



Structure of the humic acid-like compounds of raw and hydrothermally treated sewage sludge

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ABSTRACT

Humic acids are of great interest in many fields; however, they are inhibitors of fermentative processes applied to hydrothermally treated sewage sludge. Hence, the structure and composition of soluble and bound humic acid-like fractions from raw and hydrothermally treated sewage sludge were studied. Lipid, polysaccharide, protein and aromatic fractions were identified, as well as a high nitrogen content (7–10 %) and low solubility in alkaline media. Thus, they do not strictly meet the chemical definition of 'humic acids'. The soluble humic acid-like compounds had more aromatic and less protein content. Thermal hydrolysis of sewage sludge increased their aromaticity to the detriment of protein and polysaccharide fractions, while wet oxidation caused an increase in all structural fractions. Regarding the bound compounds, lipid, polysaccharide and aromatic fractions increased markedly during both treatments, although oxygen produced higher degradation of the protein fraction and, from 1 h, the partial degradation of aromatic compounds and an increase in the C/N atomic ratio (from 5.0 to 18.7 after 2 h). Therefore, hydrothermal treatments have a positive impact on the hydrolysate biodegradability due to the organic matter solubilisation, but also a negative impact linked to the higher solubilisation of the humic acid-like compounds and their structural changes.

1. Introduction

Sewage sludge, a by-product of the biological treatment processes in wastewater treatment plants (WWTP), is a semi-solid material that consists of flocs and has very poor dewaterability. The flocs consist of microbial and extracellular polymeric substances (EPS) (mainly humic compounds, proteins and carbohydrates), along with pathogens, micropollutants, heavy metals and other hazardous materials, making the proper management of sewage sludge an issue of growing importance [1–4].

As the regulations about sludge management become stricter, conventional final handling options, such as landfilling, incineration or land application, are seen to have certain important drawbacks that restrict their implementation [1]. For this reason, anaerobic digestion has been established as the preferred and most promising technique for achieving proper sludge management [5]. As well as producing biogas, anaerobic digestion also stabilises the sludge, reducing odours, pathogens and

organic matter content. Nevertheless, the initial hydrolysis of sewage sludge components is a rate-determining step that significantly limits the efficiency of the process [6,7]. In this sense, wet oxidation (oxidising atmosphere, $T = 100\text{--}325\text{ }^{\circ}\text{C}$) and, in particular, thermal hydrolysis (inert atmosphere, $T = 100\text{--}200\text{ }^{\circ}\text{C}$), have been gradually developed into a mature sludge pre-treatment technology, not only for increasing the biodegradability of the sewage sludge, but also for a variety of other purposes, such as sludge stabilisation and resource recovery [6,8,9].

At this point, it is interesting to note that sewage sludge contains many components, some of them with high added value (such as proteins and carbohydrates), which are solubilised and transformed during the hydrothermal treatments. Bearing this in mind, recent research is also being focused on using the hydrothermally treated sludge not only for biogas production, but also as a fermentation medium for other metabolites and/or as a renewable source of valuable compounds [9–13]. In this sense, the role of humic acid-like compounds during sludge management has been receiving increasing interest, for a variety

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of reasons. Firstly, these compounds have proved to have beneficial effects on soil quality and plant growth [14]. Furthermore, humic acids are also related to the retention of heavy metals and other undesirable substances, playing a key role in the land application of sewage sludge, either directly or after being hydrothermally pre-treated. As well as in agriculture, humic acids from sludge are being used in industrial production, environmental remediation and, even for medical applications [15]. However, these substances have also been identified as inhibitors during anaerobic digestion and other fermentation processes of sewage sludge, which has a significant impact on the selection of management strategies for this by-product [11,15]. Thus, taking into account that humic substances can represent up to 29 % of the total volatile solids in sewage sludge, of which at least 80 % are humic acid-like compounds [15], the need arises to deepen the understanding of how the properties of humic acids, as both soluble and bound extracellular polymeric substances, are modified during the different sludge management strategies.

In this regard, the changes in concentration and some physicochemical properties of humic acid-like substances when sewage sludge was subjected to treatments such as anaerobic digestion, gasification or land application have already been discussed [16–18]. However, it is necessary to stress that the literature dealing with the effect of wet oxidation or thermal hydrolysis on the humic acid-like compounds from sewage sludge is very scarce, despite the proven importance of these treatments during most sludge management strategies. An exception is the work of Huang et al. [19], who recently explored the compositional changes in these compounds after applying thermal hydrolysis to diluted waste activated sludge (10 % of total solids) at different temperatures (from 40 to 220 °C) for 30 min (after reaching the desired temperature), as well as their influence on the subsequent hydrolysate digestion. Nevertheless, the effect of temperature on the chemical structure of only the soluble, but not the bound, humic acid-like fraction was studied by those authors, even though the latter has proven to be more abundant in sewage sludge. What is more, the impacts of the main operational conditions of hydrothermal treatments, apart from temperature, on the evolution of the composition of the humic acid-like compounds remain unexplored.

Therefore, based on the foregoing observations, the aim of this work is to analyse and discuss how the structure and composition of soluble and bound humic acid-like fractions are modified during the hydrothermal treatment of secondary sewage sludge, paying attention to the effects of time (0, 1 and 2 h) and type of atmosphere (oxidising or inert) for the first time ever. This information will be very useful as a first step towards assessing the feasibility of potential management strategies based on an initial hydrothermal treatment of sewage sludge.

2. Materials and methods

2.1. Materials

Secondary wet sewage sludge, thickened by dissolved air flotation, was collected from a municipal WWTP in Baiña (Asturias, Spain) and subsequently stored at 4 °C until its use. The main physicochemical

Table 1
Physicochemical properties of sewage sludge.

Parameters	Mean values
pH	6.5 ± 0.1
Total Chemical Oxygen Demand (tCOD) (g O ₂ /L)	32 ± 7
Soluble Chemical Oxygen Demand (sCOD) (g O ₂ /L)	1.3 ± 0.5
Colour Number (CN)	0.11 ± 0.03
Soluble Total Organic Carbon (sTOC) (g C/L)	0.8 ± 0.4
Dry matter (DM) (g/L)	39 ± 3
Total Suspended Solids (TSS) (g/L)	37 ± 2
Volatile Suspended Solids (VSS) (g/L)	30 ± 1
Soluble Extracellular Polymeric Substances (sEPS) (g/L)	0.24 ± 0.08

properties of the raw material are shown in Table 1.

