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# Characterization of soluble and bound EPS obtained from 2 submerged membrane bioreactors by 3D-EEM and HPSEC

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## ABSTRACT

This research study deals with the quantification and characterization of the EPS obtained from two 25 L bench scale membrane bioreactors (MBRs) with micro-(MF-MBR) and ultrafiltration (UF-MBR) submerged membranes. Both reactors were fed with synthetic water and operated for 168 days without sludge extraction, increasing their mixed liquor suspended solid (MLSS) concentration during the experimentation time.

The characterization of soluble EPS ($EPS_s$) was achieved by the centrifugation of mixed liquor and bound EPS ($EPS_b$) by extraction using a cationic resin exchange (CER). EPS characterization was carried out by applying the 3-dimensional excitation–emission matrix fluorescence spectroscopy (3D-EEM) and high-performance size exclusion chromatography (HPSEC) with the aim of obtaining structural and functional information thereof.

With regard to the 3D-EEM analysis, fluorescence spectra of $EPS_b$ and $EPS_s$ showed 2 peaks in both MBRs at all the MLSS concentrations studied. The peaks obtained for $EPS_b$ were associated to soluble microbial by-product-like (predominantly protein-derived compounds) and to aromatic protein. For $EPS_{s,}$ the peaks were associated with humic and fulvic acids. In both MBRs, the fluorescence intensity (FI) of the peaks increased as MLSS and protein concentrations increased. The FI of the $EPS_s$ peaks was much lower than for $EPS_b$. It was verified that the evolution of the FI clearly depends on the concentration of protein and humic acids for $EPS_b$ and $EPS_s$, respectively.

Chromatographic analysis showed that the intensity of the $EPS_b$ peak increased while the concentrations of MLSS did. Additionally, the mean MW calculated was always higher the higher the MLSS concentrations in the reactors. MW was higher for the MF-MBR than for the UF-MBR for the same MLSS concentrations demonstrating that the filtration carried out with a UF membrane lead to retentions of lower MW particles.

## 1. Introduction

Membrane bioreactors (MBR) are being used more and more for wastewater treatment in order to produce high quality water for recycling. It is a better alternative to Conventional Activated Sludge plants (CAS) for municipal and industrial wastewater treatment because it offers advantages such as the high retention of mixed liquor suspended solids (MLSS) which means better treated water quality, higher biomass concentration, small footprint, good stability and easier control/operation than CAS [1]. One of the most important advantages is that MBRs replace secondary clarification by membrane filtration modules placed in the biological reactor. However, membrane fouling, which causes a decrease in permeate flux and an increase in operation costs [1,2], is the major obstacle for the widespread application of this technology.

Different parameters can impact on fouling propensity, for example sludge retention time (SRT) and MLSS concentrations, which has considerable effects on several sludge properties such as bound and soluble extracellular polymeric substances (EPS) content, floc size, viscosity, settling characteristics, etc. [3,4]. These bulk characteristics change according to MBR operational conditions, making the study of membrane fouling very difficult, especially when the system operates under prolonged or infinite SRT with little or no sludge discharge (as is the case in the present paper), as a consequence of the variation of MLSS concentrations and the possible change in microbial population. Many studies have found that one of the most important causes of membrane fouling is the presence of large amounts of extracellular polymeric substances (EPS) [5,6]. EPS are a complex high molecular-weight mixture of polymers excreted by microorganisms, produced from cell lysis and hydrolysis, and adsorbed organic matter from

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wastewater. EPS keep the microorganisms together in a three-dimensional gel-like hydrated matrix and are made up of different types of macromolecules such as proteins, carbohydrates, humic, fulvic, nucleic acids, etc. [7]. Thus far, most research has focused on the correlation of total EPS concentration and membrane fouling [5,6], but little attention has been given to the characterization of EPS under specific operational regimes.

Therefore, a detailed study of the structural and functional properties of EPS and their environmental behaviour is required. This is done applying numerous innovative analytical techniques such as 3-dimensional excitation–emission matrix fluorescence spectroscopy (3D-EEM) and high-performance size exclusion chromatography (HPSEC). The 3D-EEM is a sensitive and selective method which requires only very small samples and does not destroy their structure. This technique can be used to elucidate the functional groups and element compositions in EPS or microbial aggregates. EPS contain large quantities of aromatic structures and unsaturated fatty chains with various types of functional groups, which have fluorescent characteristics. As a rapid, selective and sensitive technique, 3D-EEM fluorescent spectroscopy is useful for studying the physico-chemical properties of EPS, since fluorescent characteristics are largely related to their structure and functional groups in molecules [8,9]. HPSEC can be used to qualitatively and quantitatively analyse EPS composition, and specifically to characterize the molecular weight (MW) distribution of EPS [10,11].

Nevertheless, the characteristics of sludge in conventional treatment systems differ from those in the MBR system, and most research into the characterization of EPS using these techniques uses samples from sources other than MBR technology [12–15]. Few studies into the characterisation of EPS using aerobic MBR technology and 3D-EEM and HPSEC have been found [11,16–19]. Gorner et al. [11] combined EEM spectroscopy and HPSEC when working with a MBR. Infrared results revealed the presence of one type of polysaccharide and two types of proteins. With respect to the analysis of HPSEC, a MW interval of between 45 and 670 kDa after extracting the protein from EPS using a CER was obtained. Ramesh et al. [17] characterized soluble and bound EPS from different types of sludge. For sludge digested aerobically in a pilot MBR, 3 peaks associated to soluble microbial by-product-like (region IV according to Chen et al., [12]) were found for bound EPS. Humic substances (region V) in the fraction of bound EPS was also present, although with lower concentrations than in soluble EPS fraction. Moreover, the fluorescence intensity (FI) of soluble EPS was proven to be less than for bound EPS. Jefferson et al. [16] studied the MW distribution of bound and soluble EPS at different MLSS concentrations in a MBR and found that the chromatograms related to $EPS_b$ were more uniform than for soluble EPS, which showed very different and unclear trends.

