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Additional Information

Separation and identification of microplastics from primary and secondary effluents and  
activated sludge from wastewater treatment plants

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**Keywords** – Activated sludge, Effluents, Microfiber, Microplastic, Wastewater  
Treatment Plant

**Abstract**

Although wastewater treatment plants can retain a high percentage of microplastics (MP) arriving at the facilities, no method for extracting and characterizing these microparticles has been still standardized in these units. This study investigated three protocols of chemical digestion, prior to analysis of microplastics, one directed to the effluents, using peroxidation, and two for activated sludge (peroxidation and Fenton). The samples (primary effluent, secondary effluent and activated sludge) were collected from a wastewater treatment plant (WWTP) located in Valencia (Spain). In addition, four

common types of polymers (Low density polyethylene-LDPE, Polypropylene-PP, Polystyrene-PS and Polyethylene terephthalate-PET) were used to assess the influence of reagent exposure on microparticle integrity. Peroxidation was effective in treating the studied effluents (primary and secondary) and was also identified as the ideal protocol for activated sludge. The analysis showed that the use of H<sub>2</sub>O<sub>2</sub> does not compromise the identification of the polymers evaluated by FTIR and also significantly reduced the concentration of suspended solids, resulting in an efficient visual separation of the microparticles. After been properly separated, the microparticles were characterized according to their size, colour and shape, and a fraction submitted to identification by  $\mu$ -ATR-FTIR/ATR-FTIR. In all samples, a high presence of microfibers (MF) was observed, corresponding to more than 90% of the microparticles. However, in relation to secondary effluents, only 9% of these MF were identified as plastics, the remaining ones corresponded to cotton. The fragments found in the samples were classified as secondary in origin, and were mainly PE and PP, lower than 1mm size.

### **Abbreviations**

ATR-FTIR - Attenuated total reflectance -Fourier transform infrared

MF - Microfiber (include natural and plastic)

MP – Microplastics (include all shape)

LDPE – Low density polyethylene

PE – Polyethylene

PET - Polyethylene terephthalate

PP – Polypropylene

PS – Polystyrene

## **1. Introduction**

Plastic materials have various applications and, due to their low cost of production, they are highly prominent across numerous industrial sectors (e.g. the construction, packaging, and automotive industries). In 2018, 359 million tons of plastics were produced globally - almost 62 million tons of which were produced in Europe alone [1]. The inevitable presence of synthetic polymers within the daily lifestyles of the world population, resulting in the uncontrolled release of significant plastic material, has implemented further pressure on environmental authorities.

Small plastic particles with dimensions smaller than 5mm can be considered as 'microplastics' (MP). In this context, MP can be classified as secondary when they are formed as a result of the fragmentation of larger plastics [2,3,4]. It is expected that these MP will be continuously generated in the future, due to several reasons: the high consumption of synthetic polymers (together with the inadequate management of subsequent residues), the insufficient disposal and recycling systems, and also the lack of public awareness regarding the issue. On the other hand, MP used as a feedstock in the formulation of various products, such as: exfoliating material in soaps, facial scrubs, shampoos, shaving foam and toothpaste, are denominated primary MP – and hence their presence in WWTP results from the consuming lifestyle habits of the modern population [5,6,7].

WWTPs are important barriers that act physically, chemically and biologically towards the control of pollutants in the environment. Despite their high performance in microplastic retention - reaching efficiencies of approximately 90% according to some authors [4,8,9] - these facilities still release high amounts of MP into the environment

since the volume of effluents generated by them is so large. That is, no matter how low the concentrations of MP per litre of effluent released - by extrapolating any value to daily WWTP flows, millions of particles are still released into the environment [8,10,11,12]. Studies have shown that the high retention capacity of MP in WWTPs can be attributed to the efficiency of the primary processes and to their retention in the secondary sludge – however this may lead to an additional environmental problem, as this biological material is often reapplied as fertilizer, hence resulting in soil contamination [13,14].

To prevent the occurrence of such additional issues, several researchers have proposed techniques for separating, identifying and quantifying microparticles in WWTP. Despite the significant research that has been conducted, divergence in the stages of sampling, chemical digestion and identification has made the comparison of results difficult and unreliable. This study aims to evaluate adequate protocols for the separation, quantification and identification of MP in three streams in a WWTP (investigating effluents from the primary and secondary settling, and activated sludge).

## **2. Materials and methods**

### **2.1. General definition of microparticle and microplastic**

This section aims to clarify the define and differentiate microparticles and microplastics in the context of this research. Microparticles are considered particles that have dimensions smaller than 5mm. In this group, all kinds of materials were included (natural and synthetic). In this way, the reference to microparticles was attributed to unknown materials, separated and classified by visual sorting before FTIR analysis. Once carried out, the FTIR analysis properly identified the polymers, and subsequently the microparticles were classified as microplastics if they were synthetic polymers or a mixture of synthetic polymer and natural matrix.

## 2.2. Preliminary study with MPs samples

Preliminary experiments were carried out with known MP samples to assess the interference of chemical digestion in the MP identification process. To start the procedure with MP samples, the workbench was previously cleaned with distilled water and alcohol as well as the work materials (tweezers, scissors, petri dishes) to avoid possible contamination.

MP samples were taken from commercial plastics: garbage bags (LDPE), water bottles (PET), disposable dishes (PS) and straws (PP). These four polymers corresponded to more than 50% of Europe demand in 2018 [15]. First, the plastic samples were reduced in size by using a crusher and next, the MP were separated by stainless steel sieves in a range between 1mm and 400 $\mu$ m.

Before and after the chemical digestion, MP were characterized by ATR-FTIR (Bruker) spectroscopy to confirm that polymers can be correctly identified after the chemical digestion, i.e. to check that no damage on the polymer had been produced. Four chemical digestion protocols were tested, two peroxidation processes at different concentrations (30% and 35%  $H_2O_2$ ), and two Fenton's reactions. Fenton's reactions were performed at pH 3 at the same  $H_2O_2$  concentrations used for peroxidation, plus the  $Fe^{+2}$  catalyst solution (iron sulphate heptahydrate 20mg/ml). The pH correction to 3 was performed with sulphuric acid (96%). For digestion processes, an initial mass of 20mg of plastic material was weighed and mixed with 5mL of peroxide. For the Fenton's reactions, 5mL of peroxide plus 2.5mL of  $FeSO_4$  solution (catalyst solution). The chemical digestion protocols were performed separately for each polymer.

According to prior research, temperatures above 60°C may be aggressive for polymers [16]. For this reason, peroxidation were performed at temperatures of 60 $\pm$ 2° for 4 hours and Fenton's reactions were executed at room temperature for 2 hours using ice baths,

when necessary, to maintain the temperature below 40°C due the exothermic behaviour of the reaction.

After the chemical digestion, the samples were vacuum filtered on a 1 µm glass fiber filters and dried in a laboratory oven for two hours at 50°C. Next, the filters were introduced in a desiccator for approximately 12 hours to complete the drying. After that, MP were carefully separated from the filters to petri dishes with tweezers. All digestion assays were triplicated, and the MP were randomly selected to finally be analysed by ATR-FTIR spectroscopy.

