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Thermal treatment of sewage sludge: impact of the sludge type and origin on the formation of recalcitrant compounds

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Abstract

In a municipal wastewater treatment plant, the thermal treatment of sludge can be an efficient way of increasing the final sludge cake dryness and boosting anaerobic digestion performances. However, such treatments generate refractory compounds which, once returned to headworks, can affect the quality compliance of effluent discharges, particularly concerning organic nitrogen. This study explores the effects of thermal hydrolysis (TH) and hydrothermal carbonization (HTC) of municipal sludge on the refractory organic compound production. A novel approach using ultra-performance liquid chromatography with size-exclusion chromatography and UV/fluorescence detection (UPLC-SEC-UV/Fluo) was employed to characterise recalcitrant dissolved organic matter (rDOM), which typically consists of Maillard reaction products (MRP). Specifically, UPLC-SEC-UV/Fluo was combined with principal component analysis (PCA) to enable a more thorough examination of the chemical diversity of MRPs produced. Greater temperatures during the thermal treatment step lead to increased production of rDOM and rDON. Protein-rich sludge with a great AS:PS ratio yields the greatest

rDOM levels. MRP characteristics, such as molecular weight distribution and aromaticity, are primarily influenced by temperature and plant origin. UPLC-SEC-UV/Fluo provides information on the structures of MRPs useful to optimize the thermal treatment process and in understanding their fate in subsequent processes (chemical oxidation, biodegradation). These insights have practical implications for sludge treatment processes, including optimizing TH and HTC conditions to control rDOM production and adapt the sludge treatment line of a water resource recovery facility.

Abbreviations

WWTP	Waste water treatment plant
HTC	Hydrothermal carbonization
rDOM	Recalcitrant dissolved organic matter
MR	Maillard reaction
MRP	Maillard reaction products
MW	Molecular weight
COD	Chemical oxygen demand
THP	Thermal Hydrolysis Process
TOC	Total organic carbon
DON	Dissolved organic nitrogen
PS	Primary sludge
MS	Mixed sludge
AS	Activated sludge
TKN	Total Kjeldahl nitrogen
DS	Dry sludge
VS	Volatile solids

Highlights

- Greater temperature produces more rDOM and converts more solubilized DOM to rDOM
- Protein-rich sludge (greater AS:PS ratio) leads to greater rDOM production
- MRP characteristics (MW, aromaticity) depends on thermal processes conditions
- Temperature, followed by plant of origin, had the greatest effect on MRP.
- Sludge type (PS or AS) had no impact on the MRP characteristics.

Keywords

- Hydrothermal carbonization
- Sludge thermal hydrolysis
- Recalcitrant dissolved organic matter
- UPLC-SEC-UV/Fluo

1. Introduction

Sewage sludge disposal can account for 20-50% of the operating expenses of a wastewater treatment plant (WWTP) (Yuan and Zhu, 2016). To reduce overall sludge production, sludge pre-treatments and post-treatments can be implemented to improve both anaerobic digestion performance and the dryness of the final sludge cake. These treatments can be of different natures: mechanical, chemical, thermal, or biological (Zhen *et al.*, 2017). Previous studies showed that thermal treatments are the most effective when it comes to increasing the dryness of the final sludge cake (Carrère *et al.*, 2010; Gahlot *et al.*, 2022). These thermal treatments typically consist of thermal hydrolysis (TH), placed upstream or downstream of an anaerobic digestion system (Barber, 2020), and hydrothermal carbonization (HTC) (Wang *et al.*, 2019). The primary energy benefit from thermal treatment technologies lays within the improved dewaterability of the final cake. This reduces downstream transport and processing requirements (Fernández-Polanco *et al.*, 2021). The most common placement of the thermal treatment in a sludge line is before anaerobic digestion. In this configuration, it acts as sludge pretreatment for digestion. Another approach showing promise is that of positioning the thermal treatment unit downstream of anaerobic digestion. The latter aims at maximizing biosolids dewaterability, which is especially attractive to plants incinerating or drying their sludge, while the former aims at increasing the digestion performances (Knötig *et al.*, 2021). Today, more than 100 TH plants are installed worldwide (Devos *et al.*, 2021), while HTC is still in the evolutionary stage with at least 8 industrial-scale plants (Romano *et al.*, 2023). Heating sludge to temperatures of up to 200°C transforms the solid organic matter, reducing the O/C and H/C ratios, thus improving sludge dewatering by increasing their hydrophobicity (Barber, 2016). Conventional mechanical dewatering of heat-treated sludge can then lead to an increase in cake dryness (Wang *et al.*, 2019). At the laboratory scale, up to 70% cake dryness when centrifuging HTC-treated digested sludge can be compared with the 30% dryness obtained without pre-

treatment (Escala *et al.*, 2013). Also, COD-rich liquor from thermal treatments can be biodegraded via anaerobic digestion, resulting in increased gross biogas production compared to untreated sludge (Qiao *et al.*, 2011; Wang *et al.*, 2019).

However, thermal treatments lead to the formation of complex biorefractory dissolved organic matter (rDOM) mainly attributed to Maillard reactions (MR) and called Maillard reaction products (MRP) (Tyagi *et al.*, 2019; Usman *et al.*, 2020). It has been shown that MRP are produced in large quantities and become the predominant refractory compounds in sludge treated by thermal processes (Toutian *et al.*, 2020; Yan *et al.*, 2022). Because they will be returned to the liquid treatment chain, these compounds can affect the quality compliance of a plant's effluent discharges, particularly concerning organic nitrogen. Toutian *et al.* estimated that TH implementation in an existing WWTP could lead to an increase in effluent COD of 2 to 15 mg COD/L (Toutian *et al.*, 2020). The authors estimated that a TKN increase of around 1 mg/L could be expected in WWTP effluent when adding a TH treatment at 165°C (Dwyer *et al.*, 2008). Also, the toxicity and genotoxicity of some MRPs were reported. For example, for certain microorganisms in anammox treatment, the question of their impact on overall biological wastewater treatment processes, or even on environmental discharges, needs to be examined (Cao *et al.*, 2021; Gu *et al.*, 2018). Therefore, MRP production is of crucial importance, and the potential degradation of MRP or minimization of its production by thermal treatments needs to be assessed, and further knowledge on MRP is required.

MRPs have a specific chemical structure that is responsible for their biorefractory nature. The early stages of MR form compounds called Amadori products, resulting from non-enzymatic condensation reactions between reduced sugar and proteins occurring at elevated temperatures (Chandra *et al.*, 2008). The final stages involve polymerization of the previously formed monomers, leading to the formation of MRPs known as melanoidins. The resulting bulk solution of a complex matrix such as sewage sludge can be described by the following

characteristics: highly unsaturated organic matter, greater organic nitrogen content, a wide variety of compounds (between 2,000 and 3,000) and a broad molecular size spectrum with a molecular weight (MW) of up to 40,000 Da (Hao *et al.*, 2020; Lu *et al.*, 2018). Once returned to the WWTP headworks, these molecules are soluble and resistant to biological degradation. MRs have been extensively studied in the food industry, but their formation is not yet fully understood. In the specific case of sewage sludge, little is known about the nature and quantities of MRPs. Few studies have focused on in-depth characterisation of soluble MRPs produced by thermal sludge treatment (Aragón-Briceño *et al.*, 2020; Hao *et al.*, 2020; Merzari *et al.*, 2019; Zhuang *et al.*, 2017). Concerning HTC, no literature describing the effects of sludge type on MRP production was found. This lack of knowledge limits the possibilities for reducing the production of these compounds, so they will end up at the plant outlet.