2.2. Hydrothermal treatments

Hydrothermal treatments (thermal hydrolysis and wet oxidation) were carried out in a 1 L 316 SS semi-batch reactor (Parr Instruments Co.), following the setup described in previous research [20]. The operational conditions were 700 mL of secondary wet sewage sludge, 160 °C and 40 bar, with reaction times ranging from 0 to 2 h and in the presence or absence of oxygen. These were chosen because higher thermal hydrolysis temperatures led to the formation of refractory compounds, producing inhibition in subsequent fermentative processes [10,11].

2.3. Extraction of humic acid-like compounds

Initial, stored, and the different hydrothermally treated sewage sludge samples were centrifuged at 6000g for 30 min to separate the liquid phase, which contained humic acid-like compounds as a part of the soluble microbial products, from the solid fraction, with the extracellular polymeric substances (EPS) bound to the cells, including non-solubilised humic acid-like substances. Then, bound EPS were solubilised from the solid fraction following a widely used method, based on the use of formaldehyde and NaOH [21]. Briefly, the solid phase was resuspended with distilled water to the initial volume and formaldehyde was added (60 µL per 10 mL of suspension). After 1 h, a solution of 1 N NaOH was also added (4 mL per 10 mL) and, after 3 h of incubation at room temperature, the sample was centrifuged at 10,000g for 30 min. The pellet was discarded and the supernatant, composed of bound EPS, was stored.

For humic acid-like compound extraction from the supernatants with either soluble or bound EPS, a method adapted from the International Humic Substances Society (IHSS) was followed [22]. First, the supernatant pH was lowered to 1–1.5 with 6 M HCl. After incubation for at least 12 h and, subsequently, centrifugation at 8000g for 30 min, the resulting solid fraction was washed twice with distilled water, freeze-dried and stored for further analysis. A scheme of the entire process can be seen in Fig. S.1 in the Supplementary Material.

At the same time, a sedimentary commercial humic acid crystalline powder (CAS number: 1415-93-6) from Alfa Aesar, purchased from VWR International LLC, was used as the reference material to assist in investigating the composition and structure of the different humic acid-like compounds obtained.

2.4. Analytical methods

The physicochemical properties of the sewage sludge were measured following the Standard Methods for the examination of water and wastewater [23].

The carbon, hydrogen and nitrogen contents of the soluble and bound humic acid-like samples and the reference humic acids were analysed in duplicate using a CHNS FlashSmart Elemental Analyzer (Thermo Scientific). The oxygen content was determined by difference (ash-free basis).

Attenuated Total Reflectance - Fourier Transform Infrared Spectroscopy (ATR-FTIR) spectra of the soluble and bound humic acid-like samples and the reference humic acids were recorded with a Shimadzu IRAffinity-1S FTIR over the 4000–400 cm⁻¹ range.

Solid Nuclear Magnetic Resonance (NMR) spectra of the soluble and bound humic acid-like samples and the reference humic acids were measured at room temperature using a Varian VNMR5-500 spectrometer of 11.7 T (500 MHz proton frequency) equipped with a double resonance HX Pencil probe with a sample capacity of 160 µL and zirconia rotors of OD 5 mm. The spectrometer operating software was VNMRJ 4.0. All the spectra were processed with MestreNova software v14.0 (Mestrelab Research Inc.). Carbon chemical shifts were referenced

externally to the carbon methylene signal of solid adamantane at 28.92 ppm. Proton chemical shifts were referenced externally to the amide NH proton of solid glycine that resonates at 8.5 ppm. 1D ^1H MAS (Magic Angle Spinning) spectrum was acquired at MAS 12 kHz with pulse acquisition. The inter-scan delay was set to 2 s and the number of scans was 64. The proton pulse was applied with a field strength of 48.1 kHz. ^{13}C CPMAS (Cross Polarization Magic Angle Spinning) spectrum of each sample was measured with the pulse sequence tanpdx of the Varian library. The proton-carbon cross polarization used a contact time of 3 ms and the proton contact pulse power used a linear ramp of 10 %. The inter-scan delay was 1.0 s and the MAS rate was 12 kHz. Heteronuclear decoupling during the acquisition of the Free Induction Decay (FID) was performed with SPINAL-64 with a proton B1 field strength of 79.4 kHz. The minimum number of scans acquired per sample was 3500 in ca. 1 h. ^{13}C CPMAS NMR spectra were analysed as in Fuentes et al. [24].

3. Results and discussion

First of all, it is important to note that the reaction mechanism and effects of hydrothermal treatments on sewage sludge and, even, on the evolution of humic acid concentration are very well known, but the effects of these treatments on the structure and composition of humic acids are not. For further information about these topics, the reviews of Hii et al. [8] and Suárez-Iglesias et al. [9] and the studies of Pola et al. [12], García et al. [25] and Huang et al. [19] can be consulted.

3.1. Humic acid-like compounds in sewage sludge

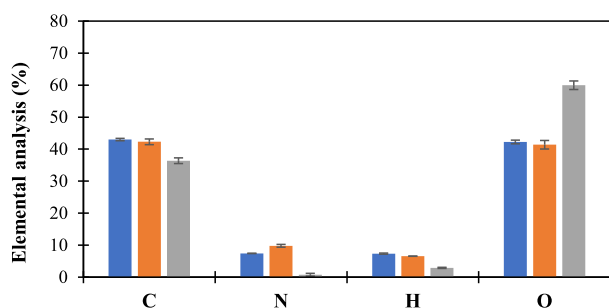
Before discussing the effects of hydrothermal treatments on humic acid-like compounds, the composition and structure of soluble and bound fractions in raw sewage sludge were analysed, using a commercial humic acid as the model in order to easily identify differences between sedimentary and sludge-derived humic acids (Fig. 1).

