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Additional Information

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Wastewater treatment plant as microplastics release source – quantification and identification techniques

Authors – C.B. Alvim, J.A. Mendoza-Roca, A. Bes-Piá

Instituto de Seguridad Industrial, Radiofísica y Medioambiental, Universitat Politècnica de València, Camino de Vera, s/n, Valencia 46022, Spain

* Corresponding author: *claal@posgrado.upv.es*

Abstract – The high presence of microplastics (MPs) in different sizes, materials and concentrations in the aquatic environment is a global concern due to their potential physically and chemically harm to aquatic organisms including mammals. Furthermore, the bioaccumulation of these compounds is leading to their ingestion by humans through the consumption of sea food and even through the terrestrial food chain. Even though conventional wastewater treatment plants are capable of eliminating more than 90% of the influent MPs, these systems are still the main source of MPs introduction in the environment due to the high volumes of effluents generated and returned to the environment. The amount of MPs dumped by WWTP is influenced by the configuration of the WWTP, population served and influent flow. Thus, the average of MP/L disposed vary widely depending on the region. In addition to MPs disposed in water bodies, more than 80% of these emerging contaminants, which enter the WWTP, are retained in biosolids that can be applied as fertilizers, representing a potential source of soil contamination. Due to the continuous disposal of MPs in the environment by effluent

treatment systems and their polluting potential, separation and identification techniques have been assessed by several researchers, but unfortunately, there are no standard protocols for them. Aiming to provide insight about the relevance of studying the WWTP as source of MPs, this review summarizes the currently methodologies used to classify and identify them.

1. Introduction

Plastic is an important material that is widely used in multiple applications. In 2016 the world production of plastic was superior to 330 million tons. Despite its numerous applications and large participation in the economy (PlasticsEurope, 2017), these polymeric materials have been presented as a hazardous source of environmental pollution. According to the United Nations Environment Program (UNEP, 2016), more than 80% of marine litter corresponds to plastic. In addition, it is estimated that if the rate of plastic litter release in the oceans does not decrease, by 2050 the oceans could have more plastic than fishes by weight (Auta et al.,2017)

Microplastics (MPs) are defined as synthetic polymers with dimensions less than 5mm (European Food Safety Authority) and nanoplastics (NPs) are those plastics with size smaller than 0.1 µm (He et al., 2018, Ng et al., 2018). Based on the origin, the MPs can be classified as primary and secondary and both have been found in freshwater systems (Simon et al., 2018). The primary microplastics are present in the formulation of products for personal care (microbeads) and fibers from laundry (Waller et al., 2017). Secondary microplastics are originated from plastic remains (mainly of discarded consumer packaging) that suffer fragmentation by photo-degradation, physical, chemical and/or

biological interaction (Auta et al., 2017; Carr et al., 2016; Li et al., 2018, Rio Mendoza et al., 2018;).

The concern for the presence of MPs in the aquatic environment occurs since the ingestion of these compounds can cause toxicity to both humans and other living organisms, in addition to their characteristic of accumulation and persistence in the environment once microbes are usually unable to mineralize and assimilate them (Li et al, 2018; Ng et al., 2018). Aquatic organisms as zooplankton, benthic vertebrates, mollusks, fishes and seabirds can ingest these MPs, leading to biological complications (reduced feeding, energetic deficiencies, injury) or even death (Cole et al., 2014).

Due to the hydrophobicity of MPs, other pollutants, such as persistent organic pollutants (POPs), can be sorbed by these particles that act as vectors for the contamination of other environments and animals, leading to biomagnification. Due to their small superficial area the amount of POPs sorbed per gram of MPs is significant, acting like a vehicle for concentration of these contaminants (Carr et al., 2016; Cole et al., 2013; Bouwmeester et al., 2015; He et al., 2018; Rocha-Santos, 2018). In the human body, POPs (for example DDT, polychlorinated biphenyls and dioxins), may increase the risk of cancer, reproductive disorders, immune system disorders, endocrine dysregulation and increased congenital defects, even at very low concentrations. Teuten et al. (2007) developed a study to evaluate the contribution of MPs in phenanthrene distribution compared to sediments. For this purpose, polyethylene, polypropylene and PVC in dimensions of 200-250µm were used to provide a high surface area for the hydrophobic organic contaminant (HOC) sorption. The results of the work revealed the sorption preference of this compound on the plastics when compared to the sediments, although there are affinity differences for each polymer. The fact that several species mistakenly feed on plastics, the sorption of organic contaminants by MPs can make them a transport vector. On sea

surface microlayer, buoyant MPs with phenanthrene sorbed can be displaced to the sediments, increasing the concentration of the latter in the benthic layer and tissues of lugworm *Arenicola marina* (a common benthic deposit feeder). The processes of sorption and desorption of organic contaminants by MPs still have many gaps. (Mato et al., 2001; Teuten et al., 2007). Little is known to what extent desorption of organic contaminants occurs within the animal organism, studies covering this aspect would provide important information regarding the actual amount of HOC that can cross the food chain.