Most studies have used fluorescence EEM and mass spectrometry to characterise PRMs (Hao *et al.*, 2020; Lu *et al.*, 2018; Usman *et al.*, 2020). Mass spectrometry provides a good description of small structures: aromatics, furans or nitrogenous heterocyclic compounds such as pyrazines, pyrroles, or pyridines were reported (Chen *et al.*, 2019). In any case, mass spectrometry remains a destructive analysis that cannot consider molecules with MW greater than 3000 Da (De la Rubia *et al.*, 2018; Hao *et al.*, 2020). EEM fluorescence has gained interest in recent years for the analysis of complex matrices such as wastewater. EEM fluorescence can provide a significant amount of information for affordable and rapid analysis, such as biodegradability estimates or the presence and semi-quantification of specific molecules and structures (Jimenez *et al.*, 2014; Usman *et al.*, 2020). Here, a UPLC-SEC-UV/Fluo method is used to study MRPs formed by thermally treated sewage sludge (Faixo *et al.*, 2024). This method is non-destructive, allows fractionation of MRPs according to their MW (Dwyer *et al.*, 2008; Gupta *et al.*, 2015), and takes advantage of the fluorescent properties of MRPs (Morales, 2001).

This work aimed to study the effect of thermal treatment and the nature of treated sewage sludge with respect to the quantity and quality of rDOM produced. The nature of treated sludge is studied through the plant origin and the mixture of primary and activated sludges (respectively PS and AS). Samples of PS and AS collected from two WWTP were thermally treated separately or in mixture (50/50 dry mass) using a lab-scale reactor. Beside conventional global parameters such as biodegradable and refractory COD, TOC and DON, UPLC-SEC-UV/Fluo was used in conjunction with PCA analysis to enable a more thorough examination of the chemical diversity of MRPs produced, depending on sludge nature and the treatment temperature. The results help to better understand the chemical transformations occurring during treatment, and to find the best operating conditions to minimize both rDOM and rDON.

2. Material and methods

2.1. Preparation of thermally treated sewage sludge

Thickened PS and AS were collected from two French WWTP (named A and B in this work). Collected sludge was stored at 4°C. Mixed sludge (MS) was obtained by mixing PS and AS at a 50/50 Dry Solids (DS)-based ratio. The characteristics of each raw sludge and liquid phase, before and after thermal treatment are given in Table 2. Sludge samples were then concentrated by centrifugation for 15 min at 7800 g. Thermal treatment was performed in a 3 L cell loaded with 200 g DS that was filled with distilled water to reach 3 L for each batch. The thermal treatments were conducted at 165°C or 200°C for 1 h. Liquid and solid phases of the thermally treated sludge were then separated by centrifugation for 10 min at 7800 g, and both fractions were stored at 4°C. A recent review of Chen *et al.*, 2023 reported that the optimal hydrothermal conditions for AD is a treatment temperature in the range of 160–180 °C and a treatment time in the range of 30–60 min (Chen *et al.*, 2023). This is in line with most of the commercial THPs such as the CambiTHP® are set up at 165°C for 30 min (Sahu *et al.*, 2022), which coincide with the optimal conditions for the Maillard reaction (Ngo *et al.*, 2021). On the other hand, for

deep dewatering using hydrothermal carbonisation (HTC), a moderate temperature (200 °C) and holding time of 30 min were suggested to optimise energy recovery (Zhao *et al.*, 2014).

The formation of recalcitrant compounds was linked to Maillard reaction as the primary causes of the hydrothermal treatment associated problems (Yan *et al.*, 2022). This reaction being time dependant, the present work considers a conservative approach, which aims at maximising the production of refractory products for both temperature treatments.

2.2. Biological degradations

Aerobic biological degradations were carried out in a 1 L reactor. Excess air was injected into the reactor and each biodegradation was inoculated with 0.2 g DS/L of activated sludge. The inoculum was a conventional activated sludge collected from a WWTP with approximatively 40 days of sludge age. Biological degradations lasted for 28 days with regular sampling and COD monitoring. If COD remains stable after 28 days, it is assumed that only biorefractory compounds remain in the samples. pH was measured and maintained between 7 and 8.5 during the degradation process. Aerobic degradation was conducted with the aim of isolating the MRP for characterization purposes. In practice, such liquor would first undergo anaerobic biodegradation. However, it has been demonstrated that refractory components under aerobic conditions remain refractory during anaerobic biodegradation as well (Ekama *et al.*, 2007). Measurements of COD, DON, and TOC for refractory samples are respectively noted as rCOD, rDON, and rTOC. Table 2 presents the obtained rCOD, rDON, and rCOT values for all biodegraded samples, both before and after thermal treatment. The samples taken before treatment can be regarded as controls.

2.3. Samples preparation

Six sludge samples were generated from two WWTP with PS:AS ratios of 1:0, 0.5:0.5, and 0:1. Nine samples were then thermally treated at either 165°C or 200°C (T1 and T2, respectively). Samples of each PS:AS ratio which originated from plant A were thermally treated at

temperatures of 165 and 200°C, and samples which originated from plant B were only treated at temperature T2. All samples then underwent biological degradation, resulting in supernatants with completely refractory COD. These samples are referred to as "refractory samples" in this study.

2.4. Global analysis

All global analyses were carried out on the dissolved fractions. Samples were filtered using 28 mm, 0.45 µm MiniSART filters. Dilutions for global analysis were carried out using water (> 18.2 MΩ.cm) from a PURELAB system (Veolia, United Kingdom). The COD was evaluated using standard methods (5220 A) using Dr. Lange cuvette tests (LCK 114) in conjunction with a Hach spectrophotometer (Hach Lange DR-5000) at a 420 nm wavelength. The TOC of the soluble reaction products was measured with a TOC analyzer (Shimadzu TOC-5050A). TOC was calculated by subtracting inorganic carbon (IC) from total carbon (TC). Total Kjeldahl Nitrogen (TKN) and Ammonium Nitrogen (N-NH₄⁺) were determined using the steam distillation method (Sommers, 1977). DON is calculated by subtracting Ammonium Nitrogen from Total Kjeldahl Nitrogen.