Focusing firstly on the comparison of humic acid-like compounds (bound or soluble) from sludge with sedimentary humic acids, the

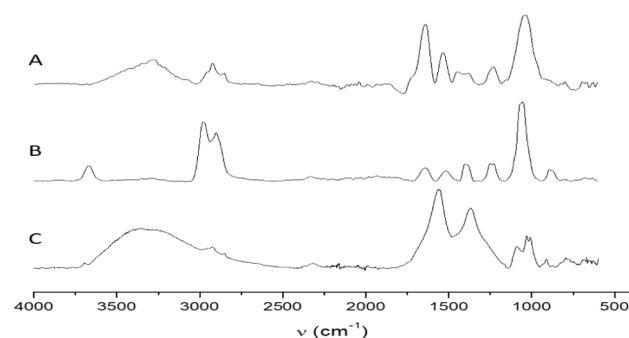
differences are clearly noticeable. The elemental analysis convincingly showed these differences, especially in the percentage of nitrogen and hydrogen. The ratios H/C and N/C of humic acid-like fractions from sewage sludge were 2 and 8 times higher, respectively, than those obtained for the commercial ones. This was expected, as similar results for humic acid-like substances from sludges had already been reported in previous studies [19,26,27]. In fact, these compositional differences can be explained by the fact that the humic acid-like compounds have not undergone maturation comparable to that of humic acids in soils or other evolved organic matter. For this reason, the high N/C ratio is due to the presence of amino acids and tissue residues of the microorganisms in the structure of these humic acid-like fractions, while the higher H/C ratio is due to the predominance of aliphatic structures [26–28]. In this regard, Hernandez et al. [26] reported a higher bioavailability for those humic acids with a higher nitrogen content, thus suggesting a lower inhibitory effect for the humic acid-like compounds than for the soil-based ones during the hydrolysate anaerobic digestion [19].

Structural features of sewage sludge-derived humic acids also differed from sedimentary ones, as can be deduced from the different spectra analyses (see Fig. 1b, c and d and their corresponding chemical shifts in Table S.1 and Fig. S.2 and S.3 in the Supplementary Material). Thus, the ^{13}C NMR spectrum for commercial humic acids showed two predominant peaks. The former, in the range of 5 to 50 ppm, corresponded to the aliphatic fraction, mainly containing lipids. The latter, between 100 and 160 ppm, was associated with aromatic structures, such as phenol-carbon and aromatic carbon. Although a small peak at 160–180 ppm, attributed to carboxyl groups, was also detected, the contribution of the protein fraction to the structure of the commercial humic acid structure was insignificant, which is in accordance with their low nitrogen content. The low absorbances at 1200–1300 and 1500–1700 cm^{-1} in the FTIR spectrum also confirmed a negligible presence of amide groups in the sedimentary humic acids. On the other hand, when humic acid-like compounds from sludge were analysed, ^{13}C NMR spectra revealed the presence of not only the lipid and aromatic fractions, but also those of polysaccharide (50–100 ppm) and protein

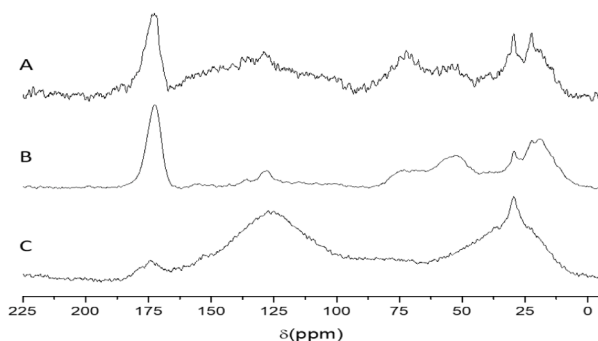
a) Elemental analysis



b) FTIR



c) ^{13}C -NMR



d) ^1H -NMR

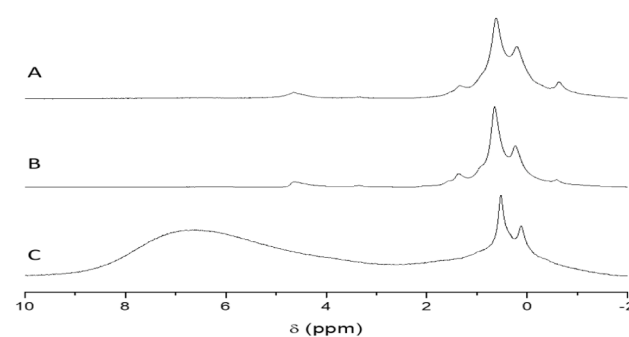


Fig. 1. Characterisation of raw soluble (●, A) and bound (●, B) humic acid-like compounds from sewage sludge and reference (●, C) humic acids.

(160–200 ppm). By FTIR measurements, the high absorbances related to aliphatic groups ($3000\text{--}2700$ and 1450 cm^{-1}) and amide and C=O groups ($1650\text{--}1535\text{ cm}^{-1}$) confirmed the presence of these structures. Moreover, the existence of peptide structures is consistent with the higher nitrogen content of the humic acid-like compounds from sludge in comparison to the sedimentary ones. In this regard, Amir et al. [29] also reported a heterogeneous mixture of etherified aromatic structures bearing peripheral long-chain lipidic, peptidic and carbohydrate structures for humic acids extracted from a mixture of 90 % sewage sludge and 10 % straw. Furthermore, the spectra obtained are in agreement with those reported by other authors for humic acid-like compounds extracted from sewage sludge [28,30]. It is interesting to note that Zhao et al. [31] reported that humic acids with low redox potential facilitated sludge anaerobic digestion, whereas those with high redox potential inhibited it. Therefore, taking into account that aromaticity involves high redox potential, commercial humic acids should have a more negative impact on anaerobic digestion than those derived from sludge.

Finally, for the fluorescence study, difficulties were encountered in redissolving the freeze-dried samples of humic acid-like compounds derived from sewage sludge in a 1 M NaOH solution (see Table S.2 in the Supplementary Material). Although the samples were obtained by the humic acid precipitation method, the fact that these substances were not completely soluble in an alkaline medium, in combination with the variations found in their composition and structure, precludes their classification as conventional ‘humic acids’ (organic matter soluble at alkaline pH and insoluble at acidic pH) or ‘fulvic acids’ (organic matter soluble at any pH). These differences, as explained before, apart from being caused by the binding of humic acids to other organic compounds present in the sewage sludge, could be explained by these humic acid-like compounds having a lesser degree of maturation [26–28]. In fact, this can be inferred from analysing the effects of storage time on the structural variation of the initial humic acid-like substances (see Fig. S.4 and S.5 in the Supplementary Material). For example, observing the FTIR spectra (Fig. S.4b and S.5b), it can be deduced that the longer the storage, the higher the aromatic content and the lower the polysaccharide and aliphatic fraction, especially in the case of the bound humic acid-like compounds.