Although conventional wastewater treatment systems can eliminate from water more than 90% of MPs, these systems are still classified as the major source of release of these emerging compounds in the aquatic environment (Gies et al., 2018; Li et al., 2018). This is due to the enormous volume of effluent discharged by the conventional treatment systems; therefore to the continuous release of these emerging contaminants into the water bodies (Talvitie et al., 2017a).

Thus, millions of MPs are disposed daily in water bodies around the world after secondary treatments (Gies et al., 2018; Ziajahromi et al., 2017; Mason et al., 2016; Gündoğdu et al., 2018). Besides the contamination of bodies of water, around 80%-90% of MPs passing through WWTP are retained in the generated biosolids that are widely applied as fertilizers, representing a substantial soil contamination (He et al., 2018; Murphy et al., 2016; Talvitie et al., 2017b).The microplastics introduced into the soil can be ingested by the terrestrial biota and transported along the food chain and finally reach human consumption. The study by Huerta Lwanga et al. (2017) showed the possible amount of microplastics ingested by a population in a region of Mexico where chicken gizzards are usually prepared for human consumption. For this, the amount of plastics ingested by the terrestrial biota earthworms and chickens (*Gallus gallus domesticus*) and finally in the preparation of chicken gizzards was evaluated. The number of MPs found in earthworms

casts, chicken feces and gizzards were respectively 14.8 ± 28.8MP/g, 129.8 ± 82.3MP / g and 10.2 ± 13.8MP/gizzard. As far as gizzard preparation is concerned, 7 out of 10 women only wash the food on the outside without cleaning it on the inside, this can be aggravating when it comes to the entry of MPs into the food chain by land, reaching the human consumption.

The current review highlights the presence and relevance of WWTP as a source of MPs in the aquatic environment and the techniques for MPs separation and identification by reviewing the current state of knowledge.

2. WWTP as a source of MPs

WWTPs are a source of MPs in both aquatic and terrestrial environment since several recent studies have demonstrated the presence of MPs in water after the secondary treatment. Table 1 shows microplastic concentrations in effluents from WWTPs depending on operational conditions, population served, and treatment processes applied (primary, secondary and tertiary). According to literature, microplastics are removed about 78%-98% after primary treatment (Murphy et al., 2016; Talvitie et al., 2017b), while secondary treatment is responsible for a smaller MPs decrease (7-20%) (Murphy et al., 2016; Talvitie et al., 2017b). Thus, the study conducted by Murphy et al. (2016) at a WWTP in Scotland, that serves a population equivalent to 650,000 inhabitants, showed that even with a 98% efficiency of removal of these emerging compounds, about 6.5×10^7 MPs are discharged daily at the aquatic environment after a secondary treatment (about 100MP/equivalent inhabitant).

One of Italy's largest WWTP, which serves a population equivalent to 1,200,000 people, receives approximately 1×10^9 MPs daily (2.5±0.3 MP/L) and even with a removal efficiency of 84% about 1.6×10^8 MP/day are disposed into the aquatic environment after

a tertiary treatment, which corresponds to a release of 133MP/equivalent inhabitant. Focusing on the MPs size, in that study the range of 0.5-0.1mm corresponds to the mayor fraction (more than 50%) found after secondary treatment, in the final effluent and in the sludge. In terms of the shape of these MPs, lines (according the authors this shape presents the same thickness in all length with sharp ends, differently of fibers that show frayed ends) corresponded to 41% of final effluent MPs (Magni et al., 2019). Otherwise, Talvitie et al. (2017b) showed that, in the WWTP's effluent studied by them, around 70% of the particles corresponded to the smallest size (0.100-0.020mm) and 60% were classified as fragments.

The study performed by Mason et al. (2016) in 17 wastewater treatment facilities in the United States (6 of them include advanced/tertiary treatment), showed an averaging of 0.05MP/L in the final effluent totalizing more the 4 million of MPs discharge in environment per day. About the physical characteristic of these MPs, most of them were fibers (59%) followed by fragments (33%). Concerning dimensions, the size of 57% of MPs ranged between 0.125mm and 0.355mm, whereas 43% was larger than 0.355mm.

The composition of the effluent of WWTPs in terms of MPs is singular. The shape, size and amount vary significantly, which make difficult to compare the results. As an example, in Figure 1, results obtained in different countries are represented. They are difficult to compare due to demographic and methodological differences (in this case shown in terms of the smallest considered MPs size) (Gies et al., 2018; Gündoğdu et al., 2018; Lares et al., 2018; Mason et al., 2016; Murphy et al., 2016; Ziajahromi et al., 2017). However, independent of these, the concerning topic is that the values of MPs discharged per day by WWTP around the world is alarming surpassing millions of particles per day.