2.5. UPLC-SEC analysis

UPLC-SEC analyses were performed on a Waters Acquity UPLC instrument. The column used was an Acquity UPLC Protein EthyleneBridge Hybrid SEC. The following work entails the use of 1.7 µm ethylene bridged hybrid (BEH) particles (125 Å) packed into a 4.6 mm × 150 mm column provided by Waters Corporation. Phosphate buffer at pH 7.4 and ionic strength 7.5 was used as eluent at a flow rate of 0.3 mL/min. Analyses were performed at 25°C. Analytical quality grade disodium phosphate and sodium dihydrogen phosphate were used. UV absorbance and fluorescence were used as detection methods. The wavelengths used and the specificity of the chemical structures they analyse are detailed in Table 1. Almost all the organic matter is quantified by UV absorbance at 210nm and unsaturated matter was associated with

UV absorbance at 254 nm (Faixo *et al.*, 2021). Both MEL and AH fluorescence can be associated with rDOM originated from MRP. Previous studies have detected fluorescence peaks near these excitation/emission pairs: Dwyer *et al.* observed fluorescence peaks at wavelengths of 340/430 nm and 360/440 nm, respectively, for melanoidins produced from sewage sludge and a glucose:glycine mixture at 165°C (Dwyer *et al.*, 2008).

Table 1 – Detection methods used with UPLC-SEC separation

Wavelength	Specificity	Notation
UV 210 nm	Sp ² bonds in DOM	UV ₂₁₀
UV 254 nm	Conjugated double bonds	UV ₂₅₄
Ex/Em = 330 nm/425 nm	Melanoidin like	MEL
Ex/Em = 390 nm/500 nm	Humic acid like	AH

For every chromatogram, the integration of each peak was carried out with Waters Empower™ Pro 3 Software. The same method was used for each chromatogram integration and took into consideration the peak overlapping. Molecular weights were associated with elution time using a calibration curve, which is a mixture (5 mg. mL⁻¹ each) of phosphorylase b (MW: 97.2 kDa), bovine serum albumin (MW: 66 kDa), albumin from chicken egg (MW: 44.2 kDa), pepsin (MW: 35 kDa), lysozyme from chicken egg (MW: 14.3 kDa), and cytochrome c (MW: 12.3 kDa). The elution profiles were reproduced with a peak area variation under 1% for every peak after 3 injections.

2.6. PCA analysis

PCA was performed in MATLAB R2023b using the Statistics Toolbox V.23.0. PCA was performed by using the transformation of variables to describe the matrix variance. The transformed variables were projected onto different components. From all the components of the correlation matrices, two or three from PC1, PC2, and PC3 were included in the analyses to perform the classification of the samples.

3. Results

3.1. Refractory compounds generation

All the analyses carried out on the different sludge samples are presented in Table 2.

Table 2 – Characterisation of each raw sludge and liquid phase, before and after thermal treatment. Batches contained fixed DM mass of 200g.

DS and VS characterise the raw sludge sampled from the two WWTP. COD_{in} is the total COD at the initial time of thermal treatments. Soluble COD, TOC, TKN, and NH₄⁺ were measured values whereas DON was calculated. COD, DON, and TOC refer to samples that have not undergone biodegradation and rCOD, rDON, and rTOC refers to samples that underwent biodegradation.

	Temperature	DS	VS	COD _{in}	sCOD	TOC	TKN	NH ₄ ⁺	DON	rCOD	rTOC	rDON	rTOC/rDON	rCOD/COD _{in}	rDON/COD _{in}
		g/L	%	g O ₂ /L	g O ₂ /L	g C/L	mg N/L	mg N/L	mg N/L	g O ₂ /L	g C/L	mg N/L	g C/g N	g O ₂ /kg O ₂	g N/kg O ₂
PS A	No thermal treatment	12.5	85	119	0.47	N/A	80	55	25	0.03	N/A	7	N/A	0.00025	0.00006
MS A		16.8	86	98	1.41	N/A	123	97	26	0.07	N/A	40	N/A	0.00071	0.00041
AS A		21.1	87	93	1.49	N/A	108	107	0	0.12	N/A	23	N/A	0.00129	0.00025
PS B		12.1	85	96	0.98	N/A	72	24	48	0.12	N/A	6	N/A	0.00125	0.00006
MS B		13.6	82	89	0.53	N/A	94	49	45	0.04	N/A	15	N/A	0.00045	0.00017
AS B		15.6	80	88	0.13	N/A	85	55	31	0.04	N/A	0	N/A	0.00045	0
PS A	165°C				17.86	5.0	1439	319	1120	1.85	0.6	45	13.3	15.5	0.4
MS A	165°C				28.72	9.0	2632	627	2005	3.98	1.3	241	5.4	40.6	2.5
AS A	165°C				34.78	12.8	3570	871	2699	6.71	2.3	281	8.2	72.2	3.0
PS A	200°C				38.48	12.3	2948	938	2010	8.40	2.9	511	5.7	70.6	4.3
MS A	200°C				44.24	14.3	3920	1198	2722	9.50	3.3	613	5.4	96.9	6.3
AS A	200°C				57.30	18.8	5600	1652	3718	16.00	5.2	980	5.3	172.0	10.5
PS B	200°C				24.90	8.4	2150	647	1504	6.14	2.0	370	5.4	64.0	3.9
MS B	200°C				29.71	10.3	2943	938	2005	8.46	2.5	501	5.0	95.1	5.6
AS B	200°C				38.94	13.6	4115	1285	2829	11.58	3.8	722	5.3	131.6	8.2

3.1.1. Refractory COD production

The comparison of refractory COD (rCOD) before and after thermal treatment indicates that thermal treatment produces almost 98% of the total rCOD (Table 2). Initial rCOD present in the wastewater is negligible.

Fig. 1 shows the quantities of biodegradable COD and rCOD obtained after thermal treatment for each thermally treated sample. Temperature has the greatest impact on refractory compound production. An increase in temperature from 165 to 200°C while keeping the PS:AS ratio constant results in a solubilized COD increase from 40 to 87%. Additionally, a lesser temperature leads to a lesser proportion of COD being converted to rCOD, ranging from 10.4% for PS to 19.3% for AS (Fig. 1).

Fig. 1 shows that rDOM is always produced in great amount for all PS:AS ratios. However, the amount produced varies considerably depending on the PS:AS ratio. At 200°C, PS alone produces 64.2 to 70.5 g rCOD/kg COD_{in} treated for plants A and B, while AS produces 131.8 to 171.5 g rCOD/kg COD_{in}. In comparison with PS, AS treatment leads to a greater COD solubilization and hence to a greater soluble rDOM production. Finally, the comparison for sludges with the same PS:AS ratio treated at the same temperature shows that plant A solubilizes 12 to 38% more total rCOD, but with a similar biodegradable/refractory proportion. The sCOD/COT ratio is between 3.0 and 3.3 g COD/g C, which is a conventional value given that this ratio is 0 for carbon dioxide and 4.66 for ethanol, for example. These values are close to those reported in the literature: 3.3 g COD/g C for digested sludge treated at 165°C (Gupta *et al.*, 2015), and 3.21±0.14 for standard melanoidins produced for different glucose:BSA ratios at 225°C (Hao *et al.*, 2020). The small value range for this ratio demonstrates the reproducibility of reactions occurring irrespective of processing temperature, PS:AS ratio, and sludge origin.