Focusing now on comparing the humic acid-like fractions from sludge according to their state of aggregation, different analyses revealed small but significant differences between bound and soluble ones. Thus, elemental analysis (Fig. 1a) showed a slight but noticeable difference in their nitrogen content, this being around 3 % higher in the bound ones. This was probably due to a higher presence of proteins caused by the erosion of the cell wall during the extraction of the bound EPS [15,26]. These findings were corroborated by both FTIR (Fig. 1b) and ^{13}C NMR (Fig. 1c) analysis of soluble and bound humic acid-like compounds from the raw sludge. As previously reported, and as can be deduced from the assignment of chemical groups to frequencies (Table S.1 in the Supplementary Material) and the assignment of C-shift intervals to C types (Fig. S.2 in the Supplementary Material), both samples presented four clear structural families: protein (characterised by the amide peak), aromatic, polysaccharide and lipid fractions; but their relative importance varied depending on the type of humic compound. In this regard, the soluble humic acid-like compounds had a much greater aromatic character, as can be deduced from the $1650\text{--}1535\text{ cm}^{-1}$ bands in the FTIR spectra (Fig. 1b) and the aromatic percentages in the ^{13}C NMR spectra (Table S.3 in the Supplementary Material), but a lower representation of the protein fraction with respect to the bound ones, although the lipid and polysaccharide fractions were of similar importance in both. The higher condensation of the soluble humic acid-like compounds in comparison to the bound ones suggests that these latter are less aggressive to sludge digestion.

It is also interesting to note the presence of a small peak at 3600 cm^{-1} in the bound humic acid-like compounds, in comparison to a higher one at 3200 cm^{-1} in the soluble humic acid-like compounds, denoting the appearance of OH, alcohols and free phenols (see the percentage of

phenolic C in Table S.3 in the Supplementary Material). According to Machado et al. [32], wavelengths in the $3700\text{--}3620\text{ cm}^{-1}$ band can correspond to impurities associated with minerals or water, or show the complexity of the chains in humic acids. Nevertheless, soluble humic acid-like compounds did not absorb in this band, but in the $3700\text{--}3200\text{ cm}^{-1}$ band, which is associated with COOH stretch on H bridges. The strength of intramolecular bonds is related to stretching and widening, so a higher number of bridges and a more acidic character can be deduced for the soluble humic acid-like compounds when compared to the bound ones [32]. This fact, together with the strong peak in the 1645 cm^{-1} region, corresponding to C=O groups, suggests a higher functionalisation of the soluble humic acid-like compounds. In fact, the lower functionalisation of the bound ones, implying a higher hydrophobic character, was to be expected for a non-soluble compound, as was this case. The high aromaticity and functionalisation of the soluble humic acid-like compounds were probably due to a higher exposure of these to the oxidising environment than the bound ones. From a practical point of view, a lower hydrophobic character of the humic acid-like compounds is usually associated with less irreversible fouling of the membranes used in ultrafiltration processes widely used for water treatment and wastewater reclamation [15].

A similar structural pattern for both humic acid-like fractions from sewage sludge was also supported by ^1H NMR spectra (see Fig. 1d and chemical shifts in Fig. S.3 in the Supplementary Material). Taking as an example the spectrum obtained for the bound humic acid-like compounds, the predominance of aliphatic protons, probably bound to lipid and saccharide moieties, can be determined. There are also two small peaks within the 1–2 ppm range that could correspond to amines and/or functionalised aliphatic structures, and other peaks at 3.36 and 4.66 ppm associated with aliphatic alcohols and phenols, respectively. The small peak at -0.5 ppm may correspond to protons bound to halogen.

3.2. Effect of thermal hydrolysis time

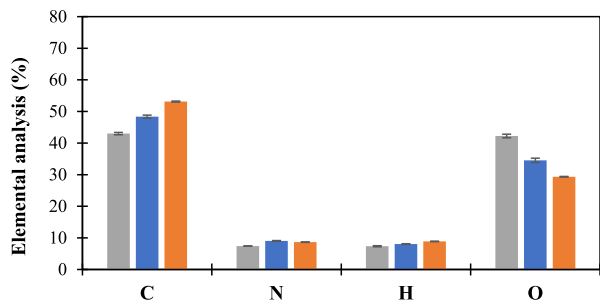
Once the initial composition and structure of the humic acid-like fractions were defined, their changes during the sewage sludge thermal hydrolysis were analysed (Figs. 2 and 3).

Regarding the chemical composition, elemental analysis (Figs. 2a and 3a) revealed an evident carbon enrichment, accompanied by an oxygen impoverishment in the chemical composition of both humic acid-like compounds during the thermal hydrolysis of sewage sludge, whereas nitrogen and hydrogen percentages scarcely changed. Thus, the O/C ratio of the humic acid-like compounds from sewage sludge decreased from an initial value of 0.74 to 0.41 after 2 h of treatment in the case of the soluble ones and from 0.73 to 0.43 in the case of the bound ones. Increases in the C/N ratio for humic acid-like compounds during thermal hydrolysis of sludge were also reported by Huang et al. [19], who attributed this to the formation of N-substituted glycosylamine by the condensation reactions between amino acids and sugars. Lower content of oxygen-containing groups in the bound humic acid-like compounds reduces the hydrophilicity of humic particles, thereby reducing their desorption and release from the sludge particles, which in turn hinders their transfer to the liquid phase [15].

Moving on to the structure, the analysis of the FTIR (Fig. 2b and 3b), the ^{13}C NMR (Fig. 2c and 3c and Table S.3 in the Supplementary Material) and the ^1H NMR (Fig. 2d and 3d) spectra obtained for thermally hydrolysed soluble and bound humic acid-like compounds revealed the presence of the four structural families previously reported (protein, aromatic, polysaccharide and lipid fractions), but with varying relative intensities during the treatment.

Focusing on the soluble humic acid-like compounds, it should be pointed out that thermal hydrolysis initially promoted the presence of soluble humic acid-like compounds with lower aromaticity and functionalisation than the initial ones (see the $1700\text{--}1500\text{ cm}^{-1}$ region of the FTIR spectra in Fig. 2b and Table S.3 in the Supplementary Material). This was due to the fast solubilisation of the bound humic acid-like