This work focuses on the characterization of the soluble ($EPS_s$) and bound EPS ($EPS_b$) obtained from 2 submerged MBRs (using different membranes) working in parallel at high sludge ages for more than 5 months. EPS obtained at different MLSS concentrations were analysed by means of the 2 innovative analytical techniques previously mentioned: 3D-EEM and HPSEC.

## 2. Materials and methods

### 2.1. Experimental setup

Two 25 L bench scale membrane bioreactors (MBRs) with micro-(MF-MBR) and ultrafiltration (UF-MBR) submerged membranes were constructed and fed with synthetic water with an average chemical oxygen demand (COD) of 650 mg/L approx. Peptone and meat extract were used as carbon sources with concentrations of 320 and 220 mg/L, respectively; urea (60 mg/L) and $K_2HPO_4$ (130 mg/L) were used as nitrogen and phosphorous sources, respectively; other salts were also added: $MgSO_4 \cdot 7H_2O$ (4 mg/L), $CaCl_2 \cdot 2H_2O$ (8 mg/L) and NaCl (14 mg/L).

The average pore size of the MF (Kubota) and UF (Toray) membranes was 0.4 and 0.08 μm, respectively. Both membranes had the same filtration area: 0.116 $m^2$.

Permeate was extracted using a pump (negative pressure) up to 0.4 bar, while the biomass rejected by the membranes was returned to the reactor. The permeate flowrate was increased from 9 to 22 LMH, and the hydraulic retention time (HRT) decreased from 25 to 10 h. In order to diminish membrane fouling, maintain the suspended biomass and provide oxygen to microorganisms, a constant air flow was pumped into each reactor.

Moreover, both MBRs worked with a filtration/relaxation cycle of 8/2 min to remove reversible membrane fouling. The systems were operated for 168 days without sludge extraction (with the exception of the sludge taken out for analysis). The plant was automatically controlled by means of a Programmable Logic Controller (PLC) in order to constantly control.

Different chemical protocols for membrane cleaning were used in the experimentation: maintenance cleaning with sodium hypochlorite (500 mg/L) to remove organic fouling and intensive cleaning with sodium hypochlorite (5000 g/L) and oxalic acid (1000 mg/L) to remove inorganic fouling.

A detailed scheme of the MBR system can be found in Domínguez et al. [20].

### 2.2. Conventional analytical methods

Mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) were analysed twice a week according to *Standard Methods for the Examination of Water and Waste Water* [21].

A pH-meter Crison (model Basic 20+) and a conductimeter Crison (model CM 35) were used to determine pH and conductivity, respectively, of the mixed liquor during the whole experiment. There were not found significant differences between the 2 reactors neither among the obtained values during the time. For MF-MBR, the mean value (value ± standard deviation) of pH and conductivity was 7.1 ± 0.4 and 1.5 ± 0.08 mS/cm, respectively; for UF-MBR, was 6.8 ± 0.5 and 1.5 ± 0.06 mS/cm, respectively.

### 2.3. EPS determination

EPS was determined according to Domínguez et al. [22] and mixed liquor for EPS determination was collected twice a week throughout the experimentation time. Soluble EPS were obtained by centrifugation using a SIGMA 4K10 centrifuge at 4000*g* for 15 min at 4 °C, in order to separate the supernatant EPS from the solid. Bound EPS were extracted using a cationic exchange resin (CER) Dowex 50 × 8, 20–50 mesh in the sodium form (Fluka ref 44445). The sludge and the CER were stirred for 16 h at 900 rpm, and the amount of CER used was 70 g CER/g VSS [10]. After that, separation of bound EPS from treated sludges was carried out by centrifugation (4000*g*, 15 min, 4 °C).

EPS determination was performed measuring the concentration of proteins, carbohydrates and humic acids by means of colorimetric methods. “Total EPS” is referred to the sum of the three compounds. For the determination of absorbance a spectrophotometer Shimadzu (UV-1601) was used. To determine the carbohydrate content, the phenol–sulphuric acid method of Dubois et al. [23] was followed, using Phenol (80%) and sulphuric acid (96%) from Merck Suprapur. Glucose (Panreac Química S.A.) was employed as standard. A Total Protein Kit (Sigma-Aldrich, TP 0330) based on Lowry's description [24], but modified by Peterson [25] was used to determine the protein content. The Kit includes a standard solution. Humic acids were measured according to Frolund et al. [10]. Humic substances from Sigma Aldrich were used as standard.

### 2.4. EPS analysis using three-dimensional excitation–emission matrix (3D-EEM) fluorescence spectroscopy

EEM spectra of soluble and bound EPS fractions collected at different MLSS concentrations were measured using a luminescence spectrometer (Jasco FP-6500). The excitation source was a 150 W Xenon lamp. All the samples were filtered through 0.45 μm filters (Millipore) before being analysed. The EEM spectra were collected with the scanning emission (Em) spectra from 220 to 460 nm at 2 nm increases by varying the excitation wavelength (Ex) from 220 to 375 nm at 5 nm sampling intervals. A new "excision, interpolation" technique was used to eliminate Rayleigh and Raman scattering peaks [26]. Windows-based software Sigma-Plot 2001 (SPSS Inc.) was used for plotting the colour maps. The detailed procedure of a sample analysis by 3D-EEM can be found in Marhuenda-Egea et al. [13].