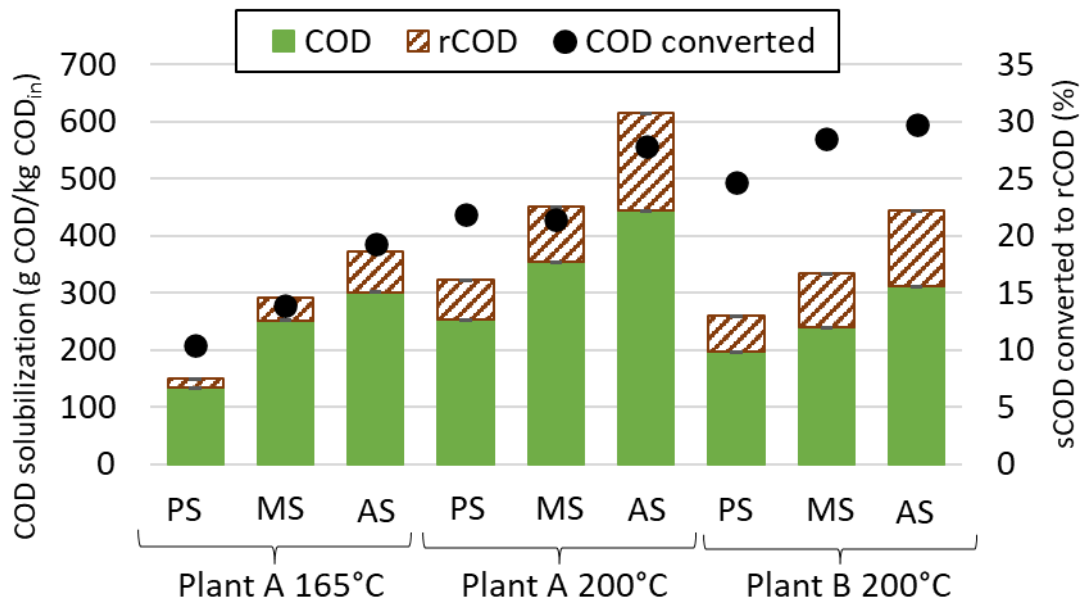


Fig. 1 – Biodegradable and refractory COD produced by thermal treatment of PS, MS, AS from plants A and B at 165 or 200°C.

3.1.2. Refractory nitrogen production

Fig. 2 shows rDON production trends very similar to those observed for rCOD: greater overall organic matter solubilization leads to greater rDON production. AS produces 91-95% more rDON than PS. Similarly, raising the temperature from 165 to 200°C increases rDON production by 3.2 times for PS and 6.7 times for AS. The DON fraction converted to rDON is shown in Figure 2, and is least at 165°C: 5-10%. At 200°C, 23 to 26% of DON is converted to rDON for each PS:AS ratio and/or original plant.

Table 2 shows that the C/N ratio is highly dependent on the thermal treatment temperature: a significant C/N ratio decrease is observed when the temperature is increased from 165 to 200°C. Sludge origin WWTP and PS:AS have little impact on the C/N ratio. Finally, very strong correlations have been observed between rCOD, rDON, and rTOC. A detailed examination of the evolution of rDON and rTOC production in relation to variations in temperature, the plant

of origin, and the PS:AS ratio would not provide any further insights beyond what is already elucidated by the study of rCOD in explaining MRP formation.

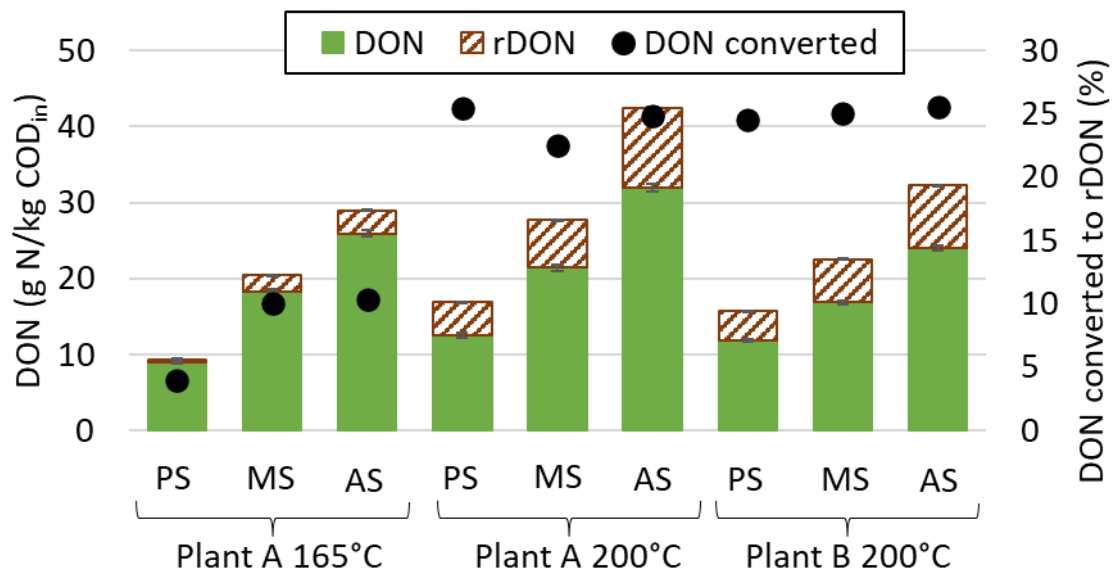


Fig. 2 - Biodegradable and refractory DON produced by thermal treatment of PS, MS, AS from WWTP A and B at 165 or 200°C.

3.2. Characterisation of rDOM

3.2.1. UPLC-SEC-UV/Fluo fractionation

Characterisation of rDOM and rDON in heat-treated effluents using UPLC-SEC with simultaneous UV and fluorescence detection provides a better understanding of the chemical structures of the molecules produced as a function of temperature and sludge type. Fig. 3 shows an example of the standardized elution profiles obtained for AS from STEP B treated at 200°C with two of the four detection methods studied (Table 1). Chromatograms of the nine refractory samples obtained with the four detection methods are available in Fig. A.1. Given the great molecular diversity of the matrices studied, the profiles obtained show good resolution of the method as a function of MW for values ranging from 1 kDa to 200 kDa. To ensure consistency in the chromatogram analyses, these profiles are divided into 4 MW classes, noted from I to IV

for MW > 40 kDa, 20 kDa <MW< 40 kDa, 10 kDa <MW< 20 kDa, and < 10 kDa, respectively. This fractionation, combined with four spectrophotometric detection methods, provides 16 different parameters to describe each sample. Integration data are available in Table S2. To quantify the relative differences between samples, ratios are calculated between all spectrometric parameters. For example, >40kDa_UV210/MEL is the ratio of UV210 fluorescence to MEL fluorescence calculated for MW compounds greater than 40 kDa. A UV210/MEL ratio indicates the relative abundance of MEL fluorescence. A total of 24 ratios are calculated. Based on this information, a statistical analysis can be performed.