a) Elemental analysis



b) FTIR

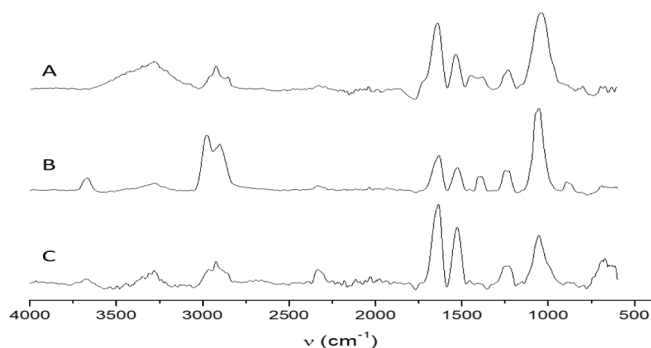
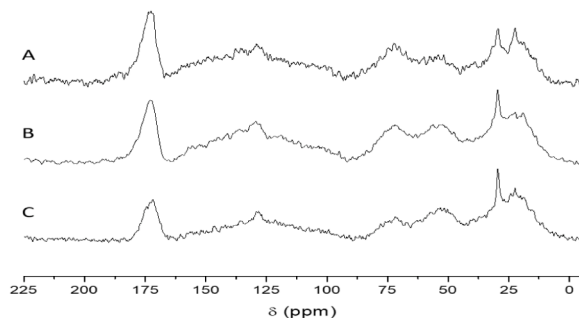
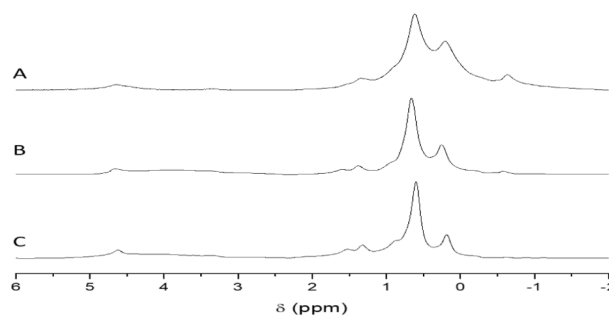
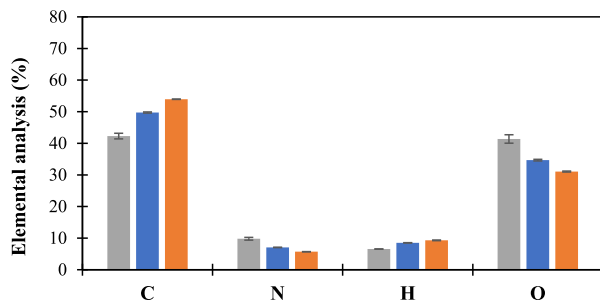
c) ¹³C-NMRd) ¹H-NMR

Fig. 2. Evolution of the structure of soluble humic acid-like compounds during thermal hydrolysis of sewage sludge at 160 °C and 40 bar (raw (●, A), 1 h (●, B) and 2 h (●, C)).

a) Elemental analysis



b) FTIR

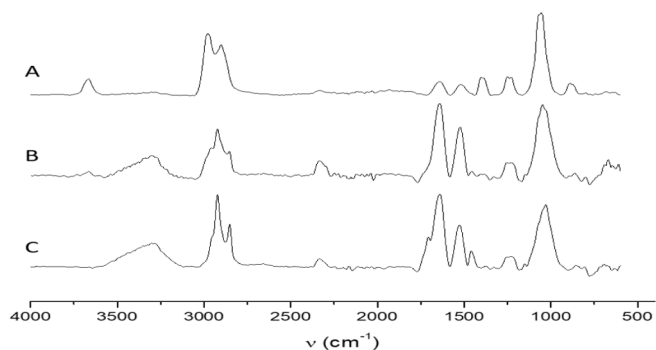
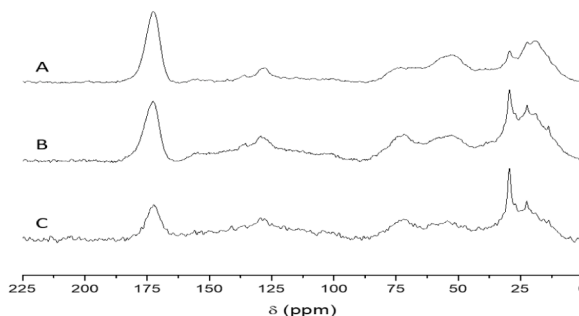
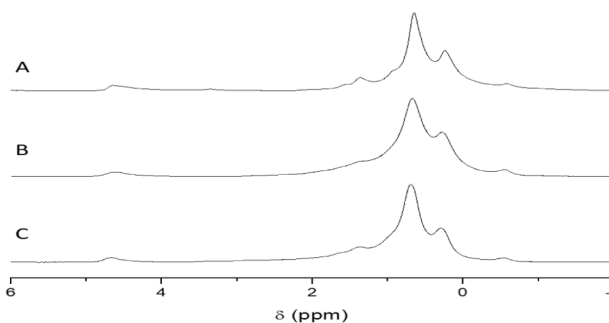
c) ¹³C-NMRd) ¹H-NMR

Fig. 3. Evolution of the structure of bound humic acid-like compounds during thermal hydrolysis of sewage sludge at 160 °C and 40 bar (raw (●, A), 1 h (●, B) and 2 h (●, C)).

compounds, with a more predominant aliphatic character than the soluble ones, as already explained above. As the reaction progressed and the solubilisation rate decreased, the predominance of aromatic

structures (1535 cm^{-1}), which were probably formed by condensation reactions, became evident. These reactions are reported to take place at temperatures above 70 °C [33] and are associated with the dehydration

of alcohols to give alkenes [34]. Although these reactions are usually acid-catalysed, the high temperature would compensate for the slightly acidic character of the hydrolysed sludge and promote the removal of water. According to the literature, the effects of acid hydrolysis on the chemical structure of the humic materials also included an increase in the aromaticity, in part due to the removal of proteinaceous components and carbohydrates, as well as the reduction of carboxylic groups, probably because of acid decarboxylation, which was consistent with the experimental results [35]. It is interesting to notice the decrease in absorbance at about 3280 cm^{-1} that was observed as thermal hydrolysis progressed, and which implied a lower content of oxygen-containing functional groups such as carboxylic, phenolic, aliphatic and alcoholic -OH groups, corroborating the previously explained mechanism of condensation and decarboxylation. Thermal decomposition of alcoholic -OH groups must not be ruled out either [36]. Moreover, the decrease in the polysaccharide fraction ($1200\text{--}950\text{ cm}^{-1}$ region in the FTIR spectra) was consistent with these explanations.

