

# Characterization of urban and industrial wastewaters using excitation-emission matrix (EEM) fluorescence: searching for specific fingerprints

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## ABSTRACT

Excitation-emission matrix (EEM) fluorescence spectroscopy has been applied to characterize several urban and industrial wastewaters (effluents from different types of industries: brewery, winery, dairy, biscuit, tinned fish industry, slaughterhouse, pulp mill, textile dyeing and landfill leachates), searching for specific fluorescence fingerprints.

Tryptophan protein-like peaks ( $T_1$  and  $T_2$ ) are the predominant fluorescence in urban and food industry wastewaters (brewery, winery, dairy/milk, biscuit and fish farm industries) but no special fingerprint has been found to discriminate among them. Protein-like fluorescence also dominates the spectra of meat/fish industries (effluents from a tinned fish industry and a slaughterhouse), but in this case tyrosine protein-like peaks ( $B_1$  and  $B_2$ ) also appear in the spectra in addition to tryptophan-like peaks. This fact might constitute a specific feature to differentiate these wastewaters from others, since the appearance of peaks B is quite uncommon in wastewaters. The textile dyeing effluent shows a characteristic triple humic-like fluorescence (peaks A,  $C_1$  and  $C_2$ ) that may represent a specific fingerprint for this kind of effluent. Leachates from medium-aged and old landfills might also show a specific fingerprint in their EEM spectra: the sole presence of the humic-like peak C with very high fluorescence intensity. This feature also allows differentiating them from young landfill leachates, which show predominance of protein-like peaks.

The fluorescence index (FI) does not seem to be very appropriate to characterize wastewaters and its usefulness might be limited to the study of humic substances in natural waters, although further studies are needed on this topic. However, the humification index (HIX) and the biological index (BIX) do seem to be useful for studying wastewaters, since they have provided consistent results in the present work. This study shows the potential of EEM fluorescence to identify the origin of some industrial effluents, although more research is needed to check these preliminary results.

## **Keywords.**

*urban and industrial wastewaters, influents/effluents, EEM spectra, fluorescence indices, fingerprints.*

## **1.- INTRODUCTION.**

Dissolved organic matter (DOM) in wastewater shows a high diversity, from simple and small molecules (amino acids, simple sugars, low-molecular weight (MW) organic acids) to high-MW macromolecules (humic substances, proteins, polysaccharides, etc) (Janhom et al, 2009). Industrial wastewaters, even after intensive treatment, still contain a considerable organic matter load remaining in the effluents. The effluents can exert detrimental effects on natural water bodies, such as toxicity and hypoxia (Yang et al, 2015). Global parameters such as chemical oxygen demand (COD), biochemical oxygen demand (BOD) and total organic carbon (TOC) do not provide any information on the structural composition of the organic matter. The analytical techniques: infrared spectroscopy (FTIR), gas chromatography-mass spectrometry (GC/MS) and <sup>1</sup>H- and <sup>13</sup>C-nuclear magnetic resonance (NMR) are very sophisticated but they usually require laborious procedures of extraction and purification of the samples.

Fluorescence has profusely been used to analyze DOM in natural and engineered water bodies (Kalbitz et al., 2000). Fluorescence spectroscopy provides useful information on the structure of the humic substances: the intensity and location of fluorescence peaks can be correlated to some structural properties, such as carboxylic acidity, degree of humification and aromatic character of the humic macromolecule (Chen et al., 2003; Rodríguez et al., 2014; Zhang et al., 2008). It is a sensitive and rapid technique requiring only small volumes and usually simple pre-treatment of the sample (filtration and pH adjustment, if necessary) and can be easily adapted to in situ automated instrumentation (Swietlik et al., 2004). Among fluorescence techniques, the conventional emission and synchronous fluorescence are the simplest ones whereas excitation-emission matrix (EEM) fluorescence (also known as total luminiscence spectroscopy: TLS) is nowadays the most interesting and complete technique. EEM (in which numerous emission scans are collected for a range of excitation wavelengths) can provide specific “fingerprints” for a mixture of fluorescent components within a complex matrix, such as wastewaters (Henderson et al. 2009; Hudson et al., 2008; Peiris et al., 2011). The position of the peaks is represented as ( $\lambda_{ex}/\lambda_{em}$ , in nm) and their maximum fluorescence intensities (Fmax) are considered to be proportional to the relative concentration of the different components (Goldman et al, 2012; Henderson et al, 2009; Yu et al, 2013).

Even though some mathematical tools (such as the Parallel Factor Analysis: PARAFAC) have commonly been used to study EEM spectra, the traditional method of “peak-picking” will be used in the present work since it is simpler, faster and provide results equally satisfactory for the identification of the fluorophores. In fact, some authors have indicated that monitoring a small number of individual peaks at the appropriate coordinates ( $\lambda_{ex}/\lambda_{em}$ ) can provide basically the same information as monitoring full EEM (Murphy et al, 2011). Additionally, one of the aims of this work is its applicability to routine measurement in industrial wastewaters and industries usually need and demand simple and fast analytical methods; PARAFAC requires a high degree of expertise and it is usually used in research applications apart from being time-consuming. Once the effluent of a specific industrial facility is characterized, the main peaks remain the same (obviously they vary in intensity from sample to sample) and the pick-picking method provides a quick tool to monitor changes in the composition of the effluent, that is what most industries usually require: a fast and simple method.

The main EEM peaks found in wastewaters are shown in Figure 1 and can be classified into two major groups:

- Protein-like peaks: peak T<sub>1</sub> (275-285/320-350, tryptophan-like), peak T<sub>2</sub> (215-237/340-381, tryptophan-like), peak B<sub>1</sub> (275-310/305-320, tyrosine-like), peak B<sub>2</sub> (220-237/305-320, tyrosine-like).
- Humic-like peaks: peak A (230-260/400-480, fulvic-like), peak C (320-360/420-460, humic-like) and peak M (290-310/370-420).

Protein-like peaks (mainly T<sub>1</sub> and T<sub>2</sub>) tend to be predominant in wastewaters (particularly in urban wastewaters), associated to the presence of biodegradable organic matter and microbial activity in water (Henderson et al, 2009; Yu et al, 2013). In fact, several studies have reported correlations between T<sub>1</sub>/T<sub>2</sub> peaks and BOD, both in natural waters and wastewaters (Baker et al, 2004; Hur et al, 2008); other authors have indicated that those correlations might be site-specific and that cannot be generalized (Hudson et al, 2008). Tyrosine-like peaks B are less abundant than tryptophan-like peaks T, both in natural waters and wastewaters; peak B<sub>2</sub> can be quenched in high-MW proteins due to resonance energy transfer (Bridgeman et al, 2013; Maie et al, 2007; Yamashita et al, 2004), that is to say, the energy absorbed by tyrosine is often transferred to the tryptophan residues in the same protein (Li et al, 2014). Additionally, peak B<sub>2</sub> can also be quenched in the presence of carboxylic acids in wastewaters (Reynolds, 2002) and peak B<sub>1</sub> can be obscured by the Raman line of water (Baker et al, 2004a; Bridgeman et al, 2013).

However, humic-like peaks (A and C) are usually the most abundant in natural waters. These peaks are indicative of the presence of humic substances in water (fulvic and humic acids), both of terrestrial

(terrestrial plant and soil organic matter) and microbial origin (autochthonous microbial processes) (Rodríguez et al, 2014). In fact, the significant presence of protein-like peaks in natural waters is considered to be indicative of anthropogenic pollution due to wastewater discharges (Borisover et al., 2011; Goldman et al, 2012; Hudson et al, 2007; Hur et al, 2008; Yu et al, 2016). Peak M, originally defined as “Marine humic-like” by Coble (Coble, 1996, 1998) indicates the presence of organic compounds from recent biological activity (soluble microbial humic-like material) (Ishii et al, 2012; Maie et al, 2007; Murphy et al, 2011; Parlanti et al, 2000; Yu et al, 2014).

In the wastewater field, EEM fluorescence has mainly been applied to the study of domestic/urban wastewaters, particularly effluents from municipal wastewater treatment plants (WWTP); however, there are very few studies of EEM application for the characterization of industrial wastewaters. The kind of organic compounds present in wastewaters clearly depends on the kind of industry and the characteristics of the wastewater treatment (in the case of effluents) (Janhom et al, 2009).

Therefore, this study is aimed at characterizing a great number of industrial wastewaters (influent/effluent from 13 different types of industries) searching for specific fluorescence fingerprints; this way, we will evaluate EEM ability to discriminate wastewaters from different origins.