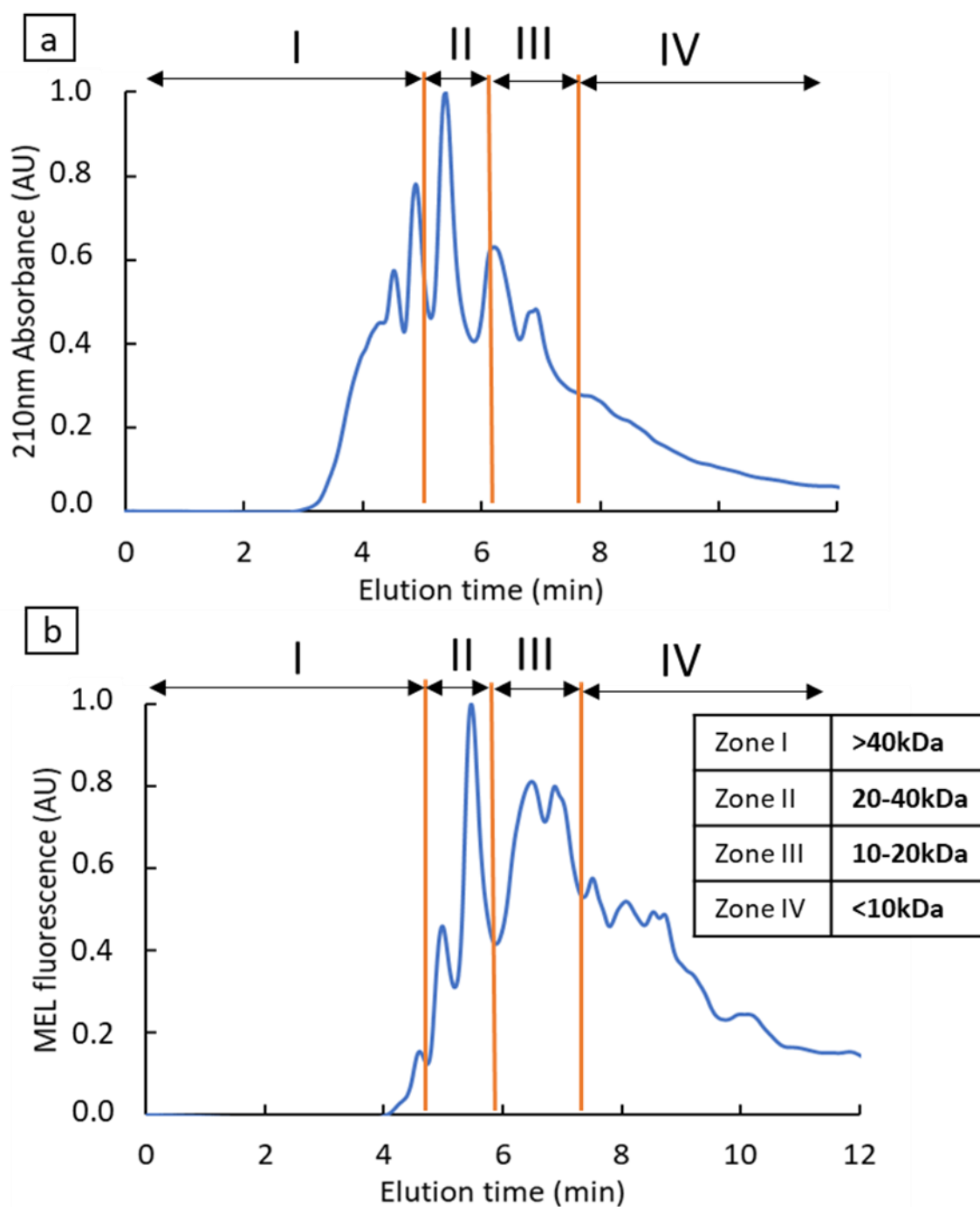


Fig. 3 – Elution profiles obtained by UPLC-SEC-UV/fluor with detection by absorbance at 210nm (a) and MEL fluorescence (b) of AS from plant B treated at 200°C. For representation, chromatograms have been normalized by their greatest intensity value. Details of the UPLC-SEC-UV/Fluo method are described in Materials and Methods.

3.2.2. Statistical analysis

Principal component analysis was used to identify small but significant differences between each chromatogram, shown in Fig. 4 for the nine refractory samples. A separation into three distinct groups is clearly observed. Component 1 contains 66% of the explainable variance and separates the samples according to temperature and WWTP origin. Component 2 contains 30% of the explainable variance and separates samples according to heat treatment temperature. Consequently, the chemical nature of refractory organic compounds appears to be primarily affected by temperature, followed by sludge origin. PCA revealed no separation of samples according to PS:AS ratio. Based on the MW distribution results, aromaticity (UV absorbances) and MEL and AH fluorescence of PRMs, the chemical differences between PS, MS, and AS do not seem sufficient to generate chemical differences after heat treatment. This result is rather surprising, since the sludge nature, assessed by samples taken from two different WWTP, showed a very significant influence on this chemical structure.