### 2.5. EPS analysis using high pressure size-exclusion chromatography (HPSEC)

The different extracts obtained from the soluble and bound EPS fractions were analysed. All the samples were filtered through 0.45 μm filters (Millipore) before being injected into the chromatograph. For HPSEC analysis, an Agilent Technologies 1100 chromatograph equipped with a G1311A pump, a G1314A UV detector, a G1322A degasser system and a Rheodyne injector with a sample loop of 2 mL were used. Chemstations software from Agilent Inc. was used for the equipment control and for acquisition and data analysis. HPSEC analysis was carried out using a borosilicate glass column (Omnifit®) 2.5 cm in internal diameter and 25 cm long. The column was packed with HW-55S Toyopearl resin (TOSOH) and calibrated using the Kit MW-GF-1000 for protein standards (Sigma-Aldrich) of specific molecular weight, ranging between 29 and 669 kDa. Blue Dextran was used for the void volume determination of the column. The mobile phase consisted of 2.5 mM $Na_2HPO_4$, 2.5 mM $NaH_2PO_4$ and 20 mM NaCl. All measurements were taken using a mobile phase flow of 2 mL/min at room temperature (≈20 °C) and the detector wavelength was 280 nm.

## 3. Results and discussion

### 3.1. EPS characterization by EEM fluorescence spectroscopy with respect to MLSS concentrations

#### 3.1.1. Bound EPS

The analytical technique of 3-dimensional excitation (Ex)–emission (Em) matrix fluorescence spectroscopy (3D-EEM) was used to characterize the fractions of soluble and bound EPS extracted at different MLSS concentrations. Fig. 1 shows the fluorescence spectra relative to $EPS_b$ in both MBRs. In the case of the MF-MBR, measurements were taken at 8 MLSS concentrations, although in the figure only the lowest ones are shown in order to compare them with those determined in the UF-MBR.

The fluorescence spectra in the MF-MBR showed 2 clearly defined peaks for all the MLSS concentrations studied (more evident at higher MLSS concentrations). Peak A is characterized by an emission/absorbance of energy at an Ex/Em wavelength pair of 270/366–376, and the second peak (Peak B) at 230/298–302. According to the classification diagram of Chen et al. [12], peak A is located in region IV and is associated with soluble microbial by-product-like, predominantly protein-derived compounds. The resulting fluorescence is mainly produced by the aminoacid tryptophan. Peak B is located in region I and is associated with aromatic protein, mainly tyrosine.

In the UF-MBR, similar peak locations were found: peak A at 270/368–374 and peak B at 230/298–304 nm. In this case, the

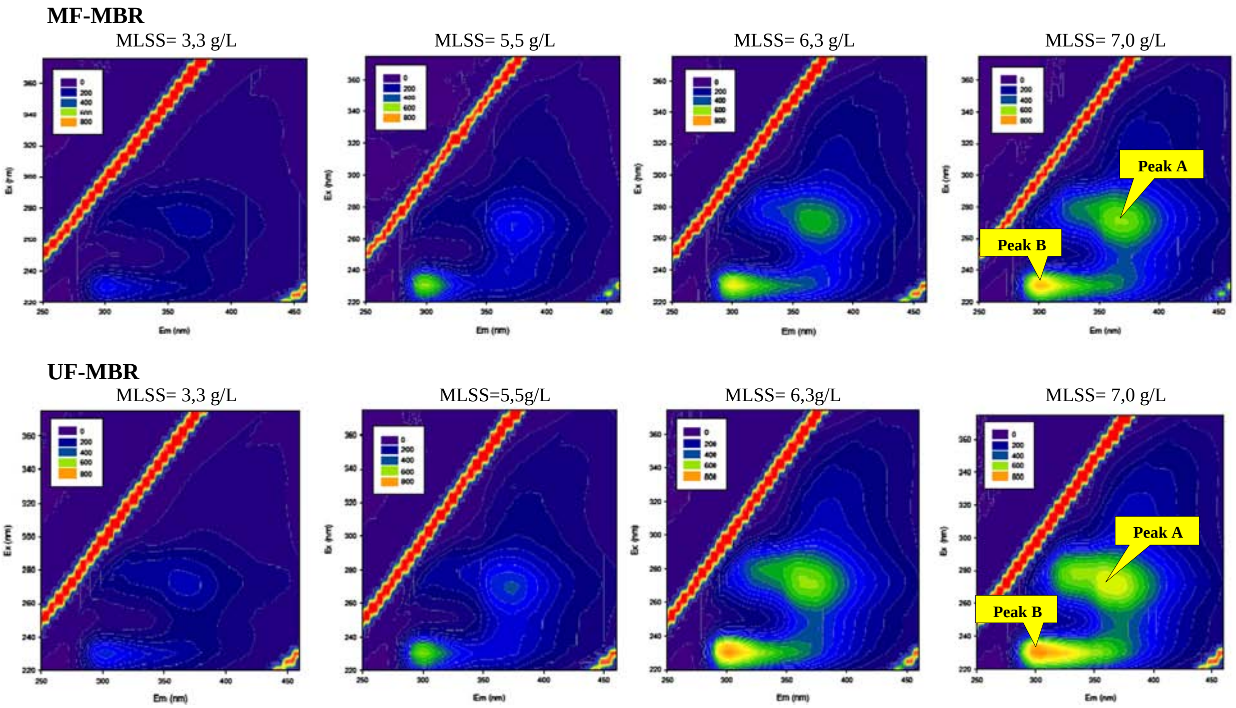

**Fig. 1.** Fluorescence spectra of $EPS_b$ fraction at different MLSS concentrations.

substances produced were also associated with proteins. These results were to be expected because both reactors were fed with an identical composition of wastewater and microorganism inoculum. The inoculum was obtained from the secondary treatment of the wastewater treatment plant at Rincón de León, Alicante, Spain.