To assess possible mass loss due to the oxidation processes or even due to the separation process, the percentage of plastic material recovery after each chemical digestion was determined using a gravimetric method, considering the MP mass before (initial) and after (final) the digestion process. The recovery percentage was obtained by Eq.1.

$$\%Recovery = \frac{MP\ Mass_{final}}{MP\ Mass_{initial}} \times 100 \quad (1)$$

### **2.3. Sampling and microplastics separation from WWTP**

Samples were collected using plastic containers from different points at an urban WWTP located in Valencia (Spain): primary settling (primary effluent), secondary settling (secondary effluent) and aerobic biological reactor (mixed liquor). All the samples were collected in May/2019 to avoid weather influences, and directly transported to the laboratory for processing. Firstly, the primary and secondary effluent samples were passed through a 150µm aperture stainless steel mesh screen. Next, the retained material was chemically digested. For mixed liquor, due to the high load of suspended solids, chemical digestion of the sample was performed first to avoid blocking the sieve opening. After chemical digestion, the digested sample was passed through a 150µm mesh (Fig.1).

Two different chemical digestion protocols were carried out to achieve a better MP separation from the organic matrix. The Protocol 1 was established for the primary and secondary effluents, whereas the Protocol 2 for the sludge (mixed liquor). The difference between the two protocols consists only in the order of the processes. The Protocol 1 was set up for samples with less total suspended solids (TSS), which can be subjected to physical separation through sieves before chemical digestion without causing their immediate blockage. However, in Protocol 2 chemical digestion step precedes the sieve separation to avoid the sieve blockage, which would be produced by the high organic load and TSS concentration in sludge samples.

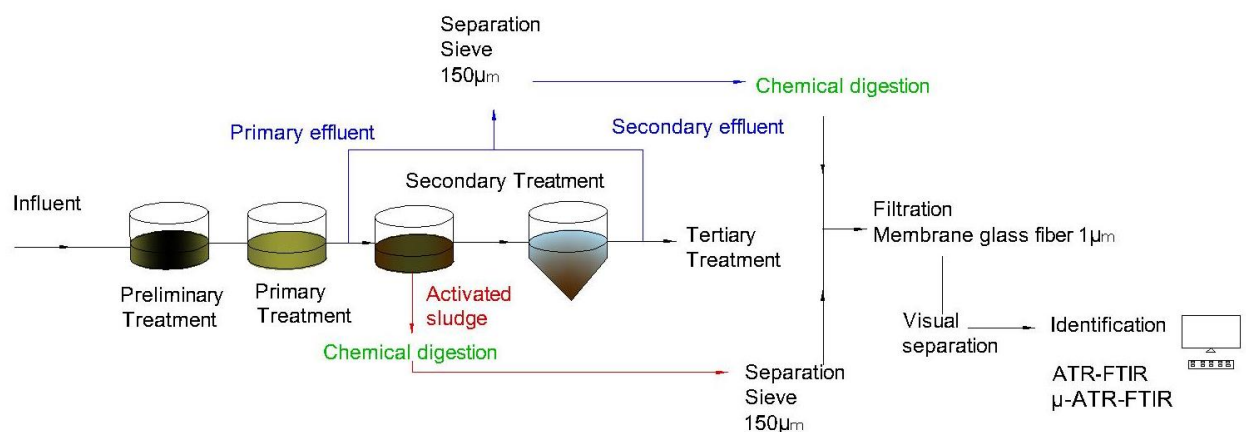


Fig.1. Flow chart of separation and identification MP from different samples

It was decided to use only a 150µm size mesh and evaluate individually the size of the retained microparticles. This methodology was thus established since the passage of microfibers through the opening sieve does not occur regularly, i.e., due to their morphology, the microfibers can be transversely retained on the meshes or pass longitudinally through them. Ziajahromi et al. [12] used a series of sieves with different apertures (500,190,100 and 25µm) and observed that the fibre size range was larger than



the size of the previous filter, fibres over 100 $\mu\text{m}$  in length were found in the 25 $\mu\text{m}$  mesh. Lee and Kim [17] also observed similar results, when using two sieves of 106 and 300 $\mu\text{m}$ . Fibres larger than 300 $\mu\text{m}$  could pass through the 300 $\mu\text{m}$  mesh sieve, and therefore fibres classified in the size range of 106-300 $\mu\text{m}$  could be underestimated. In this way, in order to avoid misclassification, it was decided to use a stainless-steel mesh of 150 $\mu\text{m}$ , and the material retained on the mesh was collected and characterized according to size, colour and shape one by one under a stereomicroscope.

As suggested by Lares et al. [18], density separation techniques were not used in this work to avoid the loss of high density microparticles. Polymers such as polyester (PET) (1.37 – 1.45g/cm<sup>3</sup>) could be underestimated when undergoing density separation with NaCl (1.2g/cm<sup>3</sup>). It would be necessary to use a higher density saline solution, such as NaI (1.8 g/cm<sup>3</sup>) to ensure effective separation [19,20]. However, when working with high matrix volumes, it could be expensive.

### **2.3.1. Protocol 1 - Primary and secondary effluent samples**

The primary and secondary effluents were previously characterized in terms of TSS concentration. The protocol was adapted from Ziajahromi et al. [12]. Five liters of each sample were filtered through the stainless-steel sieve (150 $\mu\text{m}$ ). The retained material was separated from the sieve with 100mL of distilled water and digested with 1mL of H<sub>2</sub>O<sub>2</sub> 35%wt for 2hours at a temperature of 60 $\pm$ 2 $^{\circ}\text{C}$ . When necessary, higher volume of water was used without changing the aforementioned relation. After that, the digested sample was filtered through a glass fiber filter (aperture of 1 $\mu\text{m}$ ). The filter was dried at 50 $^{\circ}\text{C}$  in a laboratory oven for 2 hours.

### **2.3.2. Protocol 2 –Sludge samples**

To find out the proper chemical digestion for mixed liquor, two peroxidation processes and two Fenton's were applied (Fig.2). The peroxidation reactions were performed with 10mL of sample and 20mL of peroxide 30% and 35% wt, for 4 hours at temperature of  $60\pm 2^{\circ}\text{C}$ . Fenton's reactions were also performed with 10mL of samples, 20mL of peroxide 30% and 35%wt and 10mL of catalyst solution (iron sulphate heptahydrate 20mg/ml), as suggested by Tagg et al. [21]. The temperature was maintained below  $40^{\circ}\text{C}$ , using ice baths when necessary. The chemical digestion efficiency was assessed in terms of total suspended solid (TSS) removal. Next, the digested sample was vacuum filtered on glass fiber filter ( $1\mu\text{m}$ ) and subsequently the filter was dried at  $105^{\circ}\text{C}$  in a laboratory oven for 1 hour and weighed for TSS determination.