## **2.- MATERIAL AND METHODS.**

### **2.1. Types of wastewaters.**

A series of municipal and industrial wastewaters have been characterized in this study, the latter coming from several industrial categories: brewery, winery, dairy, biscuit, fish farm, tinned fish industry, slaughterhouse, pulp mill, textile dyeing and landfill leachates.

In some cases the wastewater samples were raw wastewaters (influent) generated by the facility before being subjected to treatment in a WWTP (either municipal or industrial). In other cases the wastewater samples were the effluents treated in those WWTP (it can be reasonably approximated that these effluents have a similar qualitative composition to the correspondent influent but at much lower concentration). Samples were provided by the facilities staff (a final effluent sample from the releasing pipe under normal operation conditions) and were filtered before fluorescence measurements in order to remove turbidity interferences (Hudson et al, 2008; Reynolds et al, 1997).

All wastewater samples were adjusted to pH 7 using dilute HCl or NaOH (Alberts et al., 2004; Her et al., 2003).

## 2.2. Fluorescence measurements.

Fluorescence spectra were recorded with a spectrofluorometer (Varian Cary Eclipse) at ambient temperature (20-22°C) using a 1-cm cuvette. The following instrument settings were used: excitation wavelength range: 220-450 nm (step 5 nm), emission wavelength range: 350-500 nm (step 1 nm), scan speed: 600 nm min<sup>-1</sup> (Peiris et al., 2009, 2011). The spectra were concatenated in the form of an excitation-emission matrix (EEM) to produce three-dimensional contour plots of fluorescence intensity as a function of excitation and emission wavelengths (Chen et al., 2003; Kim et al., 2006). The instrument was auto-zeroed prior to each analysis and fluorescence intensity variation in triplicate experiments was less than 8%. Fluorescence intensities were normalized (Raman units: R.U) by the Raman peak of Milli-Q water at  $\lambda_{ex}=348$  nm (Yang et al., 2015). Raman emission at  $\lambda_{em}=395$  nm averaged  $27.3 \pm 1.4$  intensity units (n=18), showing the stability of the instrument. Inner-filter effects were corrected by absorbance spectroscopy (McKnight et al., 2001; Ohno, 2002).

Fluorescence “quenching” effects due to the presence of some metal ions in the samples (mostly iron and nickel) are not expected at the concentration levels that they usually present in domestic wastewaters (Baker et al, 2003; Reynolds et al, 1997). Concerning industrial wastewaters, there is no industry typically containing high amounts of heavy metals (such as surface coating industries) among those studied in this work.

In addition to EEM spectra, several fluorescence indices were calculated too (see their graphic representation in Figure 1):

- Fluorescence index (FI): this parameter was introduced by McKnight (McKnight et al., 2001) and is determined as the ratio of emission intensity at 450/500 nm measured at an excitation wavelength of 370 nm:

$$FI = I_{Em\ 450} / I_{Em\ 500}, \text{ at } Ex=370 \text{ nm}$$

- Humification index (HIX): this parameter was introduced by Zsolnay (Zsolnay et al, 1999) and determined as the ratio of fluorescence intensities for two integrated regions of the emissions scans: Em 435-480 nm divided by Em 300-345 nm, at Ex 254 nm.

$$HIX = \sum I_{Em\ 435-480} / \sum I_{Em\ 300-345}, \text{ at } Ex=254 \text{ nm}$$

Later on, Ohno (Ohno, 2002) proposed an alternative expression for HIX by dividing the emission intensity in the 435-480 nm region by the sum of total intensities in the (300-345) + (435-480) nm regions. This parameter is denominated “normalized HIX” (HIX<sub>norm</sub>) in this study, since it ranges from 0 to 1.

$$HIX_{norm} = \sum I_{Em\ 435-480} / (\sum I_{Em\ 300-345} + \sum I_{Em\ 435-480}), \text{ at } Ex=254 \text{ nm}$$

- Biological index (BIX): it was introduced by Huguet (Huguet et al, 2009) and calculated at Ex 310 nm, by dividing the fluorescence intensity at Em 380 nm by that at Em 430 nm:

$$FI = I_{Em\ 380} / I_{Em\ 430}, \text{ at Ex}=310 \text{ nm}$$

### 3.- RESULTS

#### 3.1. Urban and mixture urban-industrial wastewaters.

Figure 2a shows the EEM fluorescence spectrum of a raw urban wastewater (influent) from residential origin and Figure 2b shows that of an influent which is a mixture of urban and industrial wastewaters (the latter come mainly from the food industry). Both influents feed the wastewater treatment plant of the city of Burgos (Spain), which has two differentiated treatment lines. The location of the peaks ( $\lambda_{ex}/\lambda_{em}$ ) and their fluorescence intensities are shown in Table 1.

The domestic wastewater (Fig. 2a) is characterized by the presence of the protein-like peak T<sub>2</sub> and the fulvic-like peak A as majority peaks. The protein-like peak T<sub>1</sub> also appears but with lower fluorescence intensity. It can be noticed the presence of the protein-like peak B<sub>2</sub> but just at the end of the spectrum and it cannot be clearly quantified. That is to say, the fluorescence is controlled by the presence of protein-like substances (proteins, peptides, amino acids) and fulvic acids, the latter coming from the drinking water used in houses and which is the basis of the subsequent wastewater. Concerning humic-like fluorescence, wastewaters usually show more fluorescence associated to low-molecular-weight humic substances (fulvic-like peak A) than natural waters, which usually show preponderance of peak C (associated to humic substances of higher molecular weight) (Henderson et al, 2009).

Since tyrosine-like peaks B are quenched by the presence of tryptophan in large molecular weight proteins, tyrosine fluorescence is only observed in the absence of tryptophan (Maie et al, 2007; Yamashita et al, 2004); that is why if peaks B are detected in a sample it can be predicted the presence of peptides (and not large proteins) in which tryptophan and tyrosine do not exist together (Yamashita et al, 2003). The composition of the domestic wastewater (influent) found in this study is in agreement with the results reported in the literature (Yang et al, 2014).

However, the mixture of urban-industrial wastewaters treated in the other line of the same WWTP shows a quite different EEM spectrum (Fig. 2b), with almost the sole presence of protein-like peaks T, being the peak T<sub>1</sub> the most predominant in this case (unlike the domestic wastewater, where peak T<sub>2</sub> was the majority one). Fulvic-like peak A is also observed with a lower intensity, humic-like peak

C shows a residual presence and peaks B are not detected. Obviously, the contribution of the industrial wastewaters (mainly from the food industry) is responsible for this difference.

Concerning the relative abundance of peaks T in urban wastewaters, there is no agreement in the literature regarding which peak ( $T_1$  or  $T_2$ ) is the majority one. Some studies report peak  $T_1$  as the most abundant (Baker, 2001; Goldman et al, 2012; Her et al, 2003; Park et al, 2010; Riopel et al, 2014; Wang et al, 2009; Xue et al, 2011; Yang et al, 2014; Yu et al, 2013, 2014, 2015), others peak  $T_2$  (Bridgeman et al, 2013; Hudson et al, 2008; Lee et al, 2004; Li et al, 2014; Liu et al, 2011; Quaranta et al, 2012; Westerhoff et al, 2001; Zhang et al, 2009) and finally, there are some studies that report the same intensity for both peaks (Meng et al, 2011). It is very difficult to predict that relative abundance since most WWTP are also fed by industrial wastewaters from the surrounding facilities, therefore there is a great variability.

Regarding the origin attributed to the protein-like fluorophores containing tryptophane, peaks T can derive from both proteins present in the raw wastewater (influent) and microbial activity during the biological treatments in WWTP (SMP: soluble microbial products) (Chen et al, 2003; Henderson et al, 2009; Hudson et al, 2007, 2008; Meng et al, 2011; Parlanti et al, 2000; Wang et al, 2009). As to the molecular structure, peak  $T_2$  is likely assigned to low molecular weight protein molecules, such as simple aromatic proteins (Chen et al, 2003; Meng et al, 2001) or even free tryptophane amino acid (Wang et al, 2010). However, there is no unanimity in the literature about the molecular structure attributed to peak  $T_1$ : some studies suggest that the tryptophan-like fluorescence is due to the presence of free tryptophan amino acid in the water samples (Reynolds, 2003), whereas others suggest the opposite indicating that free tryptophan is not the dominant component of the tryptophan-like fluorescence (Baker, 2005). Maie proposed that peak  $T_1$  can be separated into two constituents: high molecular weight proteinaceous materials and humic associated phenolic moieties with small molecular weight (Maie et al, 2007). Recent studies have shown that proteins can be encapsulated by humic substances, indicating a possible combination between them through electrostatic attraction or hydrophobic effects (Li et al, 2014; Tomaszewski et al, 2014). Additionally, some polyphenolic compounds (e.g., lignin) have been shown to exhibit fluorescence like tryptophan (Hernes et al, 2009; Li et al, 2014).