Nevertheless, the peaks shifted at the different MLSS concentrations studied. The location of the fluorescence peaks provide spectral information on the chemical structural changes of the sludge of EPS samples as, depending on the emission or excitation scale, the peaks shifted toward longer (red shift) or shorter wavelengths (blue shift) [15]. Peaks A and B displayed red shifts at 7 g/L compared with 3.3 or 5 g/L, suggesting an increase in certain functional groups such as carbonyl, hydroxyl, amino and carboxyl groups [27].

Apart from identifying the type of compound, EEM spectroscopy is also useful for carrying out relative comparison of the obtained peaks by means of the fluorescence intensity (FI) [12,28]. In the graphs, the intensity of the peaks is represented by a colour scale. It can be seen that the FI of the two peaks increases as MLSS concentrations rise. Fig. 2 shows the FI values of the 2 peaks in the MF-MBR and the UF-MBR for all the MLSS concentrations studied.

The intensity of the peaks increased while MLSS concentrations increased for the two MBRs, although the FI values found for peak B were always higher than those for peak A. Thus, aromatic proteins were preponderant with respect to other protein-like substances.

Moreover, the peak B/peak A ratio was calculated using the fluorescence intensity of both peaks for each MLSS concentration studied. This ratio helps to understand how the compounds (in this case protein) evolve at different conditions (in this case different MLSS concentrations). The ratio had a decreasing trend (from 1.6 to 1.3) while MLSS concentrations increased from 3.3 to 7 g/L for the 2 MBRs. This means that the fraction of aromatic protein was degraded or other types of protein were produced as MLSS concentrations increased. As previously mentioned, other functional groups probably developed as MLSS increased. To sum up, the relative values of fluorescence intensity and the ratios calculated had similar values for the two membranes at each MLSS concentration studied. As it was expected, no influence of the type of membrane was found on the results, because they only depend on the characteristics of mixed liquor. On the other hand, in the MF-MBR at MLSS concentrations from 9 to 14 g/L, the peak B/peak A ratio remained almost constant (1.5–1.6). This means that the two proteins might be produced concomitantly, and they growth in parallel with the increase of MLSS concentration.

Taking into consideration that the spectra of $EPS_b$ only showed the presence of proteins, the relationship between the FI, bound protein and MLSS concentrations was studied. Fig. 2, apart from the FI of peaks A ($FI_A$) and B ($FI_B$) and MLSS concentrations, also shows the concentration of bound protein ($P_b$) obtained from $EPS_b$.

The location of the peaks did not depend on the EPS concentration, although a relationship between the FI of the peaks and bound protein concentrations with respect to MLSS concentrations was found. Protein concentration in both MBRs increased while MLSS concentrations increased, as with the FI of peaks A and B in the 2 MBRs studied.

A logarithmical correlation between the FI and MLSS concentrations was found in the MF-MBR for peaks A and B, with a correlation coefficient of 0.97. The equations obtained for the 2 cases are as follows:

$$FI_A[\text{MF}-\text{MBR}] = 103,481 \times \ln MLSS - 9352 \quad (1)$$

$$FI_B[\text{UF}-\text{MBR}] = 160,821 \times \ln MLSS - 14,750 \quad (2)$$

In spite of the fact that there are insufficient experimental points for the UF-MBR, no equation is provided. Nevertheless, the trend of the curves (peaks A and B) of the 2 MBRs for MLSS concentrations of up to 8 g/L was similar.

Little information about EPS characterization by EEM spectroscopy has been found. Most studies have been carried out for natural organic matter characterization from rivers, lakes or ground water [12,29], or characterization of EPS from wastewater treatment technologies [14,15] but these are different from MBR systems. Zhu et al., [15] worked with a sequencing batch bioreactor (SBR) for aerobic sludge granulation finding proteins, humic and fulvic acids in the sludge, as was found in this research study. The occurrence of each compound during sludge granulation at different MLSS concentrations differs from the present study because it is neither the same reactor nor the same technology. However, it was determined that the peak related to aromatic protein-like substances displayed a blue shift in the 50 day sample (MLSS concentration of ≈11 g/L) compared to 0 day sample (MLSS concentration of ≈3 g/L), which accounted for the reduction of some functional groups, such as aromatic ring and conjugated bond in chain structures.

### 3.1.2. *Soluble EPS*

The characterization of soluble EPS ($EPS_s$) was also performed by EEM spectroscopy at MLSS concentrations of 8 and 4 in the MF-MBR and the UF-MBR, respectively. In the case of the MF-MBR, the measurements shown belong to the first 4 MLSS concentrations, as compared with those determined in the UF-MBR. Fig. 3 shows the fluorescence spectra relative to $EPS_s$ in the 2 MBRs studied.