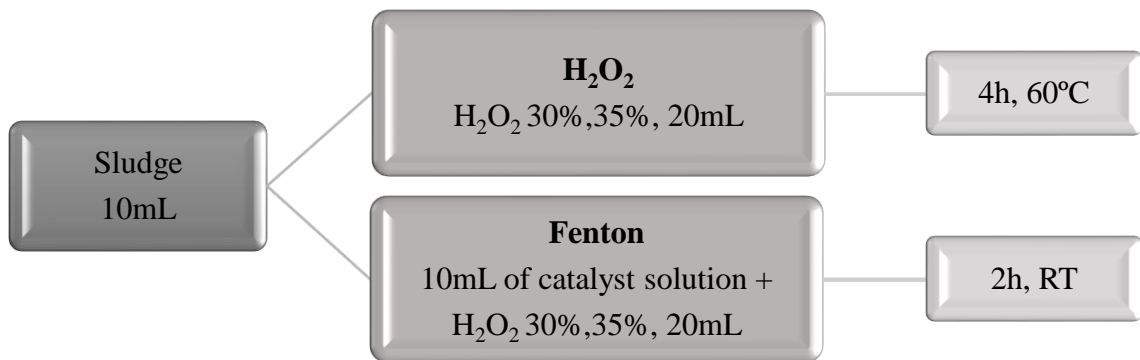


Fig. 2. Chemical digestion proposed for activated sludge

## 2.4. Microplastics detection

### 2.4.1. Visual sorting

To assist the visual inspection, a circular acetate mesh divided into octants customized by the researchers, was used to control microparticle counting, reducing the risk of microparticles over or underestimation. The materials were carefully analysed by stereomicroscope (LEICA MZ APO). The magnification was adjusted between 8X and

80X. This step allowed classification according to colour, shape and size. Three shapes were considered for the classification of the microparticles found: fibres (linear filaments with length less than 5mm), fragments (irregular shape, probably generated from the fragmentation of larger plastics) and spheres (three-dimensional circular shape).

Visual separation was carried out according to criteria addressed by Hidalgo-Ruz et al. [22]: (a) no organic materials on samples, (b) fibres should have same thickness over the entire length and (c) the colours of the samples should be evident and homogeneous. Besides that, microparticles with fragile structure, which fragmented into smaller pieces when subjected to pressure applied by the forceps were excluded from counting and classification as possible MP.

Visual identification has been used by several authors to classify microparticles [9,11,23,24]. However, an additional analysis is required to confirm the microparticle chemical structure in order to avoid misclassification, since natural fibres can be easily classified as synthetic. Consequently, microparticles identification based on polymeric analysis was key for the interpretation of results.

#### **2.4.2. Microplastic identification**

Polymeric identification was performed using attenuated total reflection (ATR) method of Fourier Transform-Infrared spectroscopy (FTIR) (Bruker) under spectral resolution of  $4\text{cm}^{-1}$ , sample scan 32 and spectrum with wavelengths between  $400$  and  $4000\text{cm}^{-1}$ , for microparticles larger than  $400\mu\text{m}$ . For smaller fragments and fibres was necessary to use the ATR-FTIR equipment coupled to a microscope ( $\mu$ -ATR-FTIR). The  $\mu$ -ATR-FTIR (Bruker) was operated under spectral resolution of  $6\text{cm}^{-1}$ , sample scan 128 and spectrum with wavelengths between  $600$  and  $4000\text{cm}^{-1}$ . All spectra were analysed using the

software Bio-Rad KnowItAll® Informatics System 2018, applying baseline correction and no-ATR correction.

From the FTIR analysis of the known MP samples (Section 2.1), an own database was set up for the subsequent polymeric characterization of samples separated from effluents and activated sludge. The spectra of known polymers, used as a reference, were directly compared with the MP extracted from the samples. To that end, the two spectra were overlaid and the analysis of each peak (starting with the characteristic peaks of each polymer) was carried out. Materials that did not match to the database spectra were compared to literature data for their classification.

Regarding the polymeric identification of fibres by  $\mu$ -ATR-FTIR, Peets et al. [25] point out that the intensity of the absorbances produced by the sample depends on the pressure applied by the crystal. In addition, fibres made of more than one material, and how these materials are organized on the fibre structure can result in spectrum with different qualities and intensities depending on the point of contact with the crystal. Thus, the existence of materials made up of copolymers may make the identification more difficult, requiring careful analysis of the spectrum. In the classification of microplastic materials, both synthetic fibres and copolymers and mixtures of natural and synthetic fibres were considered.

## **2.5. Quality assurance/Quality control (QA/QC)**

To minimize possible cross-contamination from other MP sources, glass materials were used whenever possible, avoiding the use of plastic materials as well as a cotton lab coat instead of polyester or another material made of synthetic fibres. Each new fibre glass filter was checked directly when it was extracted from the storage box with a stereomicroscope. In order to identify possible airborne contamination, a first control

glass fiber filter was left exposed on the workbench for 6 hours and another filter for 7 days.

## **2.6. Statistical analysis**

The statistical significance on the size of the microplastics counted in the primary, secondary, and activated sludge samples was assessed by Kruskal-Wallis analysis (95% confidence level) with the Statgraphics Centurion XVII. Bonferroni post hoc test was performed to identify differences between paired comparisons.

## **3. Results and discussion**

### **3.1. QA/QC**

No fibres were identified with the stereomicroscope when the glass fiber filters were taken out of their storage box. Regarding airborne contamination, a number of 8-fibers were quantified on the control filter, and sorted out as 3 blue, 4 red and 1 transparent. No fragments were observed. Another test was carried out for 7 days under the same conditions and 13-fibers were counted on the filter. As the sample handling time was approximately 6 hours, the number of 8 detected fibres was discounted from the processed samples for MP quantification. To minimize contamination of the samples, precautions were taken, such as the use of aluminium foil to cover the glassware used and the use of petri dishes with a lid to avoid leaving the filters exposed after processing the samples.

### **3.2. Effect of chemical digestion on plastic material**

Fig. 3 shows the ATR-FTIR spectra of the MP before and after chemical digestion. According to the FTIR results, after the chemical digestion protocols the FTIR spectra

did not show significant deviations from the initial spectra (without digestion), making the identification of the polymer possible. For all plastic materials, the main peaks kept their intensity allowing their identification after digestion step. In the PP spectra, after being subjected to Fenton 30% and 35%, was observed the appearance of absorption bands in the wavelength between  $3100\text{-}3600\text{cm}^{-1}$  and between  $1670\text{-}1800\text{cm}^{-1}$ , corresponding to the hydroxyl and carbonyl groups, respectively. The appearance of these bands may indicate the degradation of PP [26,27,27]. After undergoing chemical digestion with  $\text{H}_2\text{O}_2$  30%, an increase in absorbance of peaks  $1258$ ,  $1099$ ,  $1020$  and  $802\text{cm}^{-1}$  was observed. However, all the characteristic peaks of PP ( $2948$ ;  $2916$ ;  $2867$ ;  $2837$ ;  $1456$ ;  $1375$ ;  $1166$ ;  $973\text{ cm}^{-1}$ ) were easily identified and unlike the Fenton's reaction, characteristic bands of degradation were not identified. Tagg et al. [20] also assessed the impact of  $\text{H}_2\text{O}_2$  30% on the FTIR spectrum of microplastics and did not observe substantial changes in the spectra of PE, PP, PS, PVC and Nylon-6 polymers after being subjected to chemical digestion by 7 days. Hurley et al. [29] evaluated four protocols for chemical digestion and their influence on the identification of MP by FTIR. Among the protocols, peroxidation with  $\text{H}_2\text{O}_2$  30% (at  $60^\circ\text{C}$ ) and Fenton's reaction were also evaluated, and no significant spectral modification was observed for the tested polymers (PP, LDPE, HDPE, PS, PET, PA-6.6, PC, PMMA). Based on this, it was concluded that, both peroxidation and Fenton did not compromise the chemical characterization of LDPE, PS, PP and PET via FTIR, since they do not result in deviations from the main peaks necessary to identify the unique fingerprint of each polymer. Fenton's reaction seems more aggressive to PP, possible resulting in its degradation. Scanning electron microscopy (SEM) could be a method to confirm this degradation. It is important to highlight that these experiments were carried out with virgin polymers, it is also important

to evaluate the effects of chemical digestion in MP that have already suffered weathering and degradation due to abiotic factors and biodegradations.