Additionally, the location of peak  $T_1$  in wastewater samples differs (it is red-shifted) from that of tryptophane amino acid standard in the emission wavelength of the fluorescence maximum: the typical position of  $T_1$  for free tryptophane standard is 280/340-350 and the positions found in this study are 280/355 (urban wastewater, Fig. 2a) and 280/359 (mixed urban-industrial wastewater, Fig. 2b). Other studies have reported similar results for both for wastewaters (280/371, Her et al.2003) and natural waters (280/364, Borisover et al.2011). Borisover proposed two possible explanations for

this difference: the first one is that peak T<sub>1</sub> could be a mixture of proteinaceous and non-proteinaceous substances (as proposed by Maie, see above), where the latter could protect the proteinaceous component against biodegradation. The other possible explanation is that the red-shift could be induced by the polarity and composition of the local environment, for instance, an increasing exposure of tryptophan from proteinaceous matter to the solvent: it is well documented in the literature that location of the maximum peaks and fluorescence intensities of aromatic amino acid residues in the protein macromolecule are clearly affected by the protein conformation (Yamashita et al, 2003).

Protein-like fluorescence (peaks T and B) is suitable for removal by microbial biodegradation, since amino acids and proteins are used as substrates for microorganisms, whereas humic-like fluorescence (peaks A and C) shows poor biodegradability (refractory organic matter) and is more appropriate for photo-degradation and coagulation treatments (Cohen et al, 2014; Liu et al, 2011; Park et al, 2010; Wang et al, 2009; Yang et al, 2014,2015; Yu et al, 2013,2014). That is why the protein-like:humic-like fluorescence ratio (typically T<sub>1</sub>:C ratio or T<sub>1</sub>:A ratio) is a useful parameter to monitor the efficiency of the wastewater treatment (Henderson et al, 2009); for instance, Baker (Baker, 2001) reported T<sub>1</sub>:C ratios of 2.7-3.1 for untreated raw wastewaters (influent) and ratios ~ 1.0 for treated effluents. The T<sub>1</sub>:C ratios obtained in this study for the influent wastewater (1.8 for the urban wastewater and 5.6 for the urban/industrial mixed wastewater) are out of the range reported by Baker, but it must be noticed the great variability of wastewater composition (for instance, the intensity of peak C in this study is minimal in comparison with Baker's study, peak A being the main humic-like fluorescence in our case).

### **3.2.- Industrial wastewaters.**

#### **3.2.1. Food industries.**

Figure 3 shows the EMM spectra for treated effluents belonging to 4 industrial categories from the food industry: brewery (Fig. 3a), winery (Fig. 3b), dairy/milk (Fig. 3c) and biscuit (Fig. 3d).

As indicated in the previous section, the presence and relative distribution of humic-like peaks (A and C) is strongly influenced by the characteristics of the natural/drinking water used as process water in each case and it is not specific of the type of industry, therefore the discussion will be focused on protein-like fluorescence. There is no presence of tyrosine-like peaks B, so it can be argued that the proteinaceous material remaining in the effluent is not in the form of small peptides or free amino acids but in the form of larger peptides/proteins.



The brewery effluent (Fig. 3a) shows equal distribution of peaks T (similar fluorescence intensity for both peaks T<sub>1</sub> and T<sub>2</sub>), winery and milk and effluents show preponderance of T<sub>2</sub> over T<sub>1</sub> (Fig. 3b and 3c, respectively) whereas T<sub>1</sub> is the most intense peak for the biscuit industry effluent (Fig. 3d). As said in the introduction section, there is very little literature available concerning EEM spectra of industrial wastewaters in order to compare our results with other studies. For instance, Yang (Yang et al, 2015) reported some EEM spectra of industrial effluents but they are labeled as broad categories (food, drink) and not as specific industries like in our case, so the comparison is impossible. Janhom (Janhom et al, 2009) reported an EEM spectrum for a brewery wastewater quite similar to the one in this study.

Additionally, this work has only studied the effluent of one factory from each industrial category and it is not enough to characterize the above EEM spectra as specific “fingerprints” for each individual industry. Further work is necessary in order to get a complete database of EEM spectra of industrial wastewaters.

### **3.2.2. Meat/Fish industries.**

Figure 4 shows the EEM spectra for several wastewaters belonging to meat/fish industries: an effluent from a tinned fish industry (Fig. 4a) and an effluent from a slaughterhouse (Fig. 4b). As said in the previous section, the discussion will be focused on protein-like fluorescence.

Both effluents show peaks B, suggesting the presence of low molecular weight proteinaceous material (amino acids and/or small peptides) rather than high molecular weight proteins. Peak T<sub>2</sub> is the most prominent for the tinned fish effluent whereas peaks B are especially intense for the slaughterhouse effluent, showing similar fluorescence intensity to that of peaks T. Moreover, the slaughterhouse spectrum shows greater fluorescence intensity for all the peaks (T and B) than the other industries spectra (notice the intensity scale on the right side of the spectra), indicating a higher relative concentration of protein-like substances in this effluent. Louvet reported that the high concentration in serum albumin and hemoglobin of slaughterhouse wastewaters may contribute to a greater protein-like fluorescence (Louvet et al, 2013); however, Louvet detected no peaks B in his slaughterhouse wastewater, unlike our study. Finally, Wang reported an EEM spectrum very similar to that of the slaughterhouse (Fig. 4b) for a food processing factory with products of stewed meats (Wang et al, 2013).

### 3.2.3. *Other industries.*

Figure 5 shows the EEM spectra for other non-food industries: the effluent of a pulp mill industry (Fig. 5a) and the effluent of a textile dyeing industry (Fig. 5b).

Paper mill effluents are a complex mixture of acids, phenolic compounds and lignin derivatives, sugars, proteins and inorganic substances derived from the wood pulp as well as chemical additives used during the processing (Ciputra et al, 2010). The EEM of the pulp mill effluent shown in Fig. 5a is characterized by the presence of three intense peaks: T<sub>2</sub>, A, C and a less intense peak T<sub>1</sub>, which coincides with some spectra reported in the literature (Ciputra et al, 2010). It is interesting to note that the humic-like peak C has the highest fluorescence intensity in the spectrum and this is quite unusual because the fulvic-like peak A tends to be the majority one among humic substances from natural waters. This fact could be explained by the contribution of other non-humic substances to peak C, which come from the pulp mill effluent and not from the natural water used as process water. For instance, the leaching of optical brighteners or fluorescent whitening agents (such as distyrylbiphenyl and diaminostilbene types) during pulp processing has been suggested to contribute to peak C fluorescence (Baker, 2002). Other authors suggest that lignin-derived organics can contribute to peak C as well (Cawley et al, 2012; Ciputra et al, 2010) and also to peaks T fluorescence (Beggs et al, 2011; Carvalho et al, 2008; Duarte et al, 2003; Santos et al, 2000).

Textile dyeing wastewaters are characterized by high chemical oxygen demand, color, turbidity, pH, temperature and toxic chemicals (Fan et al, 2014). Fig. 5b shows the EEM spectrum of a textile dyeing effluent, which is characterized by the presence of peaks T<sub>1</sub> and T<sub>2</sub> (the most intense) and three humic-like peaks at  $\lambda_{em} = 420$  nm: one of them is peak A (225/420 nm) and the other two can be labeled as C<sub>1</sub> (250/420 nm) and C<sub>2</sub> (315/420 nm). This spectrum is very similar to one reported by Qian (Qian et al, 2013) for a biotreated textile effluent. It is interesting to note the presence of three humic-like peaks (A, C<sub>1</sub>, C<sub>2</sub>) instead of only two (A and C, the most usual situation); this fact has also been reported by other authors (Fan et al, 2014; Li et al, 2013, 2015) and it could represent a specific “fingerprint” for this kind of effluent. This triple humic-like fluorescence has been attributed in the literature to certain dyes like Orange G and its degradation products (1-amino-2-naphthol moieties), whose EEM spectra show the same pattern (Li et al, 2013, 2015). Orange G is one of the most widely azo dyes used in dyestuffs and therefore, it is one of the most common synthetic dyes released into the wastewater (Saratale et al, 2011).

### 3.3. Landfill leachates.

Landfill leachates contain a great variety of pollutants, namely inorganic macro-components, heavy metals, dissolved organic matter (organic compounds derived from degradation of plant materials, such as lignin, carbohydrates and peptides), microorganisms and various xenobiotic organic compounds from pharmaceuticals, plasticizers, pesticides and chlorinated aromatics (Baker et al, 2004b). Dissolved organic matter is a major component of leachates and consists of highly variable compounds, ranging from low-MW molecules (simple volatile fatty acids) to high-MW compounds, such as humic substances, aromatic hydrocarbons, chlorinated aliphatics and phenols (Zhang et al, 2013, see there a comprehensive list of organics found in leachates by means of GC-MS).