In the case of the bound humic acid-like compounds, significant increases in the intensities of almost all the peaks were observed during the treatment under an inert atmosphere, demonstrating an increase in their complexity in terms of both functionalisation and aliphatic and aromatic structures. Especially noticeable was the strong rise in intensities for the $1650\text{--}1535\text{ cm}^{-1}$ band, corresponding to aromatic groups, more refractory and characteristic of a high degree of humidification. As in the case of the soluble ones, this was probably caused by condensation mechanisms, leading to more condensed polyaromatic structures. Nevertheless, contrary to what was seen in soluble humic acid-like compounds, this increase in aromaticity for bound humic acid-like compounds was not accompanied by a decrease in the polysaccharide fraction. Furthermore, the simultaneous increase in aliphaticity could be associated with the integration of aliphatic components of bacteria into the humic acid structure or with the co-solubilisation of other products from cell lysis during the humic acid extraction [26,27]. Only peaks associated with the protein fraction decreased in intensities (see the percentages of carbonyl C in Table S.3 in the Supplementary Material), and this decrease was accompanied by an increase in the

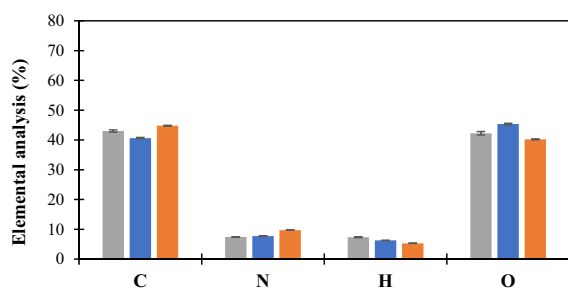
aromatic fraction, thus suggesting the merging of new cyclic aromatic structures. This can be confirmed by the appearance of a new peak at 1707 cm^{-1} after 2 h of treatment, corresponding to C=O stretching of ketones. It is also interesting to note the increase in the peak in the $3600\text{--}3200\text{ cm}^{-1}$ band, denoting the appearance of OH, alcohols and free phenols (see also the percentages of phenolic C in Table S.3 in the Supplementary Material). This involves a higher number of bridges and a more acidic character when compared to the initial bound humic acid-like compounds [32]. Moreover, the increase in aromaticity, accompanied by the reduction in the nitrogen content, indicates that the overall bioavailability of the bound humic acid-like compounds decreased during the sludge thermal hydrolysis, suggesting a negative impact on the subsequent anaerobic digestion [19].

3.3. Effect of wet oxidation time

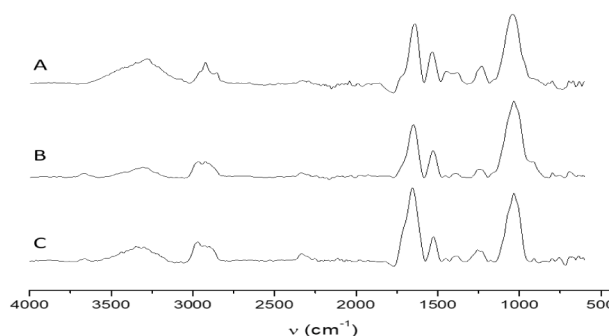
As in the previous section, the changes in the composition and structure of the two humic-like fractions were analysed and compared with their initial properties, although in this case, during wet oxidation, under an oxidising atmosphere (Figs. 4 and 5). It should be noted that NMR spectra for humic acid-like samples obtained after 2 h of wet oxidation could not be acquired as the samples were unexpectedly liquefied in the rotor of the spectrometer.

Comparing the elemental analysis of these fractions (Figs. 4a and 5a) with the initial results (Fig. 1a), different trends were observed during the wet oxidation, according to the humic acid-like fraction in question. This treatment, followed by extraction with NaOH and formaldehyde, produced bound humic acid-like compounds with high carbon but lower oxygen contents than the initial ones. This low O/C ratio is representative of humic acids with high hydrophobicity, which was to be expected given the low water solubility of bound humic acid-like compounds. Although the same trend was observed during thermal hydrolysis, the presence of an oxidising atmosphere speeded up these changes. It could be related to the treatment carried out, which could favour the removal of oxygenated functional groups, as described by Liu et al. [37]. The decrease in the nitrogen percentage of the bound humic

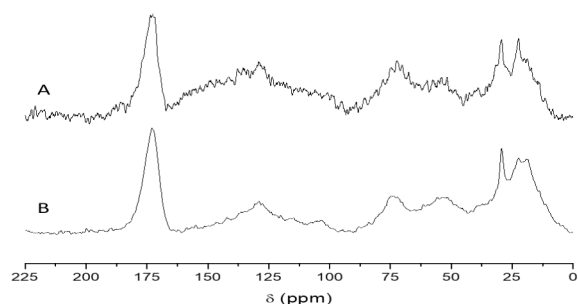
a) Elemental analysis



b) FTIR



c) ^{13}C -NMR



d) ^1H -NMR

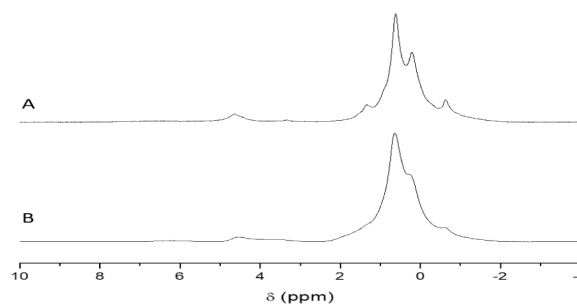


Fig. 4. Evolution of the structure of soluble humic acid-like compounds during wet oxidation of sewage sludge at $160\text{ }^\circ\text{C}$ and 40 bar (raw (●, A), 1 h (●, B) and 2 h (●, C)).

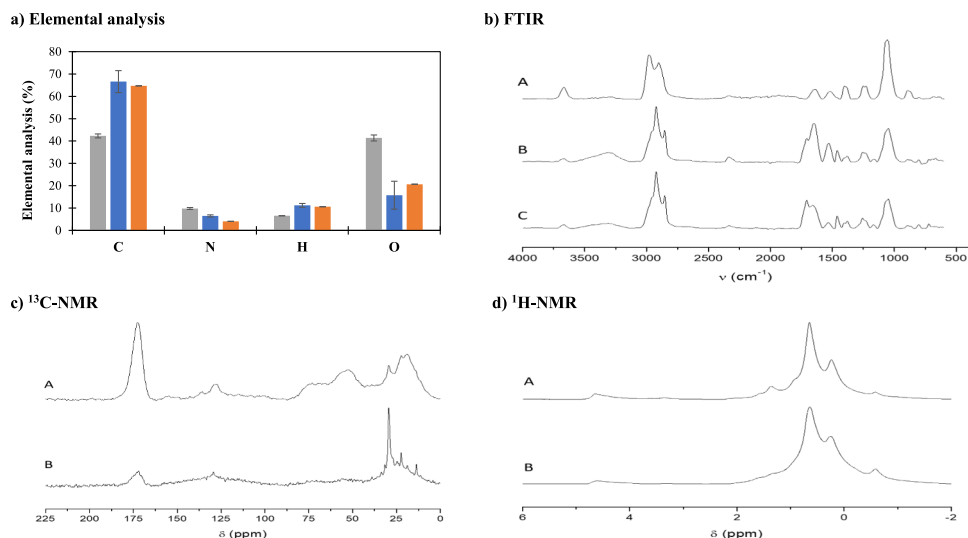


Fig. 5. Evolution of the structure of bound humic acid-like compounds during wet oxidation of sewage sludge at 160 °C and 40 bar (raw (●, A), 1 h (●, B) and 2 h (●, C)).