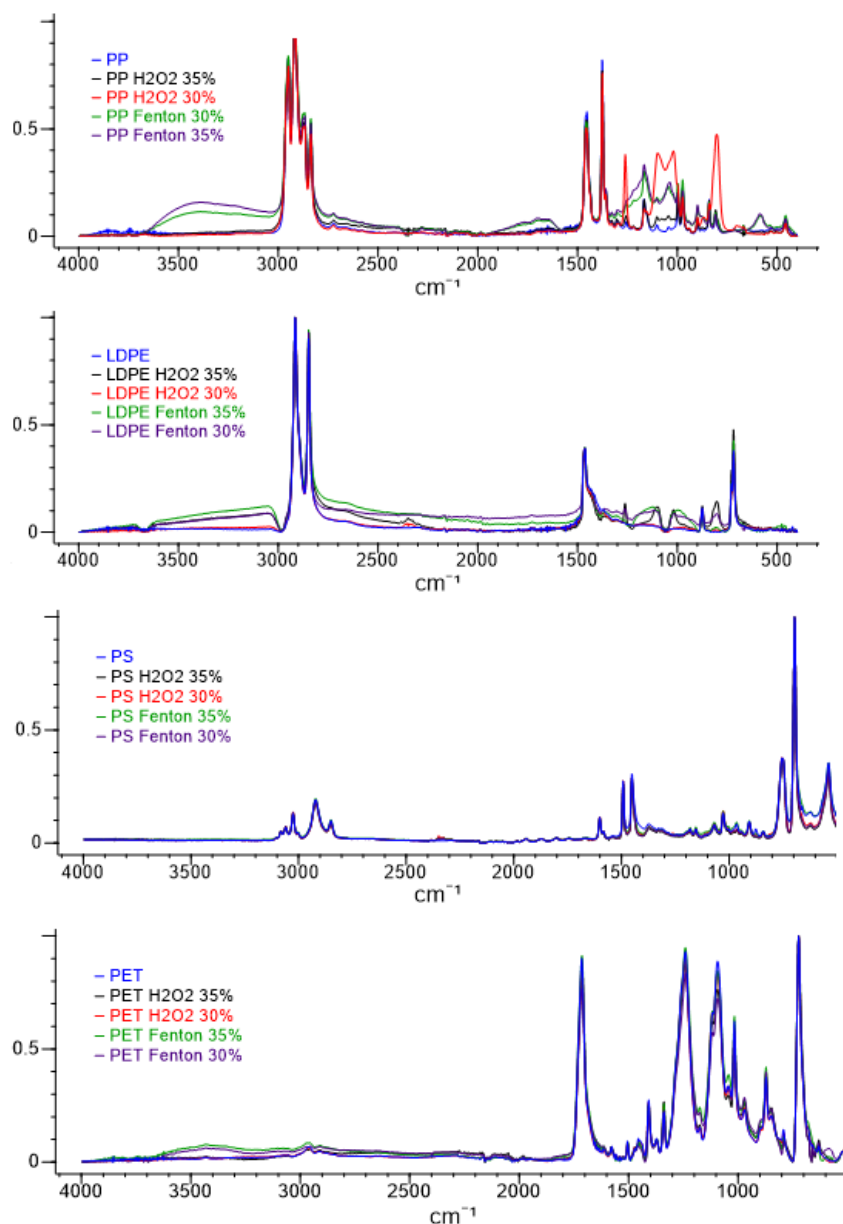


Fig. 3. MP spectra before and after the chemical digestion step

Regarding the percentage of mass recovery of the polymers after chemical digestion, PP, PET and PS reached more than 90%. Only LDPE showed a low recovery when subjected to peroxidation (Table 1). Hurley et al. [29] observed mass changes of less than 1% for PP, PET, PS and LDPE polymers when subjected to chemical digestion with H<sub>2</sub>O<sub>2</sub>

30%(v/v) at 60°C and Fenton’s reagent. Based on these results, a procedure with only distilled water was carried out for LDPE to ascertain possible losses during the digestion process, and we obtained a mass recovery of 78%. During the separation process, it was observed that the LDPE got stuck in the walls of the glass, making it difficult to be completely removed from the container. The lower recovery rate of this polymer was then associated with the separation process, and not with the chemical reagents used. Another important point is the percentage recovery obtained for the PP. As demonstrated by FTIR results, the PP could suffer some degradation when subjected to Fenton’s reaction. The higher mass recovery of PP after Fenton’s process, compared to peroxidation, could be also related to mass loss during the separation process in the peroxidation protocol. These results show that the separation process must be carried out minimizing the loss of material, since it corresponds to an important step on MP study.

Table 1 - Polymer recovery (%) after chemical digestion

Polymer	Chemical digestion method			
	H <sub>2</sub> O <sub>2</sub>		Fenton	
	30%	35%	30%	35%
PP	92	96	99	96
PET	95	91	100	100
PS	96	97	98	92
LDPE	71	64	93	82

### 3.3. Samples from WWTP

#### 3.3.1. Primary and secondary effluents samples

For the primary effluent, it was observed that digestion was necessary to provide an effective visual characterization. For this, chemical digestion with H<sub>2</sub>O<sub>2</sub> 35% wt, in the



proportion 1:100 (H<sub>2</sub>O<sub>2</sub>:water) proved to be sufficient for the reduction of suspended solids, thereby increasing visibility of the sample (Fig. 4).

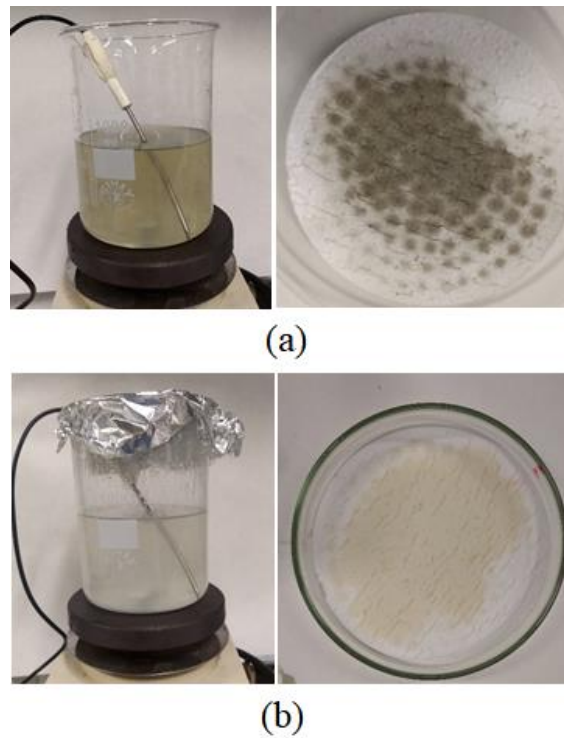


Fig. 4 . Primary effluent (250mL) before chemical digestion (a); primary effluent (250mL) after chemical digestion (b)

In the case of secondary effluent, chemical digestion will not be always necessary. Only if the sample presents a concentration of suspended solids that makes visual analysis unfeasible. In this case, digestion Protocol 1 must be properly applied. Table 2 shows the characteristics of the samples for both effluents.