Since a landfill can be considered as a giant-sized anaerobic reactor, the organic composition of the leachate is closely related to the landfill age: the leachate humic fraction aromaticity and size increases with landfill age, indicating an increase in humification (Kang et al, 2002). Older leachates have a wider range of molecular weights and with a high molecular weight fraction being present whereas young leachates have a narrower molecular weight range and lower molecular weight compounds (Baker et al, 2004b).

Some studies have investigated landfill leachates classifying them into three categories on the basis of the landfill age (Li et al, 2010): young (< 5 years), medium-aged (5-10 years) and old (> 10 years) (time limits are variable, depending on each specific study). In general, all the studies coincide on this matter and report protein-like peaks (T and B) as the main fluorescence in young landfill leachates whereas humic-like peaks (A and mainly C) are predominant in leachates from medium-aged and old landfills (He et al, 2013; Lu et al, 2009; Shouliang et al, 2008). This result is expected since an important part of the organic matter in young leachates is still made up of labile compounds (such as proteins and peptides) whereas in old leachates the organic matter has had enough time to undergo transformations through humification processes.

Fig.6 shows the EEM spectra for medium-aged (7 years, Fig.6a) and old (25 years, Fig.6b) landfill leachates (unfortunately, we have not been able to get a young leachate). As seen in Fig.6 the spectra for both leachates are similar, only showing humic-like fluorescence: a very intense peak C and a minority peak A, which is agreement with the literature reported earlier. However, there are slight differences between both leachates: the humic substances in the old landfill leachate present a greater humification index ( $HIX=28.1$  and  $HIX_{norm}=0.96$ ) than those in the medium-aged landfill leachate ( $HIX=5.9$  and  $HIX_{norm}=0.85$ ), indicating a higher degree of aromatic condensation (humification) for the humic substances of the old leachate.

Some researchers have made comparisons between the humic substances from landfill leachates and Aldrich humic acid (AHA), a commercial humic acid often used as a surrogate for humic substances in several studies. AHA is a humic acid from soil origin and is expected to be produced by the natural oxidation of low-grade coals (Monteil-Rivera et al, 2000). Kang (Kang et al, 2002) reported that the leachate humic substances were comprised of molecules of smaller size, less aromatic and less condensed than those of AHA (Baker et al, 2004b), suggesting a lower degree of humification for the leachate humic substances in comparison to AHA. That suggestion is confirmed in this study, since the values for AHA humification index are  $HIX=52.5$  and  $HIX_{norm}=0.98$ .

### **3.4.- Evaluation of fluorescence indices: FI, HIX and BIX.**

Table 2 shows the fluorescence indices measured in this work (FI, HIX and BIX) for the wastewaters studied in the previous sections.

The fluorescence index (FI) has been used to distinguish the origin of fulvic acids in natural waters (McKnight et al, 2001): FI values around 1.9 are characteristic of microbially derived fulvic acids (autochthonous origin) whereas terrestrially derived fulvic acids (allochthonous origin) show FI values around 1.4. Intermediate FI values are found in waters with NOM from blended sources (Cawley et al, 2012). Additionally, a negative correlation between humic substances aromaticity and FI was reported in the literature (Kim et al, 2006; McKnight et al, 2001; Rodríguez et al, 2014). However, little is known about the suitability of using FI in wastewaters. The FI values shown in Table 2 for the different wastewaters do not follow a pattern but show some inconsistencies instead. For instance, pulp mill wastewaters are expected to contain lignin derivatives which are rich in conjugated aromatic structures and therefore, low FI values are expected for these wastewaters given the negative correlation between FI and aromaticity commented above; however, the pulp mill effluent has a high FI (2.03) in Table 2. Another inconsistency is found for the case of the leachates: old leachates are characterized by the presence of more high molecular weight aromatics (greater degree of humification) than young and medium-aged leachates and thus lower FI values are expected for old leachates; however, the old leachate has a higher FI (1.85) than the medium-aged one (1.33). The earlier observations suggest that FI is not a suitable parameter for characterizing wastewaters. Other authors have reported that FI might not be an appropriate parameter for waters whose organic material comes from a diversity of sources and that contain significant amounts of non-humic substances (Huguet et al, 2009), which is the case of most wastewaters.

The humification index (HIX and HIX<sub>norm</sub> calculated in this study) is an indicator of a material's age and recalcitrance within a natural system, measuring the complexity and the condensed aromatic nature of DOC compounds (degree of humification) (Morling et al, 2017; Ohno et al, 2007); it is strongly correlated with DOM aromaticity and inversely correlated with carbohydrate content (Birdwell et al, 2010). Huguet (Huguet et al, 2009) reported that low HIX values (<4) are associated with autochthonous fresh DOM from biological origin or also from plant biomass and animal manure (Birdwell et al, 2010) whereas high HIX values (>10) are indicative of strongly humified organic material, mainly of terrestrial origin. The first observation that can be made from Table 2 is that the two humification indices (HIX and HIX<sub>norm</sub>) show similar behavior, therefore both of them are interchangeable. The HIX values shown in Table 2 make sense, all the wastewaters show HIX < 4 (except for the leachates) which is in agreement with their origin: fresh DOM from biological sources (fish farm, slaughterhouse, etc) or from natural products (food industry), in addition to the biological processes involved in the wastewater treatment plants (most of the samples are effluents from biological treatments). Both leachates show high HIX values, the old leachate having a greater HIX (28.0) than the medium-aged one (6.01), which agrees with the higher degree of humification showed by old landfill leachates (Baker et al, 2004b; Kang et al, 2002; Shouliang et al, 2008). The HIX of the old leachate falls within the range reported by Birdwell (Birdwell et al, 2010) for water extractable DOM from soil and soil porewater (HIX: 10-30). Finally, the HIX value obtained in the present study for the standard Aldrich Humic Acid – ALHA (HIX=52.3) is also in agreement with the literature (HIX>50) (Ateia et al, 2017; Birdwell et al, 2010).

The biological index (BIX) was introduced by Huguet to determine the relative presence of the M fluorophore, characteristic of autochthonous biological activity in water (Huguet et al, 2009). It can be roughly described as the M peak/C peak ratio and is also called “freshness index” since it represents recently produced DOC (Morling et al, 2017). BIX values ranging from 0.8 to 1.0 are indicative of freshly produced DOM of biological/microbial origin whereas BIX < 0.6 indicates little amount of organic matter from autochthonous origin (Birdwell et al, 2010). As can be observed in Table 2 all the wastewaters show BIX > 0.7 (again with the exception of the leachates) and most of them above 0.8, indicating the majority presence of organic matter freshly released into water. The slaughterhouse effluent has by far the greater BIX value (1.47). On the other hand, the leachates show low BIX (0.48 and 0.52), indicating little presence of organic matter from biological/microbial origin but from terrestrial sources. ALHA shows the lower BIX value (0.28) in agreement with its terrestrial origin.

#### **4.- CONCLUSIONS**

A series of 13 different types of wastewaters (urban and industrial) have been characterized based on their EEM fluorescence spectra. Tryptophan protein-like peaks ( $T_1$  and  $T_2$ ) are the predominant fluorescence in urban and food industry wastewaters (brewery, winery, dairy/milk, biscuit and fish farm industries) but no special fingerprint has been found to discriminate among them. Protein-like fluorescence is also the majority one in meat/fish industries (effluents from a tinned fish industry and a slaughterhouse), but in this case tyrosine protein-like peaks ( $B_1$  and  $B_2$ ) also appear in the spectra in addition to tryptophan-like peaks. This fact might constitute a feature to differentiate these wastewaters from those of the previous category since the appearance of peaks B is quite uncommon in wastewaters. Additionally, the slaughterhouse effluent shows greater fluorescence intensity for all the protein-like peaks (B and T) than the tinned fish effluent, which might also constitute a specific feature to distinguish between both wastewaters.

Concerning non-food industries, the textile dyeing effluent shows a characteristic triple humic-like fluorescence (peaks A,  $C_1$  and  $C_2$ ) that may represent a specific fingerprint for this kind of effluent.

Leachates from medium-aged and old landfills might also show a specific fingerprint in their EEM spectra: the sole presence of the humic-like peak C with very high fluorescence intensity. This feature also allows differentiating them from young landfill leachates, which show predominance of protein-like peaks.