MPs present in domestic wastewater come from different sources. Personal care and cosmetic products (PCCPs) are a substantial source of microplastics used as exfoliating material in soaps, facial scrubs, shampoos, shaving foam and toothpaste or as beauty propose in form of plastic glitter. The microplastics added to these products can be referred as microbeads or even microspheres and are mostly made of polyethylene (Fendall and Sewell, 2009; Guerranti et al., 2019; Napper et al., 2015). Although this nomenclature refers to particles of spherical geometry, this is not always true. These MPs typically have an irregular shape, but the addition of spherical particles can be performed to enhanced consumer's visual attraction (Kalčíková et al., 2017). These authors reported that MPs incorporated in PCCPs usually are smaller than 1mm (Table 1)

Kalčíková et al. (2017) estimated that in the city of Ljubljana (Slovenia) with 300,000 inhabitants, 112,500,000 microbeads are released per day into the receiver river after the WWTP. Furthermore, according to these authors 100mL of the facial scrub can be a source of more than 1,300,000 particles. According to Napper et al. (2015) around 4,600 – 94,500 polyethylene microbeads may get the sewage system per application of 5mL of a skin exfoliant and in each toothpaste application (1.6g of toothpaste) around 4,000 polyethylene fragments can be discharged as suggested by Carr et al. (2016).

Another commonly type of MPs found in wastewater samples is textile fibers. According to Talvitie et al. (2017b) fibers can represent around 70% of the MPs in WWTP's influent. Laundry of synthetic clothing may release into wastewater more than 1,900 polyester (polyethylene terephthalate) fibers per wash (Browne et al., 2011), one fleece garment could release approximately 110,000 fibers (Almroth et al., 2018) and 5kg of polyester fabrics can release about 6,000,000 microfibers (Falco et al., 2018). Even though the large quantities of fibers disposed in WWTPs, the amount depends on the properties of the

fabric, temperature, time and speed of washing, as well as products used as detergents and softeners (Almroth et al., 2017; Falco et al., 2018).

Besides the water contamination, WWTP are also a source of MPs soil pollution once the sludge generated on WWTP is processed and reused as fertilizer (Mohapatra et al., 2016). Carr et al. (2016) estimated that $1.09x10^9$ MP/day get into the environment by biosolids pathway. Nizzetto et al. (2016) estimated that yearly 63,000−430,000 and 44,000−300,000 tons of MPs are added to farmlands in Europe and North America, respectively. In Australia about 2,800 to 19,000 tons of microplastics per year are estimated to apply to agroecosystems through biosolids (Ng et al., 2018).

Regardless where MPs are found (wastewater, biosolid, water, soil), these particles must be previously separated for their quantification and identification. Basically, the sample is subjected to separation techniques, and when necessary purifications with chemical digestions that allow a better visual sorting. Later, the possible MPs are analyzed by different instrumental techniques that allow their chemical identification and classification as polymer or not.

3. Sample processing – sampling and separation techniques

Different sampling methods have been employed for collect microplastics from WWTP effluents. Sampling procedures at wastewater treatment plants can be done in conjunction with the separation step (pumping coupled with filtration, surface filtration, and auto-sampler collection), or samples can be collected in containers and taken to laboratories for separation procedures (Table 2). In the first case, where the separation is made *in-situ* (at the sampling site) has the advantage of a high outflow at the collection points, which enables the separation corresponding to a larger sample volume. Although *in-situ* separation allows for a larger sample volume, precautions should be taken regarding

cross-contamination of these samples, as separation is done in an environment with a higher exposure to contamination when compared to a closed and better controlled laboratory.

The separation process is usually performed with a series of sieves of different openings through which a continuous stream of effluent is passed. The mesh sizes of the sieves are chosen according to the size range of the MPs to be collected, but they are generally in the range of 38μm to 4750μm (Hidalgo-Ruz et al., 2012; Wang, W. and Wang, J., 2018). In this range, it is possible to separate the MPs in several sizes categories by using a series of sieves of different mesh. Afterwards, the material on each sieve can be rinsed with distilled water and then stored into glass vials (Long et al., 2019; Ziajahromi et al., 2017).

Monitoring of this procedure is important since effluents constituted with high organic load tend to quickly block the sieves. The sampled volume is a function of the effluent of interest. Tertiary effluents tend to allow for a larger volume used for separation as they have less suspended solids. In addition to the drawback of sieve blockage, the microparticle morphology also influences the separation process. Microfibers, since they have a high length to thickness ratio can be retained horizontally in the sieve or pass longitudinally to a smaller aperture sieve (Michielssen et al., 2016; Ziajahromi et al., 2017). In order to obtain more homogeneous results and fewer quantification and separation errors, the sampling step should be well evaluated and established in order to provide reproducible and comparable data.

After sampling and sieving other additional separation techniques may be applied in order to separate MPs from the sample medium. Among these, the most common techniques are visual sorting, density separation, filtration. Table 3 shows separation techniques currently used. Dyachenko et al. (2017) reject the application of methods that include

centrifugation and microwave, since they can cause the rupture and deformation of the MPs. Unfortunately, there is still no standard protocol for separation procedures and this fact makes difficult to compare the number of MPs reported in different sources. In this way, the establishment of standard protocols is of paramount importance for data comparisons.