Table 2 - Effluent characteristics

Sample	TSS (mg/L)	pH	Conductivity (mS/cm)
Primary effluent	42±8	7.57	1.673

Secondary effluent	7±3	7.91	1.757
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According to the experiments carried out, it is recommended to characterize previously the samples in terms of TSS to determine if chemical digestion is necessary. Each WWTP generates effluents in different qualities, although they must be within the permitted release standards. Thus, the method developed in this work aims to guide a viable path to be applied in the separation and characterization of MP present in WWTPs.

### 3.3.2. Activated sludge samples

The efficiency of the chemical digestion was measured in terms of total suspended solids removal (TSS). The best results were achieved with peroxidation at both H<sub>2</sub>O<sub>2</sub> concentrations (30% and 35%) and [H<sub>2</sub>O<sub>2</sub>:sludge] ratio of 2:1. For these conditions more than 90% of TSS removal was obtained (initial concentration 2500mg/L TSS, and volatiles corresponding 2127mg/L) (Table 3). However, for Fenton's reactions no more than 40% TSS removal was achieved. The process of advanced oxidation by Fenton is a complex process, whose efficiency depends on factors such as pH, catalyst concentration, concentration of peroxide and contaminants [30]. In non-optimized working conditions, both iron precipitation and loss of efficiency of oxidation of organic matter can occur. Furthermore, the Fenton's process, in addition to being able to oxidize organic matter, can also act as a coagulant due to the presence of ferric ions [31,32]. Thus, the value of lower percentage of TSS removal obtained by applying Fenton could also be attributed to the coagulation effect and/or to non-optimized working conditions.

Hurley et al. [29] also studied the efficiency of chemical digestion with H<sub>2</sub>O<sub>2</sub> (30% at 60°C; for 6 hours) and Fenton's reagent (using 30% peroxide at room temperature; for 2 hours) and achieved 44.6%±6.76 mass removal for peroxidation and 43.8%±6.61 for

Fenton. Amudha et al. [33] achieved a 22% reduction in TSS after Fenton. The efficiency of advanced oxidative processes (such as peroxidation and Fenton) regarding activated sludge digestion can be influenced not only by time, temperature and concentration of reagents, but also by sludge composition. Since extracellular polymeric substances (EPS) promote the flocculation of microbial colonies, the presence of this material in sludge can interfere on the solubilization process of organic matter. Amudha et al. [33] observed that the deflocculation of the sludge by citric acid before Fenton's reaction promotes an improvement in TSS removal, reaching 53% removal.

Table 3 – Efficiency of chemical digestion protocols for activated sludge in terms of TSS removal

Reagent	Reaction time (h)	Temperature (°C)	[sludge mL:H <sub>2</sub> O <sub>2</sub> mL]	TSS Removal(%)
H <sub>2</sub> O <sub>2</sub> 30%	4	60±2	1:2	92.35±0.58
H <sub>2</sub> O <sub>2</sub> 35%	4	60±2	1:2	93.55±2.52
Fenton 30%	2	RT	1:2	35.73±0.36
Fenton 35%	2	RT	1:2	45.56±4.64

RT: room temperature

Some authors use extensive chemical digestion times, which can take up to days for complete sample processing [8,20,34,35]. In our study, it was possible to achieve more than 90% removal of TSS in only 4 hours of digestion, which allows for efficient digestion with less time consumption.

### 3.4. Visual sorting

Visual sorting results are explained considering the shape (fibre, fragment, and sphere), size and colour of the microparticles.

#### **3.4.1. Concentration and shape of the microparticles**

Regarding the microparticles released daily, it is estimated that about  $1.12 \times 10^8$  Microparticles/day could leave the WWTP evaluated in this work in the secondary effluent. The concentration of microparticles in the final effluent will depend on both the processes used in the purification and the population served, however concentrations of the order of magnitude of  $10^8$  Microparticles/day were also reported by other authors [34,35,36]. In this research, from primary effluent (11.1 Microparticles/L) to secondary (2.8 Microparticles/L), a reduction of 74.8% was observed. This reduction could be related to the retention of microparticles in the activated sludge (280 Microparticles/L or 112.0 Microparticles/g dry weight) (Table 4). Kalčíková et al. [37], in a study carried out with SBR fed with PE microbeads, also observed that part of the MP added in the reactor was transferred to the activated sludge and deposited at the bottom of the tank. According to some authors, this affinity between MP and activated sludge could be related to the formation of biofilms around MP, which would reduce the buoyancy of this material [38,39].

When applied as biosolids, the MP present in the activated sludge can also represent an environmental problem for the terrestrial environment. Liu et al. [40] reported the occurrence of microplastics in agricultural soils in twenty vegetable farmlands in Shanghai at concentrations of  $78.00 \pm 12.91$  MP/kg and  $62.50 \pm 12.97$  MP/kg. The presence of these MP was related to both application of sewage sludge and the use of plastic mulching. MP can serve as vectors of pollutants either by leaching their additives or by transporting adsorbed persistent organic pollutant [41], so their monitoring and control deserves attention both in WWTP effluents and activated sludge.

Table 4 – Results of the quantification by shape of microparticle in the samples

Sample	Microfibers concentration	Fragments concentration	Total
Primary effluent	10.7MF/L	0.4Fragment/L	11.1Microparticle/L
Secondary effluent	2.6MF/L	0.2Fragment/L	2.8Microparticle/L
Activated sludge	264MF/L (105.6 MF/g dry weight)	16Fragment/L (6.4 Fragment/g dry weight)	280Microparticles/L (112.0 Microparticle/g dry weight)

The assessment of microparticles in primary and secondary effluents and activated sludge samples showed that most of them were microfibers (>90%). Fig. 5 shows some of the fibres found in the samples. The high presence of fibres in effluents has also been observed by other authors [9,11,12,18,42,43,44]. Gündoğdu et al. [10] found that fibres also predominated over microparticles with other shapes (fragments, microspheres), constituting 44.4% of Seyhan WWTP (Turkey) secondary effluent and 86.5% of Yüreğir WWTP (Turkey) secondary effluent. Michielssen et al. [9] calculated that the microparticles in the final effluents of two WWTPs in USA included 61% and 84.7% of fibres, with estimated concentrations of 3.58 and 5.25MF/L and 1.94 and 0.8Fragment/L. In general, methodological differences in the filtration step (different size mesh) and sample preparation (digestion process) make difficult to compare results between works. However, the presence of textile fibres in the environment has proven to be an environmental issue that deserves attention. According to Browne et al. [45], more than 1,900 polyester fibres can be released from one garment per wash. Almroth et al. [46] suggests that one fleece garment can release approximately 110,000 fibres when washed.