The fluorescence index (FI) does not seem to be very appropriate to characterize wastewaters and its usefulness might be limited to the study of humic substances in natural waters. However, the biological index (BIX) and the humification index (HIX) do seem to be useful for studying wastewaters, since they have provided consistent results in the present study.

This study shows the potential of EEM fluorescence to identify the origin of some industrial effluents. Obviously, more research is needed to complete this work: more effluents from industrial facilities belonging to the categories studied in this work must be sampled and characterized in order to check these preliminary results.

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Table 1. Main peaks identified in wastewaters

| INDUSTRY                                       | T <sub>1</sub>     | T <sub>2</sub>     | A                  | C                                     | B <sub>1</sub>    | B <sub>2</sub>    |
|--|--------------------|--------------------|--------------------|---------------------------------------|-------------------|-------------------|
| Urban wastewater (influent)                    | 280/355<br>(2.82)  | 225/362<br>(3.92)  | 225/423<br>(3.69)  |                                       |                   |                   |
| Mixture urban/industrial wastewater (influent) | 280/360<br>(9.91)  | 225/363<br>(6.01)  |                    |                                       |                   |                   |
| Brewery (effluent)                             | 280/351<br>(2.25)  | 230/348<br>(2.23)  |                    | 335/427<br>(1.48)                     |                   |                   |
| Winery (effluent)                              | 285/347<br>(0.87)  | 230/347<br>(1.47)  |                    |                                       |                   |                   |
| Milk (effluent)                                | 280/352<br>(2.06)  | 230/348<br>(3.71)  |                    | 335/421<br>(0.96)                     |                   |                   |
| Biscuit (effluent)                             | 285/354<br>(3.85)  | 225/363<br>(3.20)  | 225/421<br>(3.36)  | 335/421<br>(3.58)                     |                   |                   |
| Tinned fish (effluent)                         | 280/356<br>(3.81)  | 225/363<br>(5.22)  |                    | 340/413<br>(3.16)                     | 280/314<br>(3.47) | 225/316<br>(3.96) |
| Slaughterhouse (effluent)                      | 275/354<br>(10.48) | 225/361<br>(8.98)  |                    |                                       | 275/312<br>(9.74) | 225/310<br>(7.66) |
| Pulp mill (effluent)                           | 280/354<br>(2.94)  | 230/361<br>(4.31)  | 225/411<br>(3.82)  | 305/413<br>(4.64)                     |                   |                   |
| Textile dyeing (effluent)                      | 290/354<br>(12.36) | 230/343<br>(15.58) | 225/423<br>(12.05) | C <sub>1</sub> :<br>240/421<br>(9.55) |                   |                   |
|  |                    |                    |                    | C <sub>2</sub> :<br>305/415<br>(9.33) |                   |                   |
| Medium-aged landfill leachate                  |                    |                    | 250/461<br>(1.91)  | 375/463<br>(4.21)                     |                   |                   |
| Old landfill leachate                          |                    |                    | 250/449<br>(4.95)  | 345/425<br>(17.29)                    |                   |                   |

$\lambda_{ex} / \lambda_{em}$  (Fluorescence Intensity).  $\lambda$  in nm, Fluorescence Intensity in Raman units (R.U)

Table 2. Fluorescence indices for wastewaters.

| <b>INDUSTRY</b>                                | <b>HIXnorm</b> | <b>HIX</b> | <b>FI</b> | <b>BIX</b> |
|--|----------------|------------|-----------|------------|
| Urban wastewater (influent)                    | 0.61           | 1.70       | 1.39      | 0.87       |
| Mixture urban/industrial wastewater (influent) | 0.47           | 0.90       | 0.71      | 0.97       |
| Brewery (effluent)                             | 0.64           | 1.90       | 1.48      | 0.75       |
| Winery (effluent)                              | 0.65           | 1.96       | 1.45      | 0.95       |
| Milk (effluent)                                | 0.66           | 2.04       | 2.83      | 1.04       |
| Biscuit (effluent)                             | 0.63           | 1.77       | 1.63      | 0.87       |
| Tinned fish (effluent)                         | 0.54           | 1.25       | 1.94      | 0.98       |
| Slaughterhouse (effluent)                      | 0.44           | 0.80       | 1.86      | 1.47       |
| Pulp mill (effluent)                           | 0.57           | 1.41       | 2.03      | 0.78       |
| Textile dyeing (effluent)                      | 0.68           | 2.26       | 1.65      | 0.99       |
| Medium-aged landfill leachate                  | 0.83           | 6.01       | 1.33      | 0.48       |
| Old landfill leachate                          | 0.95           | 28.0       | 1.85      | 0.52       |
| Aldrich humic acid                             | 0.97           | 52.3       | 0.98      | 0.28       |

**HIX**: humification index, **HIXnorm** (normalized: 0-1), **FI**: fluorescence index, **BIX**: biological index

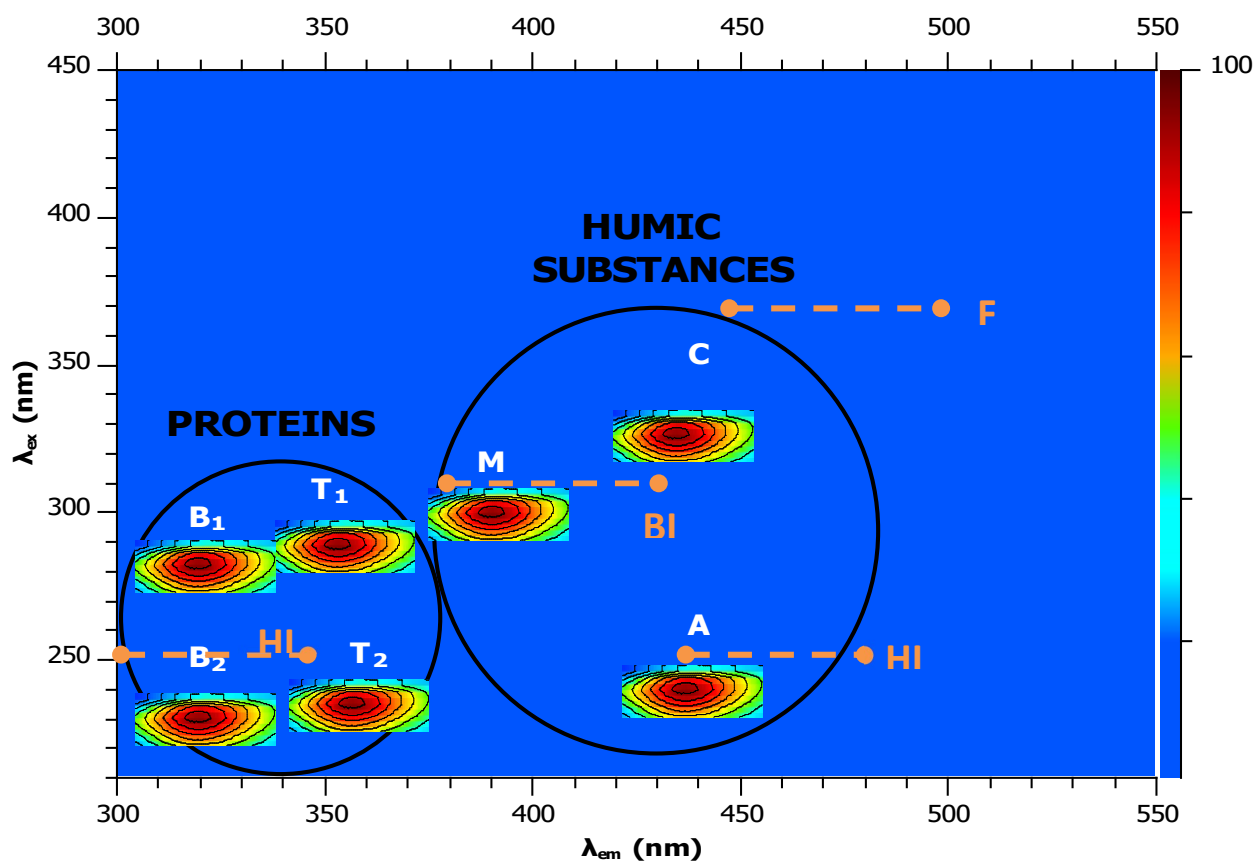


Figure 1. Location of the main EEM peaks and fluorescence indices.