In both MBRs, 2 peaks were observed but with different locations than those for $EPS_b$. For the MF-MBR, peak C was located at an Ex/Em of 345–350/424–436 and peak D at 255–260/436–442. In the UF-MBR, peak C was located at an Ex/Em of 345–350/424–434 and peak D at 255–260/436–440. According to Chen et al. [12], the location of peak C was in region IV, associated to humic acids and peak D was found in region III, associated to fulvic acids. Peaks A and B found for $EPS_b$, associated mainly to proteins, disappeared in the spectra of soluble EPS indicating that these substances were not released into sludge supernatants. The organic substances of $EPS_b$ can be partially released to mixed liquor for different reasons such as floc breakage.

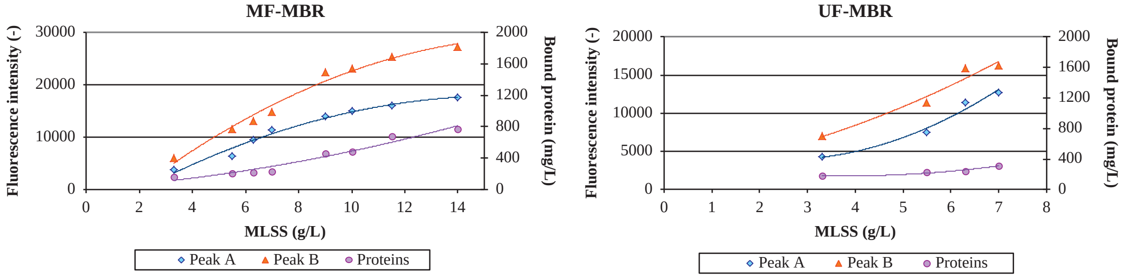

**Fig. 2.** Relationship between FI of the peaks, bound protein and MLSS concentrations.

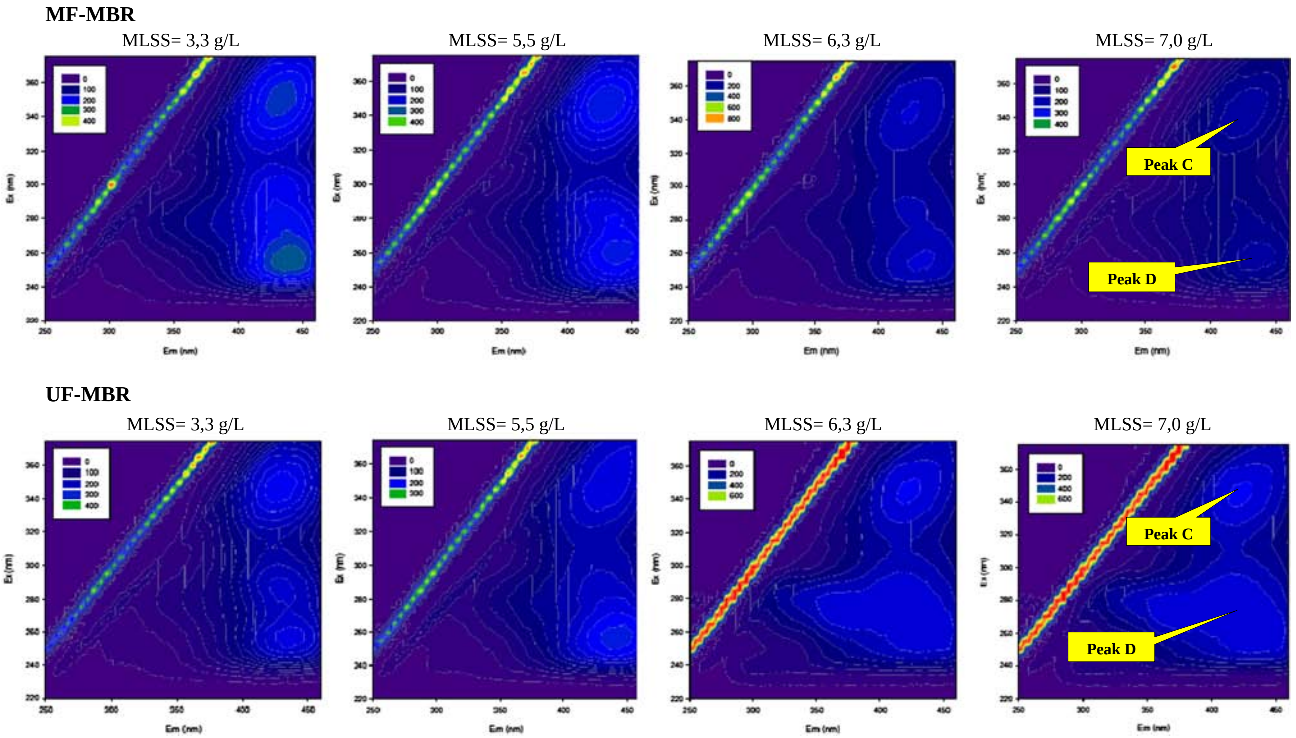

**Fig. 3.** Fluorescence spectra of EPS$_s$ fraction at different MLSS concentrations.

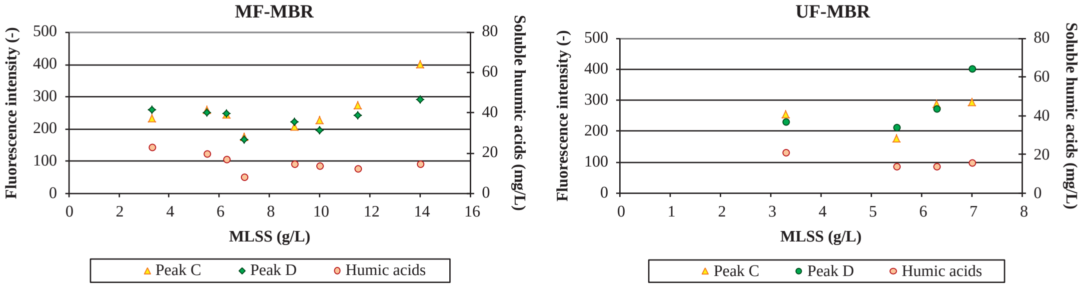

**Fig. 4.** Relationship between FI of the peaks, soluble humic acids and MLSS concentrations.

However, it is obvious that in all spectra the FI of the peaks was lower than for EPS$_b$. This is due to the fact that the concentration of EPS$_s$ is always lower than EPS$_b$. In general, the experimental data demonstrated that the fraction of soluble EPS was always smaller than EPS$_b$ and it decreased in the mixed liquor as MLSS concentrations rose. At low MLSS concentrations (up to 2 g/L), EPS$_s$ represented 25–44% of the total EPS. For higher MLSS concentrations it was less than 10% in the 2 MBRs.

Fig. 4 shows the FI of peaks C and D and the concentration of humic substances obtained from EPS$_s$ with respect to MLSS concentrations.

The FI of peaks C and D in the MF-MBR decreased as MLSS concentrations increased from 3.3 to 7 g/L. However, from 9 to 14 g/L, the FI values had an increasing trend. Something similar occurred with the UF-MBR (first a decrease in FI and then an increase) but in this case in the interval of the MLSS concentrations studied (from 3.3 to 7 g/L). In order to explain this phenomenon, Fig. 4 also shows the concentration of humic acids, because the location of the peaks was associated to humic and fulvic substances, as previously mentioned.

As can be seen, the behaviour of the FI of the peaks entirely depended on the concentration of humic acid, especially for the lowest MLSS concentrations, that is, when FI and MLSS decreased. Wang et al. [19] worked with a MBR and analysed soluble and bound EPS by 3D-EEM. Protein and humic acid in both soluble and bound EPS were present in the system that was fed with real municipal wastewater, whereas the study at hand used synthetic wastewater. However, the FI associated to humic substances in EPS$_s$ had the same trend as in this study, especially at higher sludge age. This can be explained by the fact that some non-biodegradable or slowly biodegradable substances could be generated by microorganisms at a much longer sludge age due to decay effects.