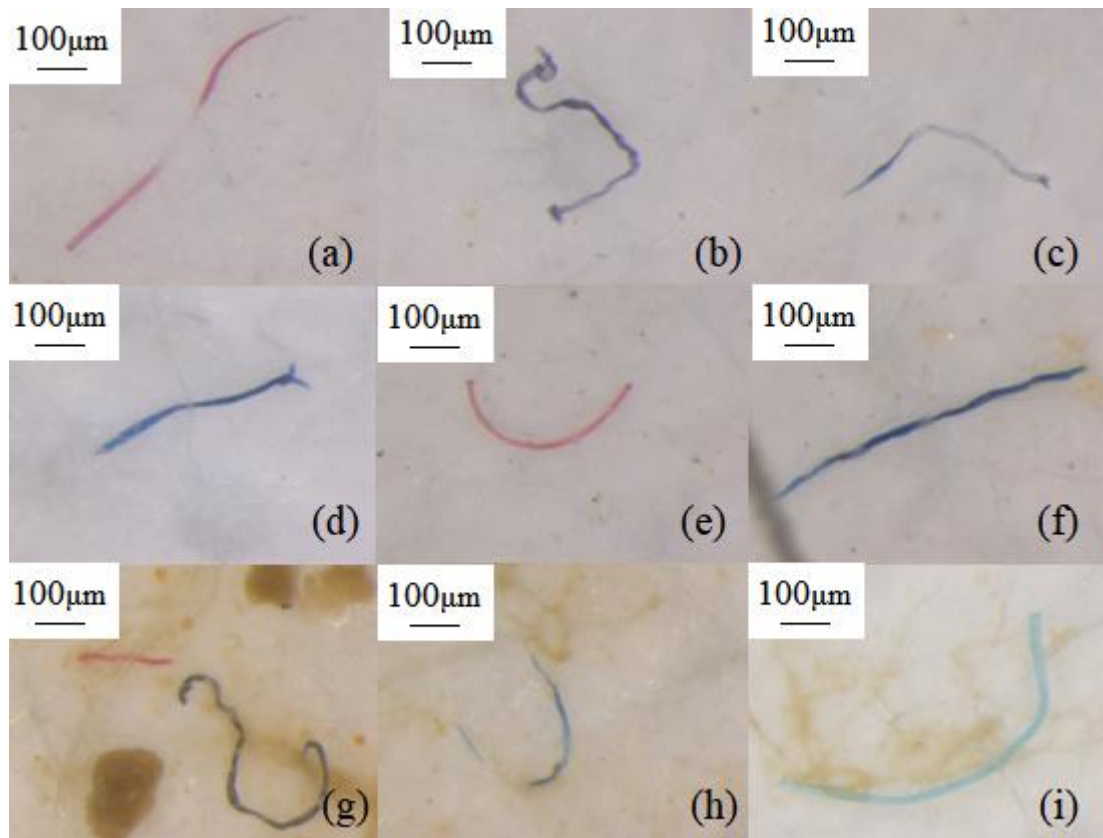


Fig. 5. Dyed microfibers: (a,b c) Primary effluent, (d,e,f) Secondary effluent, (g,h,i) Activated sludge

Fragments were also found in all samples, however in much lower concentrations compared to fibres. The identified fragments mainly had dimensions greater than  $300\mu\text{m}$  and less than  $1\text{mm}$  (Fig. 6). No fragments larger than  $2\text{mm}$  were identified. It is worth mentioning that fragments smaller than  $150\mu\text{m}$  were also visualized. The smallest fragments visualized had  $20\mu\text{m}$ , but these microparticles were not counted in this study since it was not within the lower size limit established in the methodology. Similar results were obtained by Yang et al. [44]. In this work, 14.08% of the MP identified in the effluents corresponded to fragments, spheres, granules and films, which had an average size of  $681.46\pm 528.73\mu\text{m}$ , having most MP a size of around  $300\mu\text{m}$ .

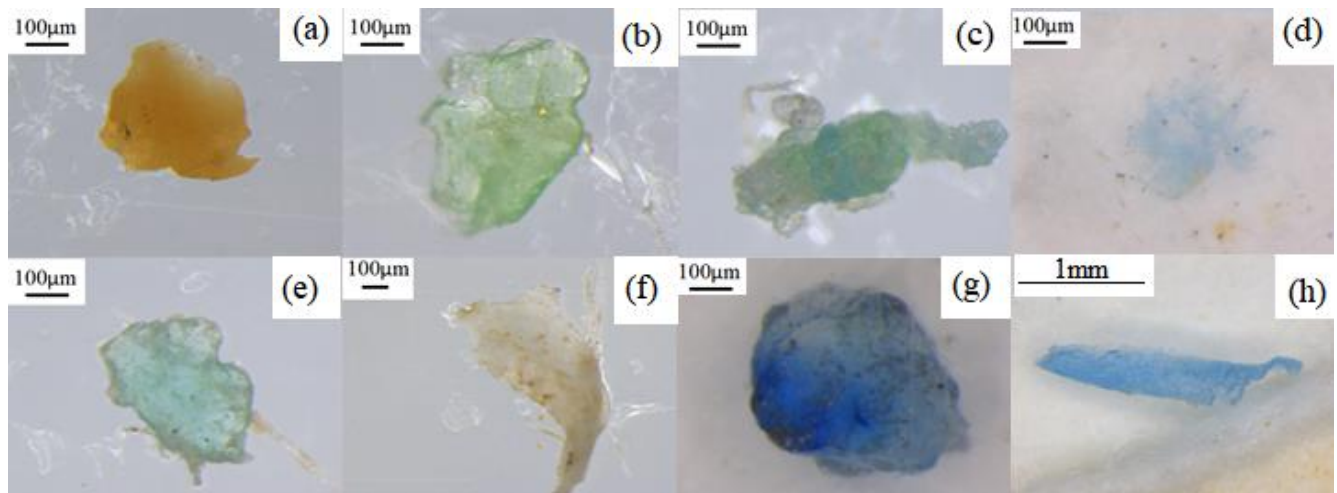


Fig. 6. MP fragments separated from activated sludge (a, b, c, e, f); secondary effluent (d, h); primary effluent (g)

No plastic microspheres were found in the processed samples. This kind of microplastics is compounded mainly by PE [3,5,7,37], and its absence of in WWTP effluents may be related to its composition. Since polyethylene material has lower density than the water, these MP tend to float on the wastewater surface with fat, grease and oil materials in the preliminary treatments of the WWTP. Similar results were found by Michielssen et al. [9] and Murphy et al. [14] who also did not find microspheres in the effluents studied. In contrast, in the study by Hidayaturrahman and Lee [23] in three WWTPs located in South Korea, all the effluents mainly contained microspheres.