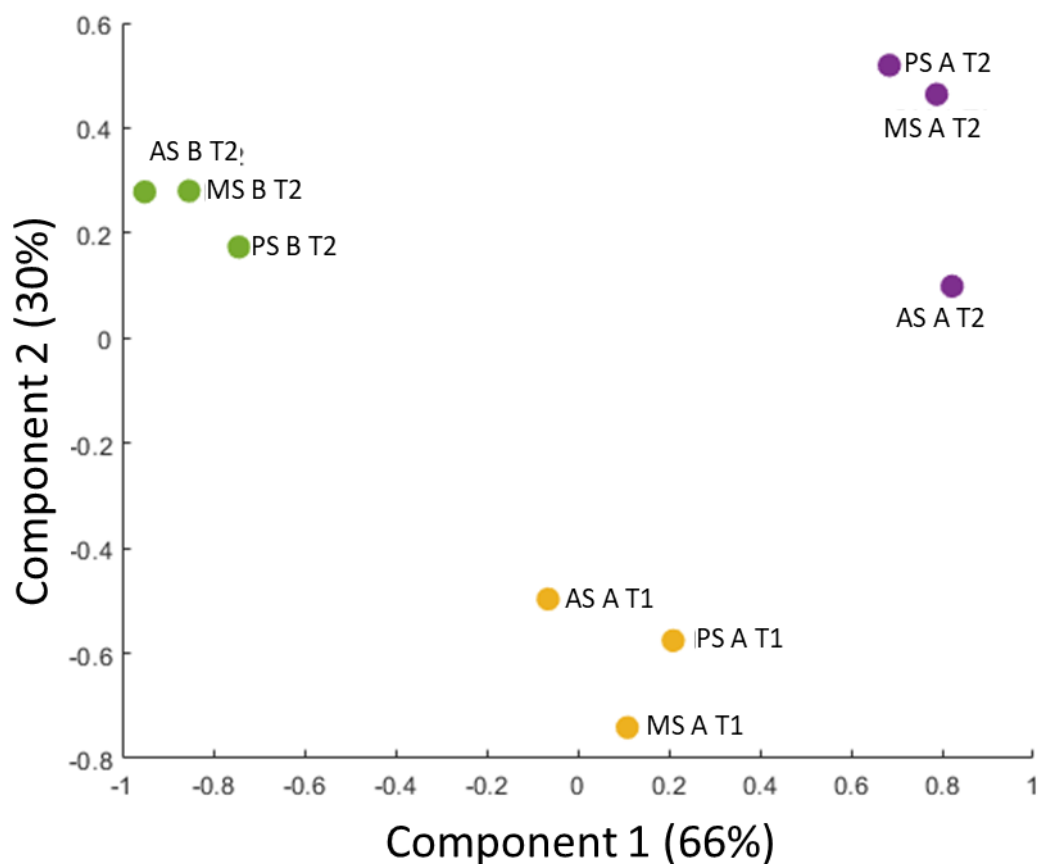


Fig. 4 - PCA based on ratios of integrated area measured by UV or fluorescence

Refractory samples were studied in more detail on the basis of their PCA separation, focusing on their aromaticity and MW distribution. UV210 is used for overall DOM quantification and UV254/UV210 for aromaticity. Fig. 5a shows the MW distribution of refractory samples based on UV210 absorbance and Fig. 5b the UV254/UV210 ratio, which characterises the aromaticity of the different MW fractions. The values represent the average obtained for the three samples coming from the same plant and treated at the same temperature with different PS:AS ratios. As the standard deviation of each result is negligible, the absence of any significant effect of the PS:AS ratio on the MW distribution of MRPs is confirmed. Fig. 5a shows that fractions II and III show no significant difference. This result was obtained independently of heat treatment temperature or sludge origin. Fraction II represents 28% to 31% of the total UV210 absorbance, while fraction III corresponds to 12% to 15% of the latter. Conversely, fractions I and IV are strongly affected by temperature, with an increase in temperature favouring a decrease in the high-MW fraction and an increase in low-MW compounds. Fig. 5b shows that the degree of aromaticity of MRPs remains similar for each MW fraction and each condition tested.

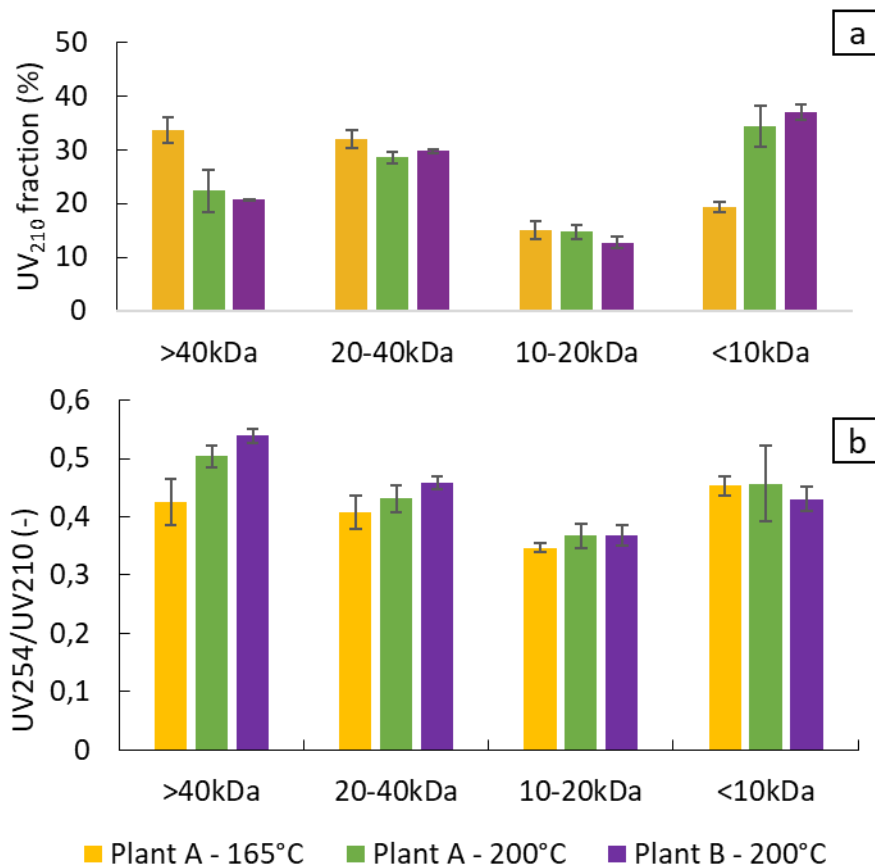


Fig. 5 – UV₂₁₀ total area distribution for each plant and temperature condition relative to molecular weights (a). Each bar represents three samples originating from the same plant and produced from averaged PS, MS, and AS at the same temperature. The associated error bars are the standard deviation.

4. Discussion

4.1. Contribution of the UPLC-SEC-UV/Fluo

Global measurements such as COD, TOC, or DON are important for obtaining a quantitative but non-specific characterisation of DOM and rDOM in a complex matrix. However, these methods provide limited information on the quality of the organic matter studied. It is thus not possible to understand the effect of heat treatment parameters on the formation of MRPs and the chemical nature of these molecules. Consequently, the reduction of MRPs and the selection of a treatment method can prove difficult. In contrast, UPLC-SEC-UV/Fluo enables us to

describe, for a given sample, the MW distribution, the overall DOM amount by UV absorbance at 210 nm, an aromaticity degree (UV at 254 nm), and the specific structures of MRPs by MEL and AH fluorescence. The challenge then lies in data processing and understanding how these features are influenced by processing parameters. By calculating the integrated area ratios for the same MW fraction for different spectrophotometric measurements, a PCA analysis (Fig. 4) enables good discrimination of refractory samples as a function of processing temperature and sludge origin. In addition, the method developed also showed that the effect of sludge type is not significant when dealing with contrasting PS:AS ratios. These results demonstrate the great sensitivity of the method.

The PCA parameters that differentiate the samples were analyzed. In previous research we carried out on MRPs produced by heat treatment at 200°C from glucose/glycine mixtures in varying molar ratios, correlations between spectrophotometric ratios that differentiate refractory samples and overall parameters were identified (Faixo et al., 2024). In particular, nitrogen-containing rDOM is detected by MEL and AH fluorescence and could be correlated with UV210/MEL and UV210/AH ratios: these ratios decrease when nitrogen-containing MRPs are relatively more concentrated and the C/N ratio decreases. Similarly, the present study focuses on establishing correlations between these different parameters. Fig. 6 shows the PCA parameters that differentiate the samples. Although a very good sample separation is obtained, the different ratios presented in this figure are difficult to analyze and there is no obvious correlation. This is certainly due to the complexity of the actual matrices and the limited sample number available. To obtain more accurate results, a very large-scale statistical study, with additional samples from various WWTP and treated at different processing temperatures, would be necessary.

The ratios in the upper right quadrant of Fig. 6 correspond to compounds with MW between 10 and 20 kDa and relatively low AH fluorescence. Ratios in the upper left quadrant also

correspond to compounds with relatively low MEL and AH fluorescence, but MW above 40 kDa. These groups relate to PRMs produced at 200°C and to ratios showing great component 2 values. In contrast, compounds formed at 165°C are associated with lesser component 2 values and more diverse MWs and ratios. This confirms that greater temperature favors a relative decrease in high MW compounds and an increase in low MW compounds.