At the same time, it can be seen that most of the points of peaks C and D overlap. The previous explanation only relates to peak C since it was compared using the concentration of humic acids. Anyway, although the concentration of fulvic acids was not measured, their behaviour could probably be explained similarly.

Moreover, the correlation between FI and MLSS concentrations, and humic acids and MLSS concentrations of EPS$_s$ was also analysed.

No satisfactory correlation between the variables was found which lead to very low values of correlation coefficients. Soluble EPS concentrations (and consequently soluble concentration of protein, carbohydrate and humic acids) vary too much with respect to EPS$_b$ because microorganisms gain access to soluble substances more easily than to bound substances, and also due to the permanent feed supply into the reactor.

With respect to the peak D/peak C ratio, it was observed that in the MF-MBR all values (except for 14 g/L) remained almost constant (0.9–1.1), indicating similar fluorescence intensities for humic and fulvic substances for each MLSS concentration studied. As it was previously said, the concentration of the substances is relatively proportional to the fluorescence intensity. For that reason, it can be inferred that there were similar amounts of humic and fulvic acids in the supernatant. For the UF-MBR, this ratio showed similar results, although for a MLSS concentration of 7 g/L, a peak D/peak C ratio of 1.4 was found. This means, comparing with other MLSS concentrations, higher fluorescence intensity or amounts of fulvic acids with respect to humic acids. Although fulvic substances were not measured, it is probable that at high MLSS concentrations, there is an accumulation into the reactor because of their high molecular weight–they could not cross the small pore size of the UF membrane (0.08 μm).

Soluble and bound EPS from different types of sludges have been characterized [17]. When analysing bound EPS peaks associated with soluble microbial by-product-like and humic acids were found. In this study no humic acids in EPS$_b$ appeared but in line with the findings of the present study, the FI of soluble EPS in their analysis was always lower than for bound EPS.

### 3.2. *EPS characterization by high pressure size-exclusion chromatography (HPSEC)*

Chromatographic analysis was carried out in order to use EPS fingerprints as EPS identity, as much for soluble as for bound EPS extracted from the 2 MBRs. Additionally, the mean molecular weight (MW) for the EPS fractions extracted at different MLSS concentrations was determined. Figs. 5 and 6 show the chromatograms obtained for bound EPS at different MLSS concentrations, for the MF-MBR and the UF-MBR, respectively.

Fig. 5 shows that the higher peaks appeared at around 13 min of elution time and other peaks at 22 min but these were negligible with respect to the former ones. The intensity of the peaks increased as MLSS concentrations increased; at 14 g/L the intensity was almost three times higher than at 8 g/L.

Fig. 6 shows the chromatograms obtained at 4 MLSS concentrations of EPS$_b$ in the UF-MBR. The first peaks were found at 13 min of elution as in the MF-MBR. Nevertheless, in the UF-MBR, there are other significant peaks at higher elution times (22 and 33 min approximately), more evident for the highest MLSS concentration.