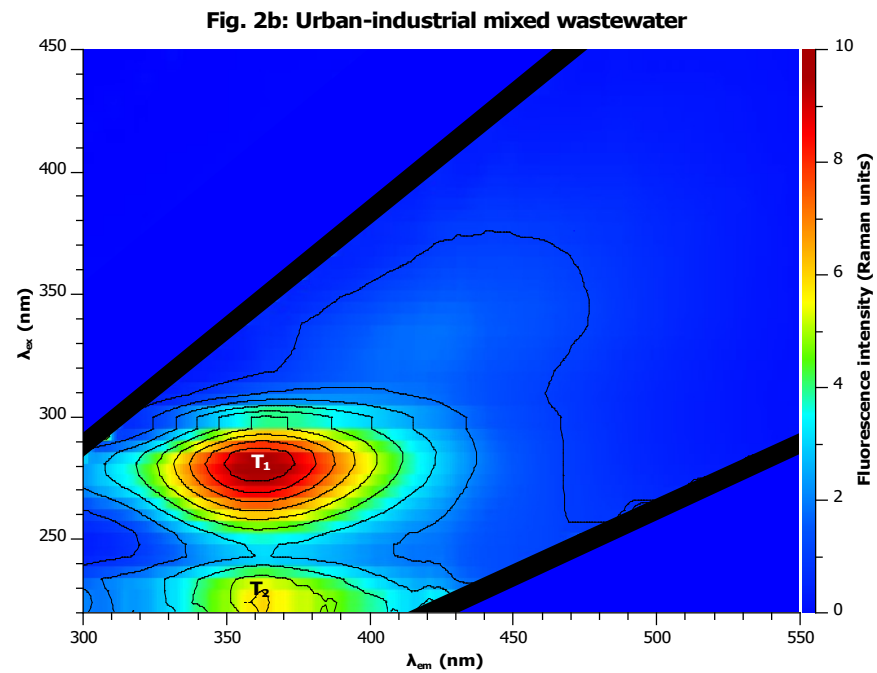
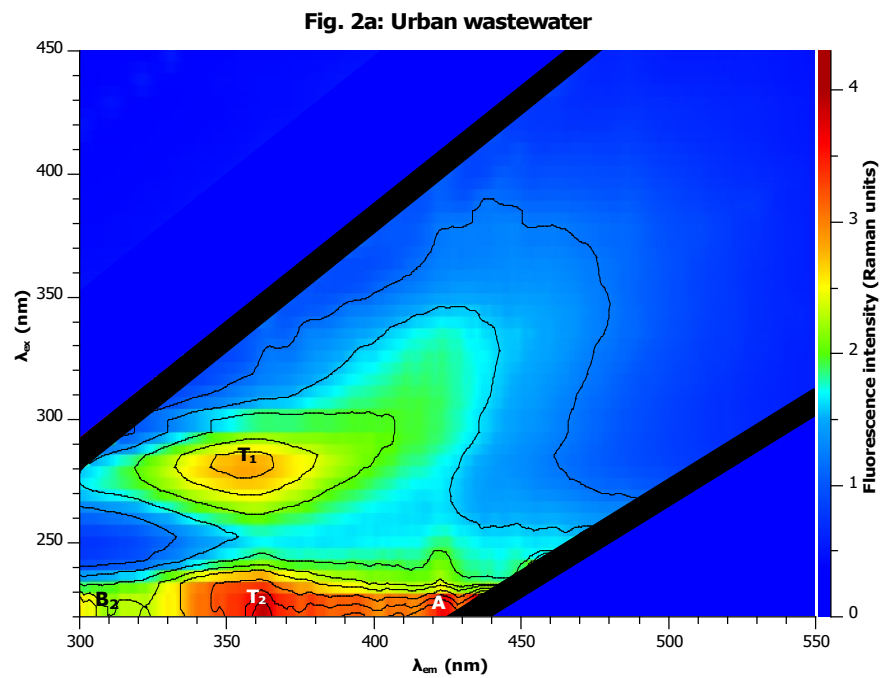


Figure 2. Municipal wastewaters influents: urban wastewater (Fig. 2a) and mixture urban-industrial wastewater (Fig 2b).

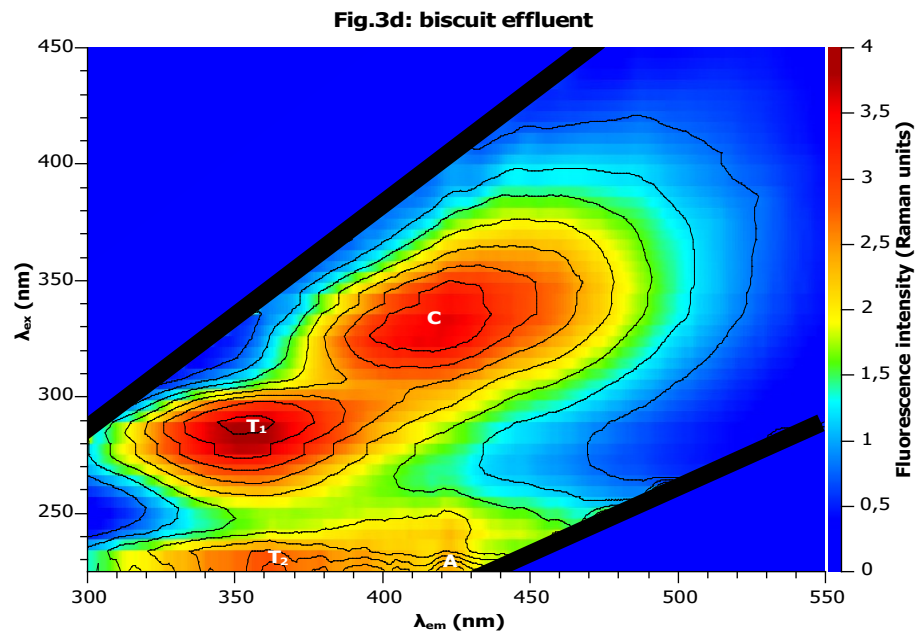
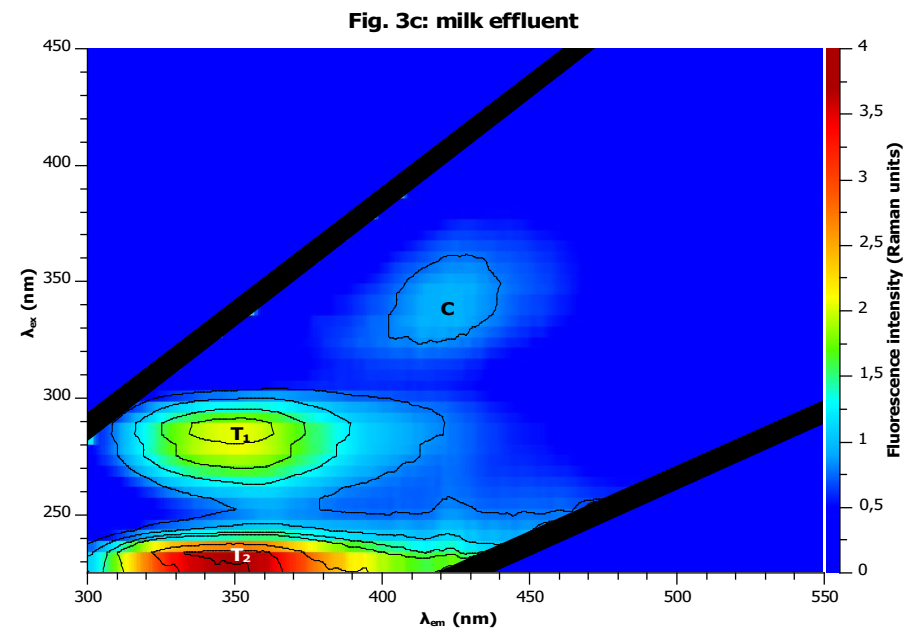
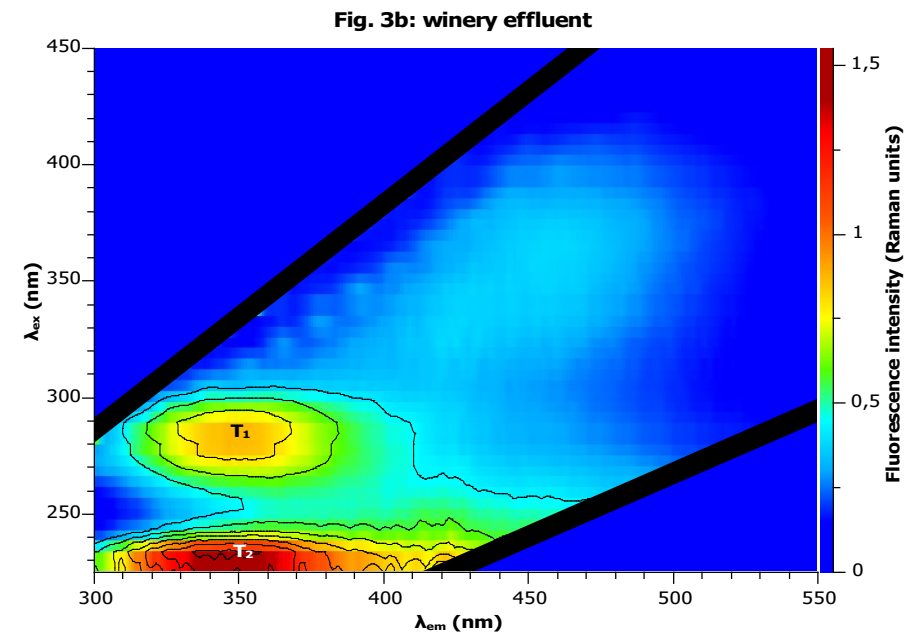
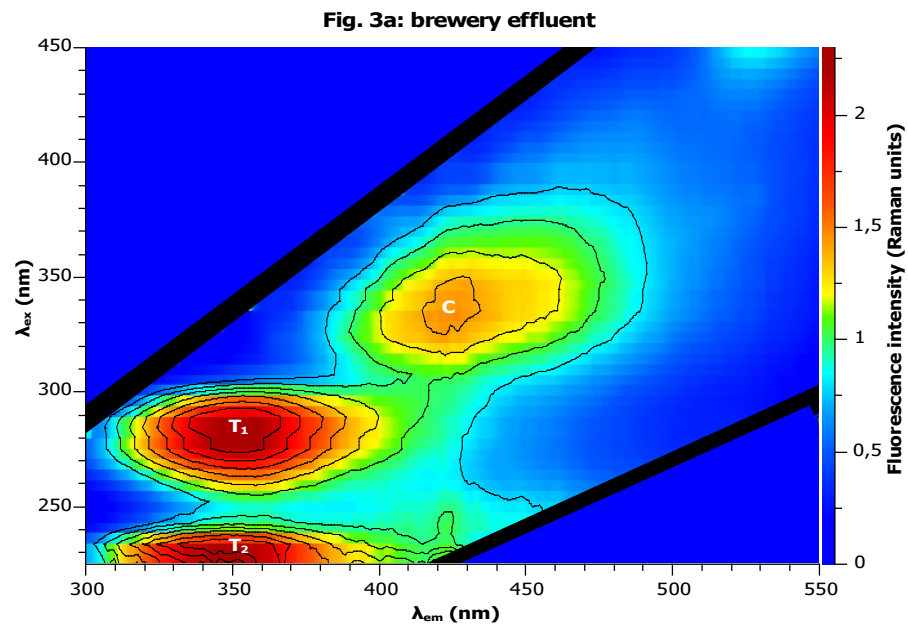
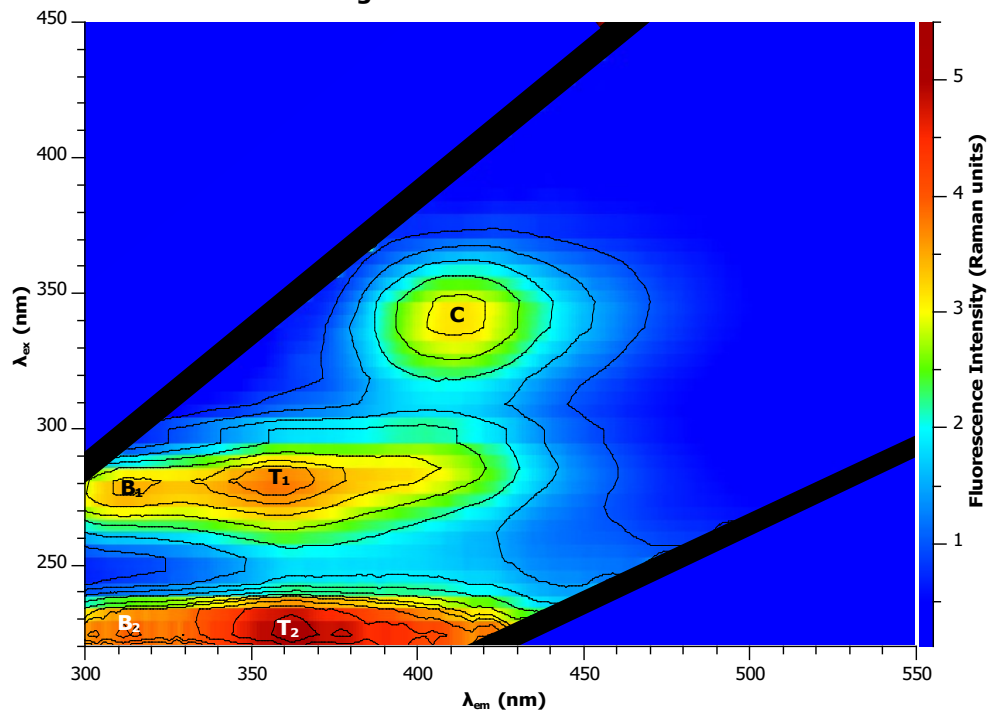