acid-like compounds observed during wet oxidation was attributed to the fast solubilisation of the bound extracellular polymeric substances, mainly composed of carbohydrates and proteins. Moreover, a slight increase in the hydrogen content was also observed. In the soluble humic acid-like compounds under an oxidising atmosphere, on the other hand, the hydrogen percentage slightly decreased and the carbon and oxygen contents remained almost unchanged. Regarding their nitrogen content, this increased during the treatment due to the fast solubilisation of proteins previously reported.

Analysing the FTIR (Fig. 4b), the ^{13}C NMR (Fig. 4c and Table S.3 in the Supplementary Material) and the ^1H NMR (Fig. 4d) spectra of the soluble humic acid-like compounds during wet oxidation, they showed high stability. The main structural fractions, instead of being degraded in the presence of oxygen, increased markedly, especially after 2 h of wet

oxidation. Moreover, at the end of the treatment, a peak in the 3700–3620 cm^{-1} band appeared, which can correspond to either impurities associated with minerals or water or the appearance of free -OH or phenols (see the percentages of phenolic C in Table S.3 in the Supplementary Material), associated with the mineralisation caused by the oxidation [32]. In the case of bound humic acid-like compounds (Fig. 5b, c and d), the combined use of oxygen and high pressures and temperatures caused almost complete solubilisation of the protein fraction, thus explaining the low nitrogen content of these after 2 h as previously reported, whereas aromatic or conjugated moieties increased. This is also supported by the reduction in the percentage of carbonyls and O- or N-alkyl C (see Table S.3 in the Supplementary Material). Furthermore, the appearance of a new peak at 1707 cm^{-1} corresponding to C=O stretching of ketones after 1 h of treatment

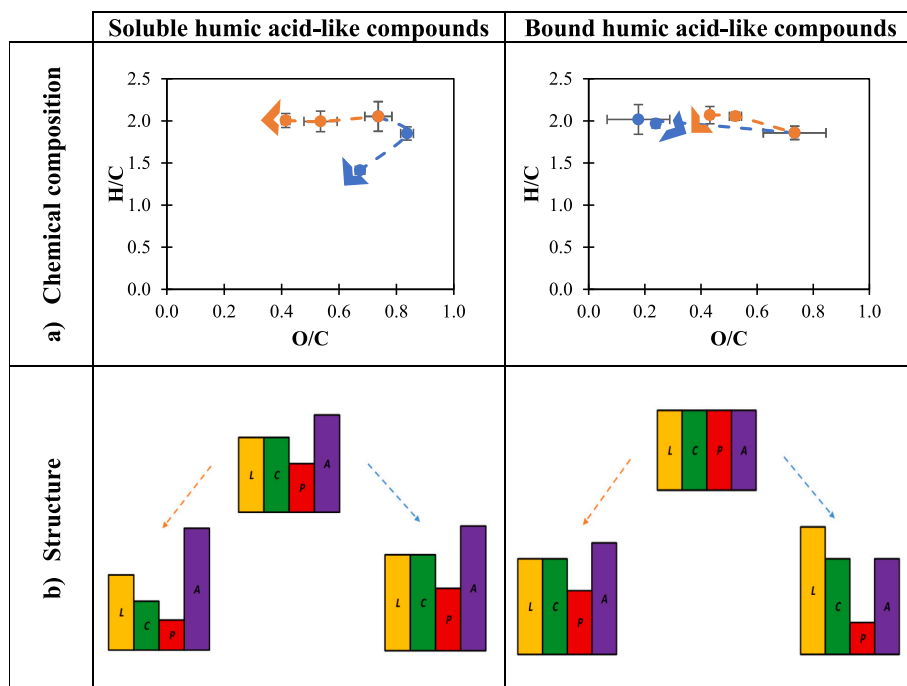


Fig. 6. Evolution of chemical composition (a) and structure (lipid (L), polysaccharide (C), protein (P) and aromatic (A) fractions) (b) of bound and soluble humic acid-like compounds during 2 h of wet oxidation (●) and thermal hydrolysis (●) of sewage sludge at 160 °C and 40 bar.

showed the formation of cyclic aromatic structures, which were also partially oxidised after 2 h of wet oxidation. Regarding the polysaccharide and lipid fractions, they increased throughout the treatment, especially the latter. Finally, an increase in the peak in the 3600–3200 cm^{-1} band (appearance of OH, alcohols and free phenols) [32] was registered, with a maximum after 1 h of treatment as well, and with the disappearance of the 3700 cm^{-1} peak.

3.4. Effect of type of atmosphere

Having commented on the changes observed in the main chemical and structural parameters of the humic acid-like compounds during the different hydrothermal treatments of sewage sludge, the effect of the atmosphere will now be discussed in more detail (Fig. 6).

The evolution of the chemical composition of the different humic acid-like fractions during the hydrothermal treatments under inert or oxidising atmospheres is presented as a Van Krevelen diagram (Fig. 6a). It is interesting to note that both soluble and bound humic acid-like compounds showed significant differences in O/C and H/C atomic ratios as compared to the corresponding values for soil-derived humic acids, regardless of the type of atmosphere or time chosen. In this regard, typical O/C and H/C atomic ratios for soil-derived humic acids range approximately from 0.4 to 0.6 and from 0.7 to 1.5, respectively [38]. For example, leonardite, which is one of the most widely used and well-known humic acids, has an O/C atomic ratio of around 0.43 and a H/C atomic ratio of 0.70 [26,27]. However, both soluble and bound humic acid-like compounds from raw sewage sludge have O/C atomic ratios of 0.74 and 0.73 and H/C atomic ratios of 2.05 and 1.86, respectively, which correspond to the carbohydrate region in the Van Krevelen diagram [39]. These notable differences were also reported by other authors who extracted humic acid-like compounds from sewage sludge. In this line, Hernandez et al. [26] analysed the composition of humic substances obtained from two different air-dried sludges, one aerobic and one anaerobic, and the results showed that their O/C atomic ratios were 0.29 and 0.31 and their H/C atomic ratios were 1.41 and 1.58, respectively. Li et al. [27] obtained bound humic acid-like compounds with O/C and H/C atomic ratios of 0.32 and 1.77, respectively, from a mixture of excess and primary sludges. Another example is Huang et al. [19], whose humic acid-like samples extracted from freeze-dried waste activated sludge had values of 0.61 and 1.92 for the O/C and H/C atomic ratios.