Factors such as WWTP treatment capacity, patterns of consumption and social behaviour could have influence on the results obtained by each author. The presence of microspheres, as well as microfibers, in wastewater is the result of domestic activities. Browne et al. [45] suggests that because people wear more clothes during the winter than in the summer, and that the use of washing machines is 700% higher in winter, more fibres can enter the WWTP during winter. Similarly, considering that microspheres are present in personal care and cosmetic products (such as exfoliants, toothpaste, shampoos,

among others) [7,37], their presence in the WWTP can be the result of different consumption patterns. This may be also one of the reasons why different results regarding the MP presence in WWTPs have been reported.

### **3.4.2. Size of the microparticles**

Fig. 7 shows microparticle size distribution found in primary effluent, secondary effluent and activated sludge samples. It can be observed that in the primary effluent 73% of microparticles were larger than 500 $\mu$ m. Similarly, the secondary effluent mostly contained microparticles greater than 500 $\mu$ m and smaller than 5mm (74%). The smallest size (150-500 $\mu$ m) involved 27% and 26% in primary and secondary effluents, respectively. Gündoğdu et al. [10] also observed that the MP separated from the secondary effluent of WWTP (Yüreğir WWTP) mostly corresponded to the size range from 1mm to 5mm (40.5%), and a 29.7% to MP between 100 $\mu$ m-500 $\mu$ m. However, the Seyhan WWTP had a size distribution corresponding to 34.9% for both 1mm-5mm and 500 $\mu$ m-1mm size ranges. The final effluent of Lares et al. [18] was also composed by larger MP (between 500 $\mu$ m and 1mm) and a smaller part corresponded to MP between 250-500 $\mu$ m.

However, for activated sludge samples, the microparticle size distribution changed, and 48% were comprised between 150-500 $\mu$ m. This result may be due to the fact that the most part of the microparticles greater than 1mm flows out of the WWTP on the secondary effluent and the smallest microparticles keep on the sludge. Similar results were reported by other authors. Magni et al. [35] found that the 54% of the sludge of a WWTP in Italy was formed by MP between 100 $\mu$ m-500 $\mu$ m and a smaller part ranged 1mm-5mm (10%). Liu et al. [36] also observed that microplastics presented in the sludge corresponded mostly (>80%) to the smallest size range evaluated by them (20 $\mu$ m-



300 $\mu$ m). The presence of smaller MP in the sludge can probably be the result of their retention on the sludge floc.

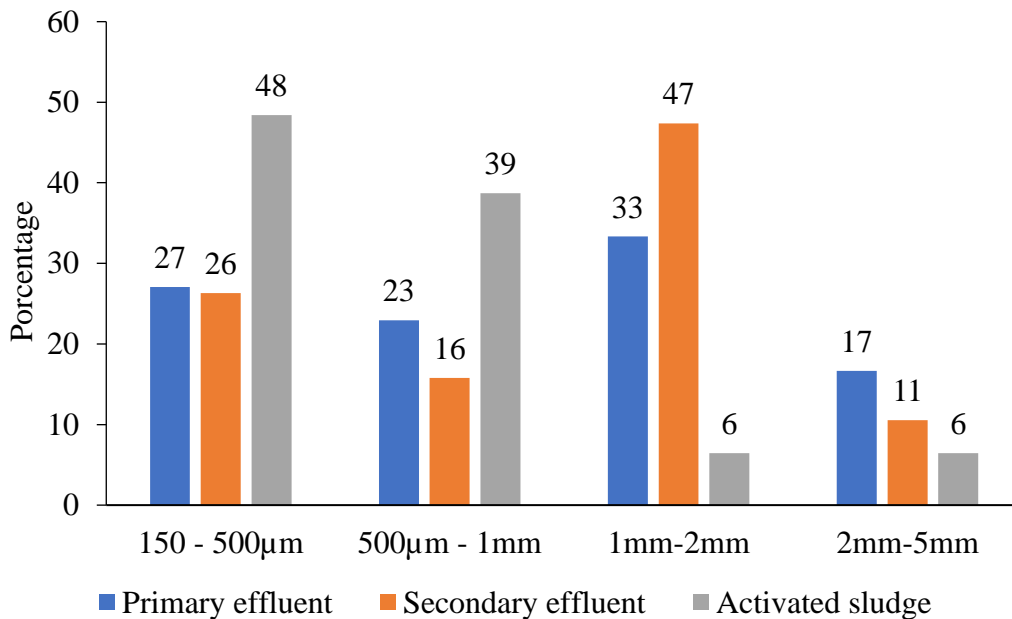


Fig. 7. Microparticle size (mm) distribution for primary effluent, secondary effluent and sludge samples

Concerning the results of the statistical analysis, a classification based on the size distribution of the microparticles (including fragments and fibres) was performed and the Kruskal-Wallis method was used (n=98). Based on the Kruskal-Wallis statistical analysis, as the P value is less than 0.05 (p-Value = 0.003) there was a statistically significant difference amongst the medians at the 95.0% confidence level. Using the Bonferroni procedure for paired comparisons, the two comparisons: ‘Activated sludge-Primary Effluent’ and ‘Activated sludge-Secondary Effluent’ were statistically significant at the 95.0% confidence level and there were no statistically significant differences between the microparticle size averages when comparing the two effluents (Table 5).

Table 5 - Kruskal-Wallis test for microparticle size by sample and Benferroni post hoc

<b>Kruskal-Wallis</b>			
Sample	Median ( $\mu\text{m}$ )	Sample Size (n)	Average Rank
Activated sludge	570	31	35.7
Primary Effluent	1050	48	54.0
Secondary Effluent	1500	19	60.5

P-Value = 0.003

<b>Benferroni post hoc</b>				
<i>Contrast</i>		<i>Sig.</i>	<i>Difference</i>	<i>+/- Limits</i>
Activated sludge – Primary Effluent		*	-18.29	15.68
Activated sludge – Secondary Effluent		*	-24.76	19.83
Primary Effluent– Secondary Effluent			-6.47	
				18.45

\*Denotes a statistically significant difference.

### 3.4.3. Colour of the microparticles

Related to the colour of the (fibres and fragments) (n=85), the majority of them was blue (48%) and black (25%), but some red (11%) and other colours like green, orange or purple were also observed (16%). In the manufacturing process of textile fibres (both natural and synthetic) different chemical compounds are applied such as dyes, surfactants, detergents, colour stabilizers, among others that can be considered hazardous substances. In this way, Remy et al. [47] identified by Raman's technique the Direct Red 28 (DR28) colorant in cellulose-based fibres ingested by macrocrustaceans. This colorant, once reduced in the human body, results in compounds classified as carcinogenic. Thus, the presence of additives, such as DR28, in natural fibres can be as worrisome as for the synthetic ones. In addition, the fact that natural fibres are more biodegradable than synthetic ones, can be an aggravating factor for the release of toxicity to the environment. [48].

### 3.5. Presence of MP in the samples

Numerous attempts were made to analyse the MP directly in the glass fiber filter but, a low signal quality was obtained. The presence of the filter made it difficult for the ATR crystal to apply pressure to the samples (especially to the fibres), and the crystal sometimes displaced them. To improve identification accuracy, it was necessary to manually extract each MP individually with tweezers and position them on the base of the  $\mu$ -ATR-FTIR equipment. This process was very time consuming; therefore, a subset of 17% of all microparticles counted were characterized (including fibres and fragments). To overcome this problem, Tagg et al. [20] propose the use of the Focal Plane Array (FPA) technique, which enables the mapping of microparticles in entire membrane filters in a short time.