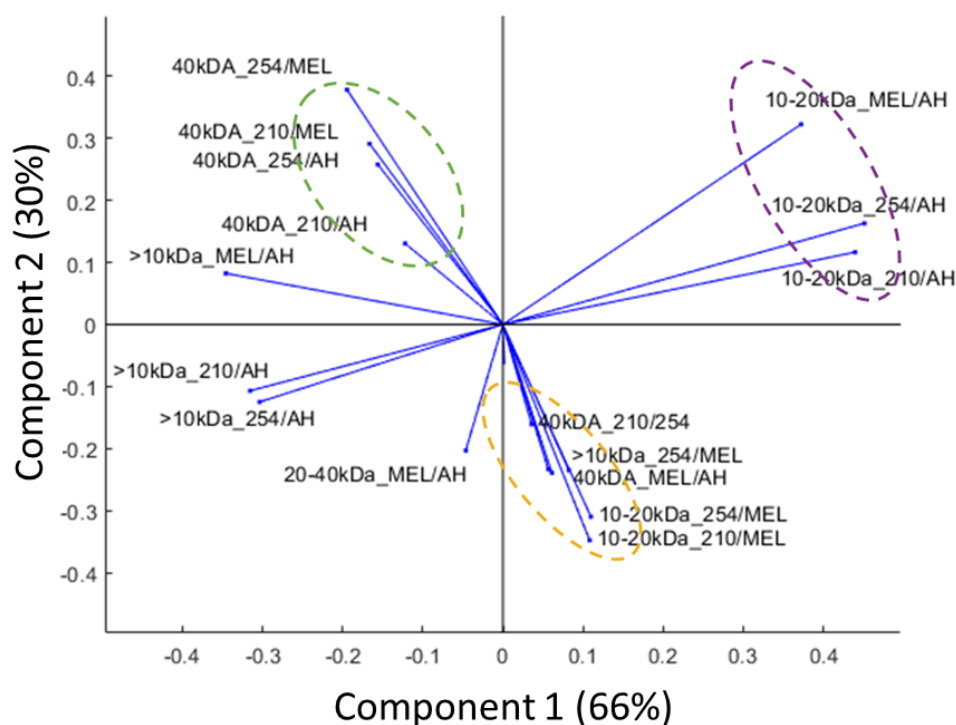


Fig. 6 – Biplot of PCA parameters shown in Fig. 4

4.2. Effect of temperature

Temperature had the greatest effect on the chemical structure and quantity of MRP produced for the parameters tested. The greater the temperature, the greater the solubilized DOM amount and the more refractory the solubilized organic matter. Furthermore, the MW distribution shifts towards lesser values as temperature increases. These observations can be studied by considering the successive reactions involved in the generation of MRPs and the differences in the reaction activation energies (Van Boekel, 2001). If, for simplicity's sake, we consider that two successive reactions represent the formation of MRPs, the first MR step forms monomers and the second step corresponds to polymerization forming greater MW MRPs. Increasing

temperature increases all reaction rates, resulting in greater production of all MRP types, whether low or very high MW. This is confirmed by the overall increase in the UPLC-SEC-UV/Fluo signal for each MW class, irrespective of the detection method used, the PS:AS ratio or the sludge origin (Fig. A.1). Furthermore, each reaction has its own kinetics and activation energy. The relative decrease in high molecular weight compounds observed in our results when the treatment temperature is increased would then correspond to a relatively lesser reactivity of the polymerization processes involved in the second step. This hypothesis is supported by the results of Martins and Van Boekel, who studied the transformation of a glucose/glycine mixture during heat treatment at temperatures between 80°C and 120°C (Martins and Van Boekel, 2005). The authors observed a more rapid formation of a low MW compound, N-(1-deoxy-D-fructos-1-yl)-glycine, in the first stage, as the temperature increased. This also helps to explain the more balanced MW distribution observed for MRPs produced at 200°C compared with 165°C. Indeed, with a limiting reaction time, the products of the initial reactions don't have time to be completely polymerized.

In addition, the increase in the reaction rate of early MR steps with increasing temperature also results in greater conversion of DON to rDON, irrespective of the PS:AS ratio (Fig. 2). These early stages involve the condensation reaction between dissolved proteins and reducing sugars. Thus, an increase in the reaction rate of the early MR steps would lead to an increase in protein uptake, and consequently in the DON amount used in MR. Similarly, protein uptake increased more than reducing sugars when the temperature was increased from 180°C to 200°C during thermal treatment of food waste (Wang *et al.*, 2018).

rDON increase may also be linked to a reduction in solid residue when the treatment temperature is fractionally greater (Román *et al.*, 2018). For example, Zhuang *et al.* observed

that an increase from 150 to 210°C results in a 2.5-1.6% decrease in nitrogen in the solid phase, recovered in the liquid phase (Zhuang *et al.*, 2017).

From the standpoint of reducing refractory compound production, it is clear that a lesser temperature is very favorable. However, 180°C seems to be the lower limit for maintaining the advantage of better sludge dewatering. Therefore, future research should focus on determining the temperature value that offers the best compromise between good sludge dewatering and refractory material production.

4.3. Effect of sludge type and origin

The effect of sludge type on refractory molecule production can be studied both quantitatively and qualitatively. Increasing the PS:AS ratio resulted in more DOM solubilization. Sludge origin also had a significant effect on solubilization. As observed with the effect of increasing temperature, the greater the amount of DOM solubilized, the greater the amount of rDOM and rDON produced. Our results confirm that the production of refractory compounds is influenced by the sludge chemical composition and probably by its protein content, since proteins are a necessary MR reagent.

Sludge biochemical properties are difficult to assess, as the determination of proteins, sugars, and lipids is subject to numerous strong interferences (Felz *et al.*, 2019). Therefore, the PS:AS ratio was chosen to represent the variation in the protein amount in sludge fed by a thermal process. Compared to PS, AS contains a greater quantity of microbial cells and therefore a greater protein concentration (Barber, 2020). Assuming that proteins are the limiting reagent in MR, it is logical that more COD is converted to rDOM and rDON for sludge enriched with AS. Similarly, in terms of sludge origin, WWTP B sludge achieved a slightly greater COD conversion (2-3%) to rCOD than WWTP A sludge. This difference could be due to the plant's organic load, with WWTP B operating at lesser load while WWTP A operates at large load.

Protein content could perhaps explain these differences, but further studies need to be carried out on several WWTP before any conclusions can be drawn on this point.

Finally, proteins could be a limiting reagent for MR in the case of sewage sludge. This can be corroborated by the constant rTOC/rDON ratio of 5.3 g C/g N measured for refractory compounds. A constant ratio indicates that all the DON available for MR is consumed to produce MRP. The effect of the limiting role of proteins (or amino acids) has also been reported, such as by Ortega-Martinez *et al.* and Usman *et al.*, who measured the production of refractory compounds for different glucose/glycine or cellulose/BSA ratios respectively (Ortega-Martínez *et al.*, 2021; Usman *et al.*, 2020).

If we look at the qualitative aspect of the molecules formed, our results showed that UPLC-SEC-UV/Fluo results, combined with PCA, do not allow us to distinguish MRPs produced on the basis of PS:AS ratio or plant origin. It therefore seems that while the rDOM quantities produced are affected by the sludge nature, the chemical structure measured by our UPLC-SEC-UV/Fluo method does not appear to be altered.