With regard to hydrothermally treated humic acid-like fractions, it can be observed that both types of atmosphere involved a decrease in the O/C atomic ratios, suggesting fewer oxygen-containing functional groups in their structure, which is consistent with the presence of condensation and decarboxylation reactions as previously commented. For the soluble humic acid-like compounds, the decrease in the O/C atomic ratio was less marked in the presence than in the absence of oxygen due to the incorporation of oxygen atoms into their structure by oxidation reactions. This was also accompanied by a reduction in the H/C atomic ratio, which suggests that wet oxidation promoted their aromatic character during the treatment time [19,27]. It was probably related to the faster solubilisation of bound humic acid-like compounds, with a more intense aromatic character than the soluble ones (see Fig. 3), due to the more aggressive conditions caused by the oxidation reactions. Finally, analysing their location on the Van Krevelen diagram, wet oxidised bound humic acid-like compounds showed O/C and H/C atomic ratios typical of lipids, while those of soluble humic acid-like compounds after 1 h of wet oxidation were similar to carbohydrates and, after 2 h, to lignin. On the other hand, under an inert atmosphere, both humic acid-like fractions showed ratios characteristic of proteins [39].

Moving now to the structure (Fig. 6b), it is necessary to bear in mind that the fractions found in both humic acid-like compounds were lipid, protein, polysaccharide and aromatic fractions and that hydrothermal treatments had a significant impact on their evolution. As previously

mentioned, raw soluble humic acid-like compounds had a much higher aromatic character but lower protein fraction than the bound ones, with lipid and polysaccharide fractions having a similar importance in both. During the hydrothermal treatment of sewage sludge, the presence of an inert atmosphere produced an increase in aromaticity in soluble humic acid-like compounds, associated with the reduction in the protein and polysaccharide fractions. On the contrary, under an oxidising atmosphere, the higher solubilisation rate led to an increase in all these structural fractions instead of their oxidation. As for bound humic acid-like compounds, lipid, polysaccharide and aromatic fractions increased markedly during both treatments, although the degradation of aromatic compounds outweighed their formation after 1 h of wet oxidation. Moreover, the presence of oxygen also produced higher degradation of the protein fraction.

Now that the composition and structure of these compounds are known in detail, the effects of their presence in subsequent sludge utilisation processes can be analysed. In view of the results obtained and also according to Huang et al. [19], the increases in aromaticity and C/N atomic ratio produced by hydrothermal treatments are mainly responsible for the inhibition of anaerobic digestion and other fermentative processes. This change in characteristics was more pronounced for bound humic acid-like compounds and after wet oxidation treatment due to the huge increase in C/N atomic ratio (from 5.0 to 18.7 and 11.0 after 2 h of wet oxidation and thermal hydrolysis, respectively). On the other hand, the C/N atomic ratio of the soluble humic acid-like compounds decreased in the presence of oxygen (from 6.8 to 5.4). Apart from this, during a subsequent anaerobic digestion of sludge hydrolysate, the hydrophobicity of the humic acid-like compounds was reported to accelerate the solubilisation of sludge particulate organic matter, acting in the same way as a surfactant, by accelerating the dissolution of organic compounds in sludge and maintaining the activity of hydrolase [17]. In addition, Zhao et al. [31] proposed that quinone groups in the humic acid structure, which are also formed by aromatic condensation, played key roles in accelerating methyl acetyl-CoA degradation and promoting methanogenesis. Moreover, the aromatic condensation of humic acids was positively related to the redox potential of the quinone groups [40]. In this regard, Zhao et al. [31] found that humic acids became inhibitory at redox potentials that were either too high or too low.

Finally, based on the results obtained, a feasible management option would be to extract these compounds in order to improve the biodegradability of hydrothermally treated sewage sludge and, simultaneously, to use these high value-added compounds in different fields depending on their structure and composition.

4. Conclusions

Knowing the composition and structure of the soluble and bound humic acid-like compounds from sewage sludge is important in view of their impact on nature and the interest in hydrothermal treatments. Results revealed that these compounds extracted from sewage sludge differed significantly from those derived from soil. The former showed higher H/C and N/C ratios, but lower O/C ratios, as well as having a more heterogeneous composition than the latter, including not only lipid and aromatic fractions, but also polysaccharide and protein ones. These clear-cut differences, which were attributed to the short maturation period and/or the presence of components from the sludge flocs, suggest that the 'humic acids' term should be used with caution.

Comparing the humic acid-like fractions from the sludge, the soluble ones had a higher aromatic character but a lower protein component in their structure than the bound ones. During the hydrothermal treatments, the protein fraction was significantly reduced at the expense of increased aromaticity and the solubilisation of bound humic acid-like compounds towards soluble ones involved an increase in the lipid and polysaccharide fractions for both. This increment was higher in the presence of oxygen due to the more severe damage to the cell wall,

resulting in greater solubilisation of flocculants. The higher aromaticity, together with the increase in the C/N atomic ratio of the bound humic acid-like fraction, especially after wet oxidation, makes them more inhibitory to biological processes.

In conclusion, the extraction of the humic acids could improve the biodegradability of the hydrothermally treated sewage sludge, but the particular properties of these substances imply that more research is needed on the possible applications of each humic acid-like sample.

CRedit authorship contribution statement

Lucía Pola: Conceptualization, Investigation, Formal analysis, Data curation, Writing – original draft. **María Movila:** Investigation, Formal analysis. **Javier Errero:** Investigation, Formal analysis. **Marta Fuentes:** Investigation, Formal analysis, Data curation, Writing – review & editing. **Sergio Collado:** Conceptualization, Methodology, Writing – review & editing. **Jose Ma Garcia-Mina:** Funding acquisition, Supervision, Methodology, Resources. **Mario Díaz:** Funding acquisition, Supervision, Methodology, Resources.

Declaration of competing interest

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijbiomac.2023.125115>.

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