3.1 Density separation

Each polymer, having a different chemical composition, behaves in a peculiar way in the environment. Regarding the separation of the microplastics from the sample medium, the density of the polymers is an important characteristic. The density of the virgin polymers (i.e. without additives incorporated during the manufacture of products), vary from 0.90 to 1.6 g/cm^3 (see Table 4). Since the typical density of sand and other sediments is around 2.6g/cm^3 (Hidalgo-Ruz et al., 2012; Rocha-Santos and Duarte, 2015; Wang, W. and Wang, J., 2018) the separation of MPs by density difference is a convenient technique to be applied.

When matrixes with high organic loads are evaluated, sieving and filtration processes before a previous separation by density can lead to the saturation of the sieves/filters, which makes it difficult to identify and separate the MPs (Lusher et al., 2017). In order to float all the microplastics, samples are mixed to a higher density solution like sodium chloride (NaCl), sodium iodide (NaI), zinc chloride ($ZnCl_2$) or sodium polytungstate (SPT) solutions and stirred for a predetermined time. Subsequently, the supernatant with the plastic particles is extracted by filtration under normal pressure or a vacuum system for further processing steps (GESAMP, 2015; Hidalgo-Ruz et al., 2012; Rocha-Santos and Duarte, 2015). The saturated solution of NaCl (1.2g/cm^3) is usually used to extract the low-density polymers as PE, PS and PP. The advantage of NaCl is that it is an

inexpensive and eco-friendly salt. Otherwise, due to their higher density, NaI solution (1.8 g/cm^3), ZnCl$_2$ (1.5-1.7 g/cm^3) or SPT (1.4 g/cm^3) have to be applied to remove high-density MPs (such as PET and PVC) (Rocha-Santos and Duarte, 2015; Wang, W. and Wang, J., 2018;).

3.2 Filtration

Filtration systems are commonly used for the recovery of MPs from liquid samples or from the supernatant of the density separation, which is passed through paper filters of pore sizes of 1 to 2 μm (Crawford and Quinn, 2017a; Hidalgo-Ruz et al., 2012). The particles retained on the filters can be separated using tweezers for identification (Rocha-Santos and Duarte, 2015). Among the filter media used, glass fiber, nitrocellulose and polycarbonate filters can be cited. Although it is a simple process, the presence of particulate material can block the pores of the filter reducing the efficiency of the process (Wang, W. and Wang, J., 2018). To reduce this drawback, practices as reducing the volume to be filtered or adding chemicals to provide previous flocculation of solid particles can be performed (Crawford and Quinn, 2017a).

4. Sample processing – Digestions

The microplastics separated from the sample medium may contain organic particles which can interfere on the subsequent identification, requiring the removal of these materials from the MPs surface (Enders et al., 2017; GESAMP, 2015; He et al., 2018). Furthermore, the organic material in the sample can be confused with MPs leading to the overestimation of polymers (Prata et al., 2019). When a protocol for digestion is assessed, the effect of the process on the integrity of MPs is an extremely important factor. In the following sub-sections, the main types of digestion methods are reviewed.

4.1.Digestion by hydrolysis with acidic substances

Studies have shown that chemical digestion with acids, such as H_2SO_4 and HNO_3 and alkaline treatments can destroy or damage polymers (He et al., 2018). In this way, the concentration of the acidic solutions used has to be low, jeopardizing the efficacy of the digestion process, i.e. the percentage of removed organic matter. Cole et al. (2014) achieved over 80% removal of biological material using 1M HCl. Enders et al. (2017) performed different chemical digestions on 21 polymers and evaluated the resistance of plastics to the applied reagents. Among these tests, one corresponded to an acid mixture (HNO_3:$HClO_4$ (4:1)). Possible visual modifications (with assignments of different impact levels) and the Raman spectrum after digestion were observed. Digestion was performed for 5 hours at room temperature and then the samples (still immersed in the reagent) were heated at 80°C for 20 minutes. The polymers that suffered the most from chemical digestion were polyamide (PA), polyurethane (PU) and a black tire rubber elastomer, which were completely dissolved. Other polymers that were not completely dissolved showed some degree of colour loss, such as polycarbonate polymer (PC), expanded solid polystyrene (EPS, PS) and polyethylene terephthalate (PET). For polypropylene (PP), high- and low-density polyethylene (HDPE, LDPE), ethylene-vinyl acetate (EVA), and polytetrafluoroethylene (PTFE) no effect was observed. In addition to the chemical reagent, the negative effects on the polymers were also attributed to the application of heating after the digestion periods. The Raman spectra after chemical digestion, in general terms the polymers showed no severe modifications except for acrylonitrile butadiene styrene (ABS). All other polymers had a similar spectrum to the original (without digestion), some showing some signs of degradation or peak deviations, like PS and PVC, that showed fluorescence after chemical digestion, the latter also revealed weakening of the main peaks indicating degradation process. Naidoo et al. (2017) also performed acid

digestion assays using HNO_3(55%), the plastics (Nylon, high density Polyethylene, Polystyrene, Poly 1,4-butylene terephthalate and Polyvinyl chloride) were immersed in acid for one month at room temperature. The polymer mass was monitored throughout the digestion period and in the first 24 hours of testing all Nylon was completely disintegrated. The other plastics were resistant to digestion. Catarino et al., 2017 also observed that the acid digestion with HNO_3(35%) affect the integrity of plastic especially Nylon, that was completely dissolved after time reaction. PET and HDPE also showed melding effects. These studies corroborate the importance of evaluating the resistance of polymers to the digestions proposed in each study, prior to their application in the real sample to be digested. The use of acidic agents showed aggressive to some polymers what may result in the disintegration of some plastics, leading mistaken results regarding the sample count.