Figure 3. EEM from food industries effluents: brewery (Fig. 3a), winery (Fig. 3b), milk (Fig. 3c) and biscuit (Fig. 3d).

**Fig. 4a: tinned fish effluent**



**Fig. 4b: slaughterhouse effluent**

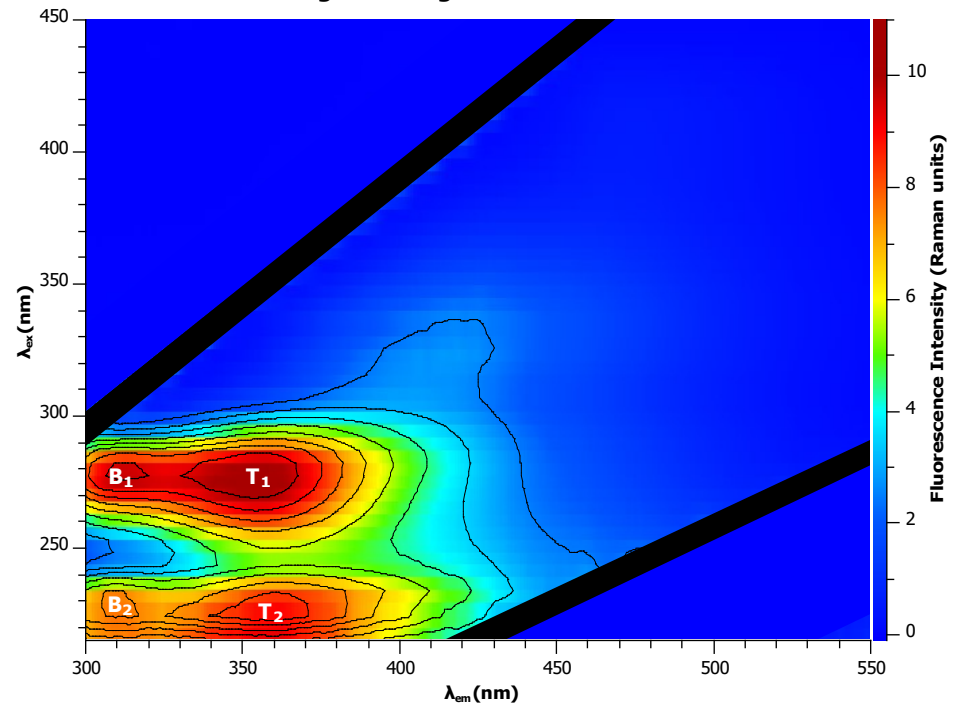


Figure 4. EEM from meat/fish industries effluents: tinned fish (Fig. 4a) and slaughterhouse (Fig. 4b).

