensity of the band at 1720 cm\(^{-1}\) is observed (Figure 4), which indicates that slurry determines the accumulation of HAs with a decreased proportion of carboxyl groups. The same significantly occurs with the intensity of the aromatic bands (1620 and 1510 cm\(^{-1}\)), which display a lower proportion of aromatic components with the dose of slurry (Figure 4). This is in agreement with the concomitant increase of aliphatic groups, 1460 cm\(^{-1}\) (Figure 4) and similar to carbohydrates or methoxylated lignin units, 1030 and 1130 cm\(^{-1}\) (Figure 5). The proportions of N-containing groups (amides, 1510 cm\(^{-1}\)) also tend to increase with the application rates of slurry (Figure 5).

As a whole, FTIR results suggest that the slurry has involved the incorporation into HA of organic matter of a low degree of maturity, in which there has not been an intense selective biodegradation of more easily biodegradable constituents, as in the case of the HAs of the original soil with mineral fertilization.

The above intensity enhancement of the aliphatic peaks with pig slurry fertilization (Figure 4) has previously been reported (García-Gil et al., 2004; Plaza et al., 2002). The maximum allowed N dose (S211) in this rainfed agricultural system also signals the evolution of the importance of the different peaks as slurries are applied.

The quantitative increase obtained in HTS by slurry fertilization (Figure 1) should be considered carefully from the point of view that these materials can produce a long-term increase in the C pool. Aliphatic structures prevail in the new organic matter incorporated to the soil, which would be degraded if conditions were favourable because of their faster mineralization rate, as reported in other experiments (Almendros et al., 2018; Madrid et al., 2004). Nevertheless, the sustained annual application of slurry should be taken into account since the benefits in soil physical quality (Bosch-Serra et al., 2017; Mateo-Marín et al., 2021; Yagüe, Bosch-Serra, Antúnez, & Boixadera, 2012) can be related not only to the SOM increase but also to changes in its composition (Álvarez et al., 2013; Sacomori et al., 2021).

5 | CONCLUSIONS

Pig slurry application, using average application rates higher than 1.6 Mg organic matter ha\(^{-1}\) yr\(^{-1}\) and after a period of 12 cropping seasons, increased SOC levels compared with mineral fertilization. In addition, significant changes in the composition of the original SOM are observed. The application of slurry determined the accumulation of HAs with a lower degree of maturity than the pre-existing ones in the soil: in all doses of slurry there was a decrease in the aromaticity and condensation of the HA fraction and an accumulation of aliphatic constituents, which are generally considered indicative of SOM with comparatively low resistance to biodegradation. In this sense, the decrease in amended soils of the optical density of HA at 465 nm is significant, but also the characteristic patterns of the IR spectra reflect a decreased proportion of aromatic structures (e.g. IR band at 1620 cm\(^{-1}\)) and concomitant increase in the proportion of aliphatic structures (e.g. 1460 cm\(^{-1}\)), which depended on the slurry application rates. Regardless of the reduction in the degree of maturity of the SOM after the application of slurry, its effect
on the studied soils should be considered very positive, since there are dose-dependent increases in fertility and increases in crop yields, although no improvement is observed by applying levels higher than 2.5 Mg ha\(^{-1}\) year\(^{-1}\).

**ACKNOWLEDGEMENTS**

This research was supported by the Spanish Ministry of Science, Innovation and Universities and the Spanish National Institute for Agronomic Research (MICINN-INIA) through the project RTA2017-88-C3-3. D.E. Jiménez-de-Santiago also acknowledges the JADE-Plus scholarship from Bank of Santander-University of Lleida for her PhD studies. The laboratory assistance of M. Antúnez and S. Porras from University of Lleida and the edition assistance of M.M. Boixadera-Bosch are fully acknowledged. We would like to thank the Editor and to three anonymous reviewers for their suggestions and comments.

**CONFLICT OF INTEREST STATEMENT**

The authors declare no conflict of interest.

**DATA AVAILABILITY STATEMENT**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

**ORCID**

**Ángela D. Bosch-Serra** https://orcid.org/0000-0003-0428-2036

**REFERENCES**


**SUPPORTING INFORMATION**

Additional supporting information can be found online in the Supporting Information section at the end of this article.

**How to cite this article:** Jiménez-de-Santiago, D. E., Almendros, G., & Bosch-Serra, Á. D. (2023). Structural changes in humic substances after long-term fertilization of a calcareous soil with pig slurries. Soil Use and Management, 00, 1–13. https://doi.org/10.1111/sum.12896