4.2. Digestion by hydrolysis with alkaline substances

As the acidic substances, alkaline solutions may damage the morphology of the MPs. Similar to acid treatment, sample loss in the basic treatment can lead to particle underestimation leading to erroneous results. The use of 10M NaOH at a temperature of 60ºC, for example, showed aggressive for some polymers resulting from the partial destruction of nylon fibers and the fusion of polyethylene fragments (Cole et al., 2014). Hurley et al. (2018) carried out a study using 6 different chemical digestions to find out which reagent reaches the highest removal of organic matter. These authors observed that the use of alkaline solutions (1M and 10M NaOH and 10%KOH at 60 ºC) are not appropriate for the removal of organic material once they did not reach 70% organic matter removal for sludge matrixes and soil matrixes. The other tested methods, which were based on oxidation achieved higher digestion efficacies. Nuelle et al. (2014) also

reported that solutions of 30% H_2O_2 and 35% H_2O_2 promoted greater removal of organic matter in comparison with NaOH (20, 30, 40 and 50%) and HCl (20%) solutions in sediment samples.

4.3. Digestion by oxidation

Digestion with hydrogen peroxide

Peroxidation is currently being used and differences in contact time and operating temperature of the chemical digestion can be found in the bibliography (Table 5).

WWTP effluents from the primary, secondary and tertiary treatments studied by Ziajahromi et al (2017) were passed through sieves of different mesh sizes and the retained material was removed with distilled water and subjected to chemical digestion with 30% hydrogen peroxide. The filtered volume (between 3 and 200 L) was stablished according to the degree of blockage of the sieves caused by each effluent. The H_2O_2 solution was added in different volumes (0, 10mL, 20mL and 50mL) depending on the type of effluent. The digestion was performed under heat (60ºC) until the H_2O_2 fully evaporated.

Gies et al. (2018) used 30% H_2O_2 at room temperature for the chemical digestion of influent, primary and secondary effluent and sludge (biosolids). For the liquid samples the supernatant was separated from the solid organic material by decantation, the settled organic layer was then subjected to chemical digestion for 7 days with 20mL of hydrogen peroxide. For the sludge, it was concluded that a mass of 5g (wet weight) requires 10 days for chemical digestion. The sludge samples were mixed with distilled water and the settled sludge was digested at room temperature before filtration through a 1μm

polycarbonate membrane filter under vacuum. The supernatant of both, liquid sample and sludge were processed with a protocol of liquid-liquid separation with canola oil to extract MPs.

In contrast to Gies et al. (2018) who performed the chemical digestion for settled organic matter, Magni et al. (2018) used 15% H_2O_2 to perform chemical digestion of the supernatant (both wastewater and sludge) obtained after separation by density with NaCl ($1.2g/cm^3$) and filtered through 8μm cellulose nitrate membrane filters. Li X. et al. (2018) also digested chemically the supernatant obtained after a density separation (NaCl 1.2 g/cm^3) of 20g of sludge. In this study the supernatant was passed through a 30μm sieve and the retentate was digested with 100mL of 30% H_2O_2.

Digestion with Fenton's reaction

Peroxidation to remove organic matter (Table 5) requires a high reaction time, which can reach days depending on the amount of organic material in the sample. An alternative method for reducing the need for long exposure times is the use of Fenton reagents, as stated above.

Fenton reaction consists of the use of an inorganic salt solution of Fe^{2+} which has the function of activating the peroxide (usually hydrogen peroxide, H_2O_2) acting as catalyser and leading to the formation of hydroxyl radicals (Equation 1), which has a high oxidation potential (2.80V) (Babuponnusami and Muthukumar, 2014; Bautista et al., 2008; Tagg et al., 2017). In addition to the shorter time required, the reaction does not require the addition of any external energy, i.e., the activation of peroxide occurs under ambient conditions of temperature and pressure (Babuponnusami and Muthukumar, 2014; Bautista et al., 2008).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + H0^{\bullet} + HO^{-} \tag{1}$$
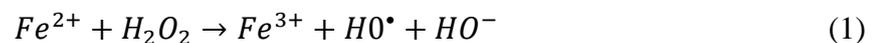
An important parameter to be controlled with respect to the Fenton reaction is pH. It has been shown that pH 3 is the ideal condition for the reaction. The pH adjustment can be performed with sulphuric acid. However, at higher pH, the precipitation of the generated Fe^{3+} as ferric hydroxide occurs and as consequently the availability of iron ions to catalyse the formation of hydroxyl radicals is lower (Babuponnusami and Muthukumar, 2014; Bautista et al., 2008).