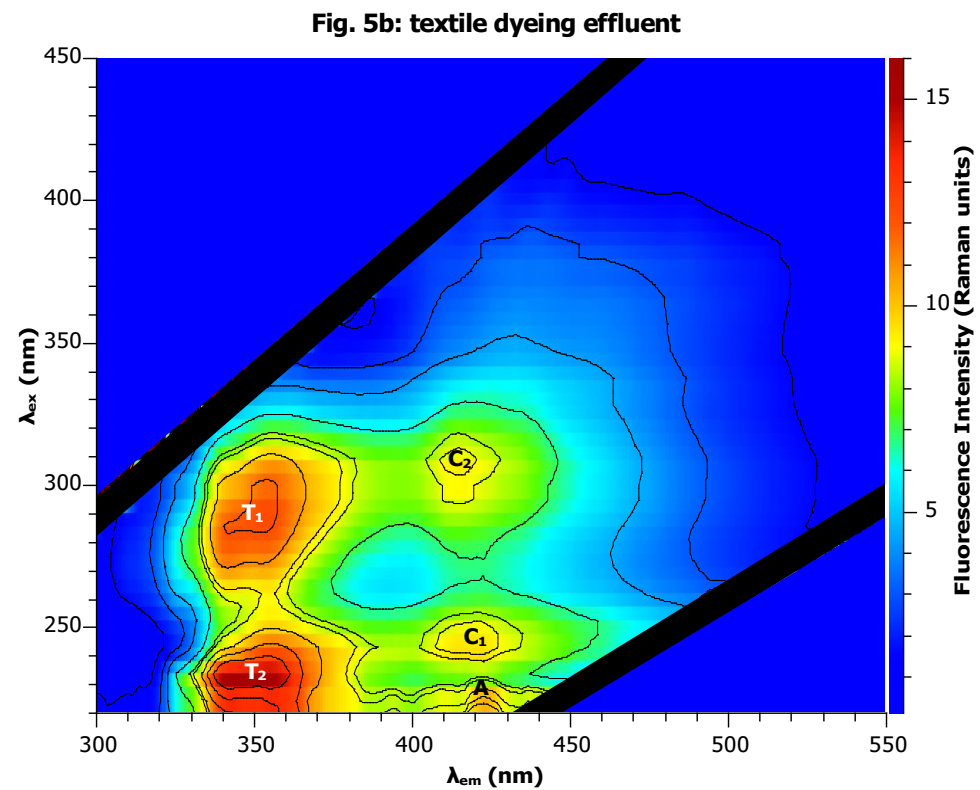
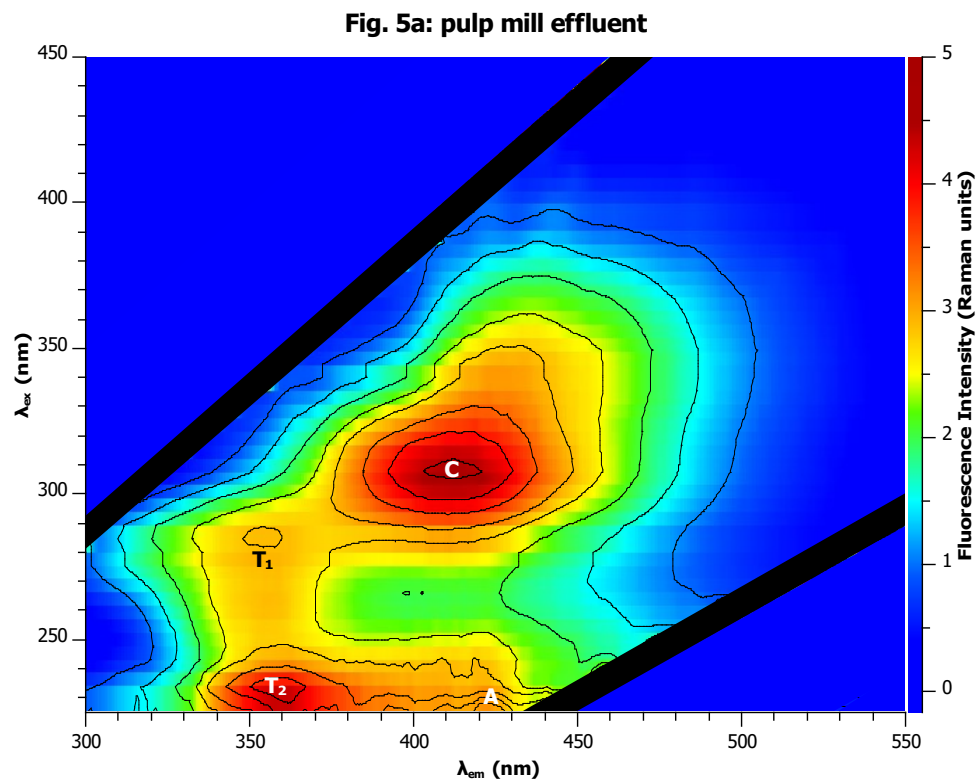


Figure 5. Other industries effluents: pulp mill (Fig. 5a) and textile dyeing (Fig. 5b).

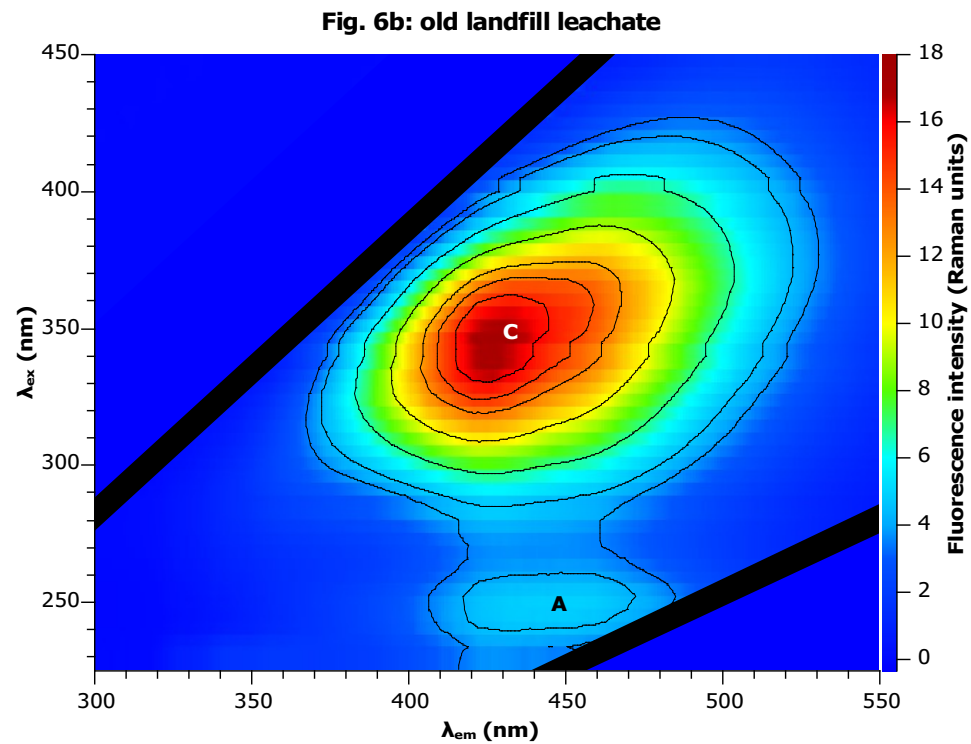
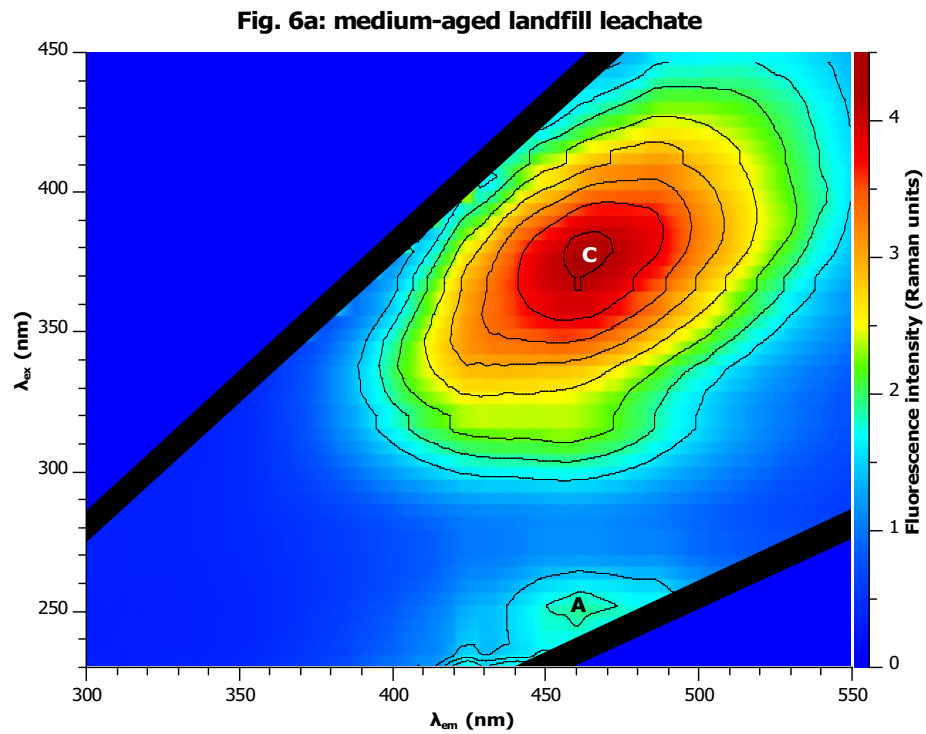


Figure 6. EEM spectra of landfill leachates: medium-aged leachate (Fig. 6a) and old leachate (Fig. 6b)