MP fragments were characterized mainly as PE and PP (63% and 25% of total MP fragments, respectively). The separated fragments were likely originated from the fragmentation of commercially used plastics such as bottles, plastic bags, etc. and therefore were classified as secondary origin. Lares et al. (2018) [18] found similar results: 65.9% of MP fragments were characterized as PE in a WWTP in Finland. According to PlasticEurope [1], PE accounted for 29.8% of European plastics demand in 2017 and PP 19.3%, being the main polymers used in packaging manufacturing. Society's behaviour regarding waste disposal (recycling and waste separation processes) and solid waste management systems are relevant factors concerning the presence of these polymers in wastewater treatment plants.

Regarding the microfibers, polyester was the most prevalent synthetic polymer, corresponding to 75% of plastic MF. The results of this study are in accordance with previous studies conducted in WWTPs, which identified a large presence of polyester fibres. Browne et al. [45] also reported polyester as most frequent plastic MF in final effluent (67%). In the work carried out by Lares et al. [18], polyester MF corresponded

to 79.1% of the total amount of MP collected. In our research, both polyester and cotton were found in primary effluent samples and in the activated sludge polyester, cotton, polyacrylic and polypropylene were identified. A percentage approach was performed for the secondary effluent since 52% of the fibres counted in the secondary effluent were characterized by  $\mu$ -ATR-FTIR. In this sample, only 9% of MF were identified as microplastic (polyester), the remainder corresponded to natural cellulose derived MF. Based on this, from 2.6MF/L counted, only 0.24MF/L were identified as synthetic. Talvitie et al. [49] also observed a high presence of natural MF in the analysed effluent. 44% of the fibres were characterized as cotton, followed by polyester fibres (33%). However, as mentioned above, natural MF can lead to an environmental problem due to the presence of additives and, therefore, should not be ignored.

According to the results obtained in this research, about 0.44MP/L (including MF and fragments) could be released in the environment after secondary treatment. Murphy et al. [14] and Bayo et al. [50] found similar results 0.25MP/L and  $0.31\pm 0.06$ MP/L, respectively, were estimated in secondary effluents. Table 6 collects the results of the MP concentrations in the WWTP effluents in different parts of the world. The discrepancy in the results obtained between authors can be attributed to the different processes of separation and identification of MP applied and the lack of standardized protocols. However, due to the high volume of effluents generated by the WWTP, millions of MPs can be released into the environment daily.

Table 6 - MP concentrations in secondary effluents of different WWTPs

Reference	Facility	Facility capacity (m <sup>3</sup> /day)	MP/L (secondary effluent)	%MF Secondary effluent	Smallest mesh size ( $\mu$ m)	Sample pretreatment	Identification	Country
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This study	WWTP Valencia	40,000	0.44	95	150	H <sub>2</sub> O <sub>2</sub> 35%, 60±2° for 2h	Visual/μ- ATR-FTIR	Spain
[8]	Metro Vancouver	490,000	0.5±0.2	60	64	H <sub>2</sub> O <sub>2</sub> 30%, RT; 7 days	Visual/μ- ATR-FTIR	Canada
[10]	Seyhan WWTP	182.78 ± 5.97	6.999±0.764	44.4	55	H <sub>2</sub> O <sub>2</sub> 30% + FeSO <sub>4</sub> •7H <sub>2</sub> O; 75°	Visual / μ- Raman	Turkey
	Yüreğir WWTP	87.49 ± 0.97	4.111±0.318	86.5	55			Turkey
[18]	Kenkaveronniemi WWTP	10,000	1 ± 0.4	53	250	H <sub>2</sub> O <sub>2</sub> 30% + FeSO <sub>4</sub> •7H <sub>2</sub> O; 75°	Visual/μ- ATR- FTIR/μ- Raman	Finland
[23]	WWTP-A	26,545	710	8	1.2	30% H <sub>2</sub> O <sub>2</sub>	Visual	South Korea
	WWTP-B	469,249	7863	1				
	WWTP-C	20,84	433	15				
[34]		45,000	10.7±5.2		25	H <sub>2</sub> O <sub>2</sub> (33% w/v) at 50°C for 20-24 h		Spain
[35]	WWTP in Northern Italy	400,000	0.9±0.3	28	63	H <sub>2</sub> O <sub>2</sub> , 15%; RT; 3 days	Visual/μ- FTIR	Italy
[36]	WWTP in Wuhan City	20,000	34.1 ± 9.4	56.7	47	H <sub>2</sub> O <sub>2</sub> 30%, 6hours; FeSO <sub>4</sub> •7H <sub>2</sub> O; 12hours	Visual / μ- Raman	China
[43]	East Bay Municipal Utilities District (EBMUD) Central Contra Costa		0.071	57	125	H <sub>2</sub> O <sub>2</sub> 30% + Fe(II)	Visual	USA
	East Bay Dischargers Association (EBDA) San Francisco Airport Sanitary (SFO)		0.072	59				
			0.022	91				
			0.19	90				
[50]	WWTP Cartagena	35,000	0.31±0.06		0.45		Visual / FTIR	Spain

<sup>a</sup>Including all microfibers.

#### **4. Conclusions**

This study has developed a methodology for the separation and characterization of MP in different streams of a WWTP. Since there are no standardised procedures, deep discussion regarding the pre-treatment and characterisation of the samples is of paramount importance in the foreseeable future.

Concerning the chemical digestion of the samples, the use of peroxidation proved to be effective with samples of primary effluents and activated sludge - since it did not compromise the identification of polymers via ATR-FTIR, and considerably reduced the concentration of suspended solids; allowing for the effective separation and visual classification of possible microplastics. For secondary effluent samples, its application will depend on the concentration of suspended solids. In addition to this, the spectra obtained via FTIR showed no interference from organic materials, which corroborates the efficiency of the digestion method based on peroxidation. Furthermore, the separation process is so important as chemical digestion and must be done carefully to avoid material losses.

In all analysed samples (primary and secondary effluent, and activated sludge), MF was found to be the most abundant fraction, constituting more than 90% of the microparticles. Despite the fact that the highest fraction of MF in the secondary effluent was characterized as cotton, these natural fibres can be classified as an environmental problem akin to microplastics, since they carry several additives in their composition, and their faster rate of degradation could hence release harmful compounds more quickly into the environment; affecting various forms of life. A high quantity of microparticles was detected in the activated sludge (280Microparticles/L or 112.0Microparticle/g dry weight) compared to effluents. Significant differences were observed when comparing

the size of the microparticles in the effluents to those within the activated sludge - which mainly consisted of smaller particles (100-150 $\mu$ m). In this regard, most of the microparticles entering the WWTP accumulated in the sludge - especially the smaller microparticles. In the future, great efforts must be made towards establishing standard protocols that help make methodological unification feasible, therefore resulting in more effective comparisons across research studies, in order to better identify possible points for improvement and minimise the impact of microplastics on the environment.

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