In this way, many recent references focus on the combination of hydrogen peroxide solutions with heat and catalysts (like Fenton reagent). This represents an effective procedure to reduce the digestion time (Devi et al. al., 2016; Gies et al., 2018; Gündoğdu et al., 2018; Lares et al., 2018; Lusher et al., 2018; Magni et al., 2018; Sujathan et al., 2017; Ziajahromi et al., 2017).

Tagg et al. (2017) also reported that the polymers (PP, PVC, PE and Nylon) investigated by them showed no significant changes in the spectra generated by ATR- FTIR after chemical digestion with Fenton reaction. In terms of the use of Fenton reagents in the separation of MPs from organic materials Hurley et al., (2018) achieved more than 86% in organic matter removal of sludge sample, whereas the application of NaOH or KOH did not surpass 67%. In this context, Gündoğdu et al (2018) worked with some samples from the influent and secondary effluent. The samples were first sieved (at 55μm) and then, the retained material was subjected to a chemical digestion with Fenton's. Lares et al (2018) also carried out this technique to remove the organic matter from the material retained on the sieves before visual inspection.

Current lliterature points to that high temperatures (higher than 60º) can lead to negative results in the digestion process (Carr et al, 2016). A study realized by Munno et al. (2018) showed that the use of heat (above 60ºC) can melt microbead, which can underestimate

the quantities of this MPs in samples. Napper et al. (2018) also reported that the use of heat could lead to an underrepresentation of microbeads in a typical cosmetic product. Considering the negative effects of high temperature on the MPs, Hurley et al. (2018) proposes a chemical digestion based on the Fenton reaction where the temperature is kept below 40º.

4.4.Enzymatic digestion.

Enzymatic digestion may become an alternative for the organic matter elimination extraction from MPs samples since it is no aggressive for them. In this way, Cole et al. (2014) obtained a digestion efficacy of 88% using Proteinase-K for plankton-rich seawater samples. This efficacy was raised above 97% by increasing the incubation period, the enzyme concentration and the active temperature to 50°C. No degradation of the samples was observed. However, Proteinase-K is very expensive and the procedure is complex. In this way, other authors have proposed alternative enzymes, though the application was for bivalve tissues (Catarino et al., 2017; Courtese-Jones et al., 2017; von Friesen et al., 2019) or for plankton, sediment and biota (Löder et al. 2017). As an alternative to reducing the costs incurred in enzymatic digestion, Coustese-Jones et al. (2017) proposed the use of an enzyme considered less expensive than proteinase-K, in the study trypsin, collagenase and papain were used. Catarino et al. (2017) also used an enzyme considered most economically viable (Corolase 7089). In terms of the effects of enzymatic digestion on the subsequent identification of polymers von Friesen et al. (2019) evaluated the impact of pancreatic enzymes on ten polymers from the FTIR result matching before and after digestion and concluded that there were no significant changes in exposed polymers. As for the other digestion procedures, the enzyme still presents variability in procedures used and, besides, little is known about the enzymatic use for degradation organic matter of WWTP samples. Simon et al. (2018) used cellulase enzyme

for a prior cellulose fiber degradation from wastewater samples, however, the oxidation of organic matter was performed with Fenton reaction. In a study by Mintenig et al. (2017) an enzymatic digestion was performed in WWTP effluents. Protease, lipase and cellulase enzymes were used in the treatment. Despite the satisfactory results in the removal of organic materials and MPs separation, the process took more than 10 days to be performed and involved several steps that may have led to contamination and sample loss according to the authors. In this way, studies about application of this technique to wastewater samples are needed to compare the enzymatic digestion with other techniques.

5. Identification techniques

The characterization of MPs can be divided into physics and chemistry. Physical identification is done visually using a microscope and the microparticles are categorized by size, type (fiber, film, foam, pellets or fragments) and color (Crawford and Quinn, 2017b). Visual examination is a mandatory step to identify MPs separated from the matrix and this step have been wildly used by researchers (Table 6). According to Hidalgo-Ruz et al. (2012) preliminary discrimination between plastics and non-plastics can be made from some initial observations: the samples cannot have organic matter, the fibers must have the same thickness throughout its length and the particles must show homogeneous color along the structure. Hidayaturrahman and Lee (2019) based on the observations suggested by Hidalgo-Ruz et al. (2012) to visually identify MPs. Besides the use of spectroscopy for visual identification scanning electron microscopy (SEM) is a method to study the morphology of particles. Due to its generation of high-resolution images, SEM can be applied for the identification of impurities and possible MPs (Wang, W. and Wang, J., 2018). In addition, after a chemical digestion procedure, SEM can detect possible modifications on the MPs surface.

Despite the application of strict protocols and a very detailed visual evaluation, the number of error increases with the decrease of the particle, even so, this stage is of great importance for previous identification of MPs. In Figures 2(a) and 2(b) a great similarity between fibers is observed, one corresponds to cotton 100% (a) and the other one corresponds to a polyester fiber (b). Without an evaluation of the chemical structure of these fibers both could be erroneously classified as MP.