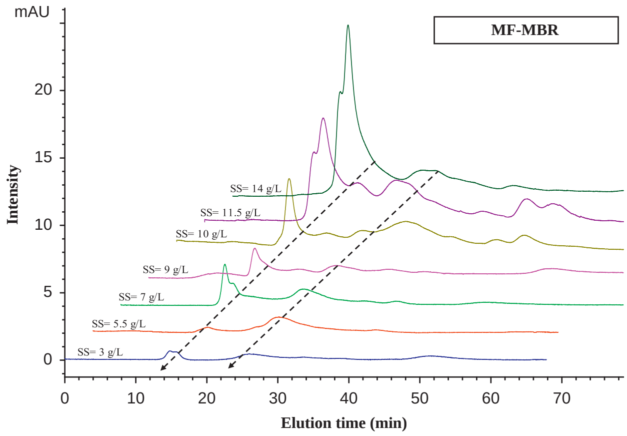

**Fig. 5.** Chromatograms of EPS$_b$ from MF-MBR at different MLSS concentrations.

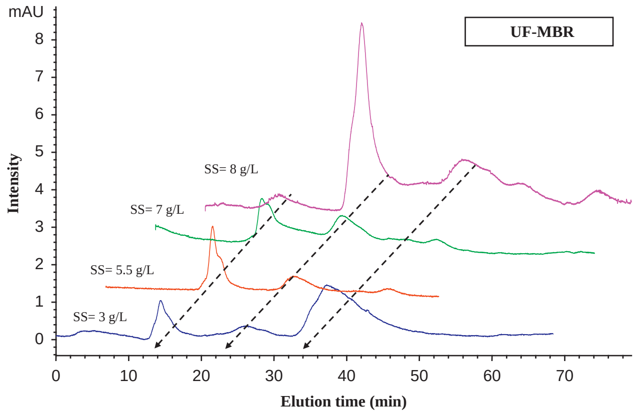

**Fig. 6.** Chromatograms of EPS$_b$ from UF-MBR at different MLSS concentrations.

Differences in elution time are based solely on the volume the analyte "sees". On one hand, a small molecule that can penetrate every corner of the pore system of the stationary phase "sees" the entire pore volume and the interparticle volume, and will elute late. On the other hand, a very large molecule that cannot penetrate the pore system "sees" only the interparticle volume and will elute earlier when this volume of mobile phase has passed through the column. The presence of peaks at high elution times indicates that EPS$_b$ extract in the UF-MBR also occurred because of small particles that were retained by the UF membrane with pore sizes less than 0.08 μm. However, for the MF-MBR, small particles were not found because they could pass through the MF membrane due to the higher pore size (0.4 μm).

In general, the intensity of the peaks increased as MLSS concentrations increased. This is proportional to the concentration of proteins in the EPS$_b$ which also rose as MLSS concentrations increased (see Fig. 2). For the measurement of EPS extracts, protein standards were used because EPS are for the most part made up of these substances. Moreover, the UV detector measured at a wavelength of 280 nm because the quantification of peptides and proteins in solution by HPLC with detection by UV absorbance has proven to be suitable and accurate [30].

Soluble EPS were also analysed but their chromatograms showed irregular behaviour at different MLSS concentrations. This is probably because proteins were found at very low concentrations ( < 37 mg/L), showing intensity signals below 1 mAU (data not shown). AL-Halbounia et al., [18] also found that the macromolecular peak (low elution times) was not UV-detectable, concluding that very low amounts of or no protein appeared in the samples.

Additionally, the mean molecular weight (MW) of all samples was calculated by means of Agilent Chemstation software. Table 1 shows the distribution of MW of the EPS$_b$ fractions and their mean MW for the 2 MBRs at 3 and 7 g/L MLSS concentrations.

It was decided to detail the behaviour of MW distribution and to calculate the mean MW at 3 and 7 g/L because they were, respectively, the minimum and maximum MLSS concentrations at which the UF-MBR worked. First, it can be seen that at higher MLSS (7 g/L), the values were always greater than those obtained for 3 g/L. For example, for the MF-MBR, it was found that 50% of molecules had a MW lower than 13 and 64 kDa for 3 and 7 g/L, respectively. Another interesting thing is that the values obtained for the UF-MBR were always lower than for the MF-MBR. For example, at 7 g/L, 50% of the molecules showed a MW of 64 and 29 kDa for the MF-MBR and UF-MBR, respectively.

**Table 1**
Comparison of MW distribution and mean MW of the two MBRs at two MLSS concentrations.

| | MW distribution (kDa) | | | |
|---|---|---|---|---|
| | MF-MBR | | UF-MBR | |
| | 50% | 70% | 50% | 70% |
| MLSS (g/L) | | | | |
| 3 | < 13 | < 44 | < 0.17 | < 1 |
| 7 | < 64 | < 740 | < 29 | < 398 |

| | Mean MW (kDa) | |
|---|---|---|
| MLSS (g/L) | MF-MBR | UF-MBR |
| 3 | 446 | 272 |
| 7 | 515 | 439 |

Consequently, the mean MW calculated indicated that they were higher for the MF-MBR and for the highest MLSS concentration. At 3 g/L, the UF-MBR showed a mean MW of 272 kDa compared with 446 kDa obtained for the MF-MBR. As previously explained, the chromatogram of the UF-MBR, apart from having a peak at 15 min of elution, also presents a high peak at 35 min which was not the case with the MF-MBR, which showed a lower MW. Evidently, the filtration carried out with a UF membrane lead to retentions of lower MW particles. This technique determined the behaviour of the MW of extracted EPS at different MLSS and the influence of the different membranes used was verified.

## 4. Conclusions

Soluble and bound EPS obtained from the 2 submerged MBRs (using different membranes) operating at high sludge age were characterised by 2 innovative analytical techniques: 3D-EEM and HPSEC.