4.4. Practical implications

rCOD and rDON presented in this study are significantly greater than those reported in the literature. Indeed, Oosterhuis *et al.* measured the inert COD fraction in an anaerobic digester fed with thermally hydrolysed waste activated sludge (Oosterhuis *et al.*, 2014). Measurements were carried out on the anaerobic liquor obtained following the addition of poly-electrolyte and dewatering. The authors reported a figure of 11 kg rCOD /tDS as refractory material. Considering a 70% VS content and assuming a COD:VS ratio for activated sludge of 1.5, this figure is approximately 15.7 kg rCOD /tVS or 1.05% rCOD/COD_{in} (i.e. 1% of the COD flowrate processed through the THP).

Similar results were obtained by Kjørlaug *et al.* who studied the degradability of Post AD thermal hydrolysis centrate in a UASB and a CSTR (Kjørlaug *et al.*, 2017). Based on a measured 90% degradation of soluble COD, the refractory material production from this work varies between 1.1% and 5.8% rCOD/COD_{in}.

Svennevik *et al.* quantified both the soluble colloidal COD and DON of the digestate from seven full-scale THP facilities (Svennevik *et al.*, 2020). These compounds are associated with recalcitrant compounds. Based on the reported VSR for each site, the refractory material production from this work varies between 2.9% and 6.4% rCOD/COD_{in}, with an average value of 4.2% rCOD/COD_{in}. This was calculated assuming a VS content of 75%DS for the fresh sludge as well as a COD:VS ratio of 1.5 and 1.7 for waste activated sludge and primary sludge respectively. Similarly, assuming the DON measured in this study is entirely associated with recalcitrant compounds, rDON production varies between 0.71% and 1.04% rDON/COD_{in}, with an average value of 0.90% rDON/COD_{in}.

Similarly, Toutian *et al.* evaluated the effect of thermal hydrolysis temperature on WAS biogas yield and on the following aerobic biodegradability of its centrate in a series of batch tests (Toutian *et al.*, 2020). The authors defined a parameter named “conversion factor” (CF) as the rCOD amount remaining in the digestate liquor after aerobic degradation based on the total VS amount in WAS fed to the AD system. At 160°C, the authors reported a CF varying between 4.09% and 5.80% rCOD/VS_{in}. Based on the measured COD:VS ratio of 1.66, this leads to a rCOD production ranging between 2.47% and 3.50% rCOD/COD_{in}. When the temperature is increased to 170°C, the rCOD does not exceed 7.25% rCOD/COD_{in}.

Three possible explanations for the above-mentioned discrepancy with the values measured in this study (Table 2) can be proposed.

First, all the above-mentioned studies were carried out at 165°C and 30 minutes reaction time, whereas the present study was based on a one-hour reaction time. As previously reported, a

longer reaction time results in an increase in soluble refractory materials and inert compounds (Ngo *et al.*, 2023, 2021). The reaction time, along with thermal treatment temperature, is thus generally accepted as one of the levers for minimising the formation of refractory and colour compounds, which are produced during the MR (Yan *et al.*, 2022). Indeed, even if more efforts are required to elucidate the extent of the latter in sludge thermal treatment, reaction time was important in High Molecular Weight (>10 kDa) accumulation, which are associated with refractory compounds (Zhang *et al.*, 2020).

Second, besides the reaction time impact, biomass adaptation in terms of biodegradation capacity is an explanation for the differences observed with the aforementioned refractory compound figures. Indeed, the biomass used in the Zahn-Wellens tests of this study was not adapted to thermally treated sludge. Previous studies demonstrated a reduction in various parameters associated with refractory components following anaerobic digestion, with adapted biomass (Higgins *et al.*, 2017). In a recent study, Mailler *et al.* also reported the acclimatization capacity of a biomass treating a filtrate produced from a sludge hydrothermal carbonisation process located at the Seine Aval WWTP (Achères, France) treating up to 1500 MLD (Mailler *et al.*, 2023). The HTC unit serves as an ultra-dewatering process, in which sludge is thermally treated at 195°C (20 bar) for 45 min and then dewatered using filter presses. The authors analysed 7 years of operational data of the membrane bioreactor (Biosep® process) treating the filtrate produced by the HTC and observed up to 89.5% and 95.7% of COD and TKN removal rates respectively. The full-scale and stable COD and nitrogen removal rates could be explained by the acclimatization of the aerobic biomass to a diluted feeding compared to the batch aerobic Zahn-Wellens tests conducted in this study.

Finally, refractory compounds adsorption on the biomass is another lever for reducing the refractory content at the plant discharge. This was demonstrated for Pharmaceutically Active Compounds (PhACs), which fail to be efficiently mineralized in a traditional wastewater

treatment process (Cui *et al.*, 2022). The authors showed that much PhACs is adsorbed on WAS flocs due to their refractory and hydrophobic properties. For instance, when assessing the fate of 2,4,4'-trichloro, 2'-hydroxy-phenylether (Triclosan) in a German sewage treatment plant, Bester demonstrated that 30% of the triclosan was adsorbed to the sludge (Bester, 2003).

Typically, the returned liquor from the thermally treated and digested sludge is recycled to activated sludge tanks. The latter part of the refractory compounds can still be removed by adsorption to the activated sludge or by biodegradation of the adapted biomass. Therefore, the figures reported in the present study are considered as the worst-case scenario rather than design parameters.

5. Conclusion

The refractory compound production was studied for municipal sludge subjected to different thermal treatments, commonly known as thermal hydrolysis (165°C) and hydrothermal carbonisation (200°C). The influence of the treatment temperature, the origin and the nature of the sludge, was assessed based on nine refractory samples containing MRP. The origin and nature of the sludge was represented by varying the PS to AS ratio (1:0, 0.5:0.5 and 0:1) for sludges sampled from two different WWTP. The latter mainly differed in terms of the organic load received. The following conclusions were drawn:

- Increasing the temperature from 165 to 200°C increases rCOD and rDON production by 237-454% and 254-349% respectively.
- Decreasing the PS:AS ratio from 1:0 to 0:1 increases rCOD and rDON production by 188-367% and 191-195% respectively.
- The chemical nature of refractory organic compounds (MW distribution, aromaticity) following a thermal treatment is primarily affected by temperature, followed by the plant origin. It is not impacted by the PS:AS ratio in the case of our study, but this aspect should be further studied using a greater sludge number and PS:AS ratios.

- Lesser thermal treatment temperature resulted in a greater proportion of very high MW refractory compounds (>40 kDa), while a greater temperature led to a greater fraction of lower MW refractory compounds (<10 kDa).
- Temperature optimum between MRP production and dewatering improvement need to be further investigated
- DON may be the limiting MRP reactant, and their optimal management may reduce rCOD and rDON production
- UPLC-SEC-UV/Fluo provides information on the structures of MRPs, which can be useful in understanding their fate in subsequent processes (chemical oxidation, biodegradation).
- Protein amino acids are probably the limiting reagent for refractory molecule the production.

This study demonstrates that temperature and sludge composition are critical parameters to consider. Evaluating various sludge types in a broader campaign could provide a more holistic approach, potentially enhancing the ability to predict rDOM production in specific cases. However, when the final cake dryness dictates the latter, a dedicated treatment of the rDOM produced becomes necessary, mainly to reduce the nitrogen compounds. In which case, the present UPLC-SEC-UV/Fluo characterisation method can provide valuable information in this context.

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