Literature reports some analytic methods as Pyrolysis gas chromatography-mass spectrometry, FTIR or Raman spectroscopy that can be applied for studying the chemical structure and to identify kinds of MPs. These techniques allow a precise identification of the chemical structure of the samples, and in addition to allowing segregation between MPs and non-MPs, it provides the polymer base and even the presence of additives. This information is important as it can be related to society's behavioural patterns and waste dispositions.

Apart from identifying the chemical structure of MPs, dyeing techniques have been employed in order to separate natural polymers and organic materials from synthetic polymers (MPs). In this context, the Rose-Bengal and Nilo Red reagents may enable this separation (Erni-Cassola et al., 2017; Maes et al.,2017; Ziajahromi et al.,2017). The first reactive acts by staining natural materials and non-MPs allowing visual separation and the second is adsorbed on the surface of plastic materials and requires the use of fluorescence microscopy. Although dyeing protocols are an alternative to rapid separation, a very effective chemical digestion process should be performed as the presence of organic materials can provide false results.

5.1 Fourier transform infrared (FTIR) spectroscopy and Raman

Among the existing techniques for MPs chemical identification Fourier transform infrared (FTIR) and Raman are the most commonly used. Both are vibrational spectroscopic techniques, which involve the molecular excitation of the sample and sequentially the generation of a characteristic spectral fingerprints. With the spectra generated is possible identify the substance by comparing with the spectra of known materials.

FTIR consists in irradiating the sample by IR light. Part of the radiation is absorbed depending on the molecular structure of the sample and then it is measured in transmission or reflection mode. Since each material has different chemical bonds, the spectrum generated by each sample can be compared with a database, which makes possible the identification. FTIR has two possible measurements to identify MPs: transmittance and reflectance setting, including the attenuated total reflectance (ATR) configuration, where the crystal must be in contact with the sample. Table 6 shows the variability of the operating conditions used in the technique for identification of microplastics whether in read mode (reflectance, transmittance), number of scans used and resolution. Fitting of reading conditions will be a function of the sample, as their size, shape and color may interfere with the analysis, requiring adjustments in equipment to provide an adequate spectrum with less noise and noticeable peaks. The ATR-FTIR proved to be efficient for the identification of larger particles (>500μm). To analyze smaller particles, ATR-FTIR coupled with a microscope (μ-ATR-FTIR) has been applied, and for these, even membrane filters can be visualized directly. Unfortunately, by this method little areas can be searched by time, which makes inviable visual sorting on the entire surface membrane area (Li et al., 2018; Käppler et al., 2018). To solve this drawback the FTIR with focal plane array (FPA) can analyze entire areas (Huppertsberg and Knepper, 2018; Li et al., 2018; Qiu et al., 2016;).

In our preliminary study, MPs were separated from the secondary effluent samples of a WWTP located in Valencia (Spain) and then identified by ATR-FTIR (Bruker) (Figure 3(a)) and μ-ATR-FTIR (Bruker) (Figure 3 (b)). Due the small thickness of the fiber (around 20µm) it was necessary the coupled microscope to place the crystal on the targeted MP.

The difference between FTIR and Raman is that the spectrum generated by the first technique depends on the change in the permanent dipole moment of the chemical bond, while the Raman depends on the change in the polarizability of the chemical bond (Käppler et al., 2016).

Operationally, while FTIR spectroscopy uses the incidence of IR light, Raman spectroscopy applies a monochromatic laser and this energy is absorbed by the sample before generating a spectrum. Käppler et al. (2016) suggest the use of both FTIR and Raman to obtain more complete and accurate results of the analyzed particles. In this report, authors compared different range of size and composition of MPs by these advanced techniques. Once Raman can provide more information about non-polar structure, this technique improves the information about the particle. (Lenz et al., 2015)

Despite the high sensitivity of Raman analysis to identify small particles (<20 μm), the method may suffer interference from the additives present in commercial plastics, resulting in considerable modifications in the base polymer's spectrum, which makes it difficult to identify them (Araujo et al., 2018; Lenz et al., 2015; Qiu et al., 2016). These interferences include the presence of foreign band and fluorescence. In this way, the additives present in the matrix can overlay the fingerprint spectrum of the base polymer and the MPs counted can be overestimated. Another problem, in terms of Raman analysis, is the fluorescence. Dyes and pigments may strongly emit fluorescence in the presence of

visible light precluding the identification of the polymer spectra (Lenz et al., 2015; Li et al.,2018; Massonnet et al., 2012, Jochem and Lehnert, 2002). Furthermore, the Raman performance depends on the equipment, laser wavelength applied and the operator. Massonnet et al. (2012) showed that a same dye fiber can provide different spectrums depending on the instrument used and mainly the excitation wavelength. Additionally, the pigment concentration and the combination of pigments affect directly on the result obtained.