As far as 3D-EEM is concerned, fluorescence spectra of EPS$_b$ and EPS$_s$ showed 2 peaks in both MBRs at all the MLSS concentrations studied. The peaks obtained for EPS$_b$ were associated to soluble microbial by-product-like and to aromatic protein and those obtained for EPS$_s$ to humic and fulvic acids. In the 2 MBRs, the FI of the peaks increased as MLSS and protein concentrations increased. The FI of the EPS$_s$ peaks was much lower than for EPS$_b$. It was verified that the evolution of the FI clearly depends on the concentration of protein and humic acids for EPS$_b$ and EPS$_s$, respectively.

Chromatographic analysis showed an increase in the intensity of the EPS$_b$ peaks as MLSS concentrations rose. Additionally, the mean MW calculated was always higher for higher MLSS in each reactor. For the MF-MBR, higher MW was found than in the UF-MBR for the same MLSS concentrations showing that the filtration carried out with a UF membrane lead to retentions of lower MW particles.

## 5. Aknowledgments

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## References

[1] T. Stephenson, S. Judd, B. Jefferson, K. Brindle, Membrane Bioreactors for Wastewater Treatment, IWA Publishing, London, 2000.
[2] B. Lesjean, S. Rosenberger, C. Laabs, M. Jekel, R. Gnirss, G. Amy, Water Sci. Technol. 51 (6–7) (2005) 1–8.
[3] B. Wu, Y. Shan, A.G. Fane, Bioresour. Technol. 102 (2001) 2511–2516.
[4] N. Yamato, K. Kimura, T. Miyoshi, Y. Watanabe, J. Membr. Sci. 280 (1–2) (2006) 911–919.
[5] J. Cho, K.G. Song, H. Yun, K.H. Ahn, J.Y. Kim, T.H. Chung, Water Sci. Technol. 51 (6–7) (2005) 9–18.
[6] Z. Ahmed, J. Cho, B-R. Lim, K-G. Song, K-H. Ahn, J. Membr. Sci. 287 (2) (2007) 211–218.
[7] J. Wingender, J., T.R. Neu, H.C. Flemming, What are Bacterial Extracellular Substances: Characterization, Structure and Function, Springer, Berlin, 1999.
[8] M. Esparza-Soto, P.K. Westerhoff, Water Sci. Technol. 43 (6) (2001) 87–95.
[9] G. Sheng, H. Yu, Water Res. 40 (2006) 1233–1239.
[10] B. Frolund, R. Palmgrem, K. Keiding, P. Nielsen, Water Res. 30 (8) (1996) 1749–1758.
[11] T. Gorner, P. de Donato, M. Ameil, E. Montarges-Pelletier, B. Lartiges, Water Res. 37 (2003) 2388–2393.
[12] W. Chen, P. Westerhoff, J.A. Leenheer, K. Booksh, Environ. Sci. Technol. 37 (2003) 5701–5710.
[13] F.C. Marhuenda-Egea, E. Martínez-Sabater, J. Jordá, R. Moral, M.A. Bustamante, C. Paredes, M.D Pérez-Murcia, M.D., Chemosphere 68 (2007) 301–309.
[14] I. Bourven, G. Costa, G. Guibaud, Bioresour. Technol. 104 (2012) 486–496.
[15] L. Zhu, H.-Y. Qi, M-L. Lu, Y. Kong, Y.-W. Yu, X.-Y. Xu, Bioresour. Technol. 124 (2012) 455–459.
[16] B. Jefferson, A. Brookes, P. Le-Clech, S. Judd, Water Sci. Technol. 49 (2004) 237–244.
[17] A. Ramesh, D.J. Lee, J.Y. Lai, J.Y, Appl. Microbiol. Biotechnol. 74 (2007) 699–707.
[18] D. Al-Halbounia, J. Traberb, S. Lykoc, T. Wintgensc, T. Melinc, D. Tacked, A. Janote, W. Dotta, J. Hollenderb, J., Water Res. 42 (2008) 1475–1488.
[19] Z. Wang, S. Tang, Y. Zhu, Z. Wu, Q. Zhou, D. Yang, J. Membr. Sci. 355 (2010) 151–157.
[20] L. Domínguez, V. Cases, Ch. Birek, M. Rodríguez, D. Prats, Chem. Eng. J. 181–182 (2012) 132–143.
[21] 17th ed., American Public Health Association, 1989.
[22] L. Domínguez, M. Rodríguez, D. Prats, Desalination 261 (2010) 19–26.
[23] M. Dubois, K. Gilles, K. Hamilton, P.A. Rebers, F. Smith, Anal. Chem. 28 (1956) 350–356.
[24] O. Lowry, N. Rosebrough, A. Lewis, R. Randall, J. Biol. Chem. 193 (1951) 265–275.
[25] G. Peterson, Anal. Biochem. 83 (2) (1977) 346–356.
[26] R.G. Zepp, W.M. Sheldon, M.A. Moran, M.A., Mar. Chem. 89 (2004) 15–36.
[27] J. Chen, B.H. Gu, E.J. LeBoeuf, H.J. Pan, S. Dai, Chemosphere 48 (1) (2002) 59–68.
[28] A. Baker, Environ. Sci. Technol. 35 (2001) 948–953.
[29] N. Her, G. Amy, D. McKnight, J. Sohn, Y. Yoon, Water Res. 37 (2003) 4295–4303.
[30] F. Moffatt, P. Senkans, D. Ricketts, J. Chromatogr. A 891 (2) (2000) 235–242.