5.2 Thermo-analytical methods

Apart from the application of commonly techniques, as FTIR and Raman, thermo-analytical methods also have been used on MPs characterization (Fries et al., 2017; Hermabessiere et al., 2018). Samples treated by thermo-analytical methods release gaseous compounds that are transferred to gas chromatography-mass (GC) for identification of chemical compounds (Li et al., 2018). Pyrolysis-gas chromatography-mass spectrometry (Py-GC-MS) is a thermo-analytical method that employs pyrolyzation to identify MP (Hermabessiere et al., 2018) and its additives simultaneously by the direct introduction of the sample with minimal pre-treatment (Crawford and Quinn, 2017b). The method is capable to identify a single particle, and a small amount of sample $(0.1 - 0.5mg)$ is suitable for one measure (Dümichen et al., 2017). The disadvantages of this method are because it is destructive, the sample must be manually placed in the instrument (Crawford and Quinn, 2017b) and compounds with high molecular weight (400 g mol^{-1}) can be condensed into the capillary from the pyrolysis to GC-MS system (Dümichen et al., 2017).

To overcome these drawbacks of Py-GC-MS another thermo-analytical method has been studied for the identification of microplastics. The TED-GC-MS combines the thermal

extraction with thermogravimetric analysis (TGA) with thermal desorption gas chromatography mass spectrometry (TDS-GC-MS) which allows to identify MPs in environmental samples (Dümichen et al., 2017). The TGA provides information about the mass change of polymers during heating and when coupled to MS or FTIR is possible to identify the decomposition products of the process. (Duemichen et al., 2014). Based on this, TED-GC-MS initially uses heating of MPs sample under inert atmosphere and the use of a thermogravimetric balance. The decomposition productions are adsorbing on a solid-phase located on the air outlet of the oven and these are transferred to the thermal desorption unit. The organic compounds desorbed are separated through a chromatographic column and identified by mass spectroscopy. The generated spectrum can be compared to spectral libraries and use to create database (Elert et al., 2017).

6. Quality assurance/Quality control (QA/QC)

A workshop report published by the Environmental Protection Agency (EPA) highlights the importance of procedures that ensure Quality assurance/Quality control (QA/QC) in the processes of sampling, separation and identification of MPs in order to generate reliable data and reduce underestimation or overestimation of MPs. The purpose of the QA/QC procedures is to reduce any type of contamination of the sample, to establish inherent errors in the separation techniques (during the processes used, such as filtration, sieving and density separation) as well as during the chemical digestion of MPs.

In addition, the instrumental techniques used (Raman, FTIR, thermo-analytical methods or other) must also be carefully studied for method determination, limit of detection and establishment of a reliable database (Fisher and Scholz-Bottcher 2017; Hermabessiere et al, 2018). Procedures as avoiding synthetic clothing, using glass materials instead of plastics, cleaning the work surface with alcohol and the use of blanks to evaluate sample

contamination and losses are reported by some authors (Gies et al., 2018; Lares et al., 2018; Lenz et al.,2015; Li X et al., 2018; Liu et al., 2018; Magni et al, 2019). In order to assess airborne contamination petri dishes with a membrane filter can be placed on the workspace for some hours and then can be analyzed as black control (Lenz et al, 2015).

7. Conclusion and perspectives

The presence of MPs in water bodies is increasingly evident around the world. It is noticeable that in the case of effluents from WWTPs millions of MPs are released per day all over the world. Because of that, these facilities are considered as significant sources of MPs even when they have a high percentage of retention. Despite the intense efforts that have been directed towards the elaboration of methodologies of separation, quantification and identification of these emerging contaminants, no standard protocol is still applied in WWTPs. These methodological differences presented by researchers are seen even in the initial stages of sampling and in the selection of size ranges of MPs to be analysed, which makes difficult the comparison of the results among researchers. Therefore, the determination of efficient and rapid protocols for the study of MPs is extremely important, always considering steps that evaluate cross-contamination, either in the transport of samples or during analysis. In addition, the standardization of sizes (sieving, nets and filters), chemical digestion (acidic, basic, peroxidation or other), density separation (best solution to be used), visual separation (addition of staining dyes) and analytical techniques for chemical identification of the polymer, need to be optimized and applied in a standard manner.

Based on current knowledge of WWTP as sources of microplastics and the remaining gaps, the following aspects deserve attention for future research:

- Since microplastics have shown high sorption potential of organic pollutants, the better understanding of the desorption process is relevant to the understanding of how these pollutants can actually be transported in the food chain.

- Given the accumulation of MPs in biological sludge, future studies on the incorporation of these particles into terrestrial animals should be performed.

- In terms of digestion procedures, we understand that when evaluating WWTP, factors such as effluent compositions (amount of organic matter, solids, pH, among others) and the fact of treatment procedures employed in the stations differs sometimes, the standardization of a methodology can present difficulties. However, since some digestion procedures have already been shown to be extremely aggressive to MPs, a basic methodology with at least the reagent and its concentration to be used should be designed and established to facilitate reproducibility of the results.

- With the review of the literature performed in this paper, it was observed that several authors use only visual identification to distinguish microplastics from non-plastics. This step is of fundamental importance for an initial screening of possible MPs however, this may imply a wrong sample count and classification. For example, without the use of chemical identification techniques, natural fibers can be mistakenly called MPs when performing only visual separation. For better validation and comparison of results, the application of polymer identification methods should be more widely used and not just the visual method.

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