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Determination of microplastics in municipal wastewater treatment plant effluents and sludge using micro-Raman spectroscopy

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Abstract

Microplastics (MPs) increase global awareness due to their ubiquity, high concentration levels, and devastating effects on the aquatic environment. Wastewater treatment plants (WWTPs) are recognized as a significant source of microplastics in aquatic and terrestrial environments, particularly for plastics used in personal care products and textile fibers from the clothing industry. The focus of the present study was the determination of MP in wastewater and sewage sludge samples of WWTP of Ioannina city, according to their shape, size, concentration, and polymer type. A wet peroxide oxidation (WPO) method using 30% H_2O_2 and 0.05 M ferrous (II) solution was applied to the water effluent, while an enzymatic digestion method combined with WPO was employed to eliminate the organic matter and extract MPs from sludge samples. Micro-Raman spectroscopy coupled with appropriate software was applied to detect and quantify the microplastic particles. The outcomes from this study showed that the most representative shape of MP in effluent wastewater and sludge was fragments (66.7% and 75%, respectively), followed by fibers, spheres, and films. Polyamide (PA), *p*-acrylic acid (PAA), and *p*-acrylamide-co-*p*-acrylic acid (PAM-co-PAA) were the most abundant polymers (100% frequency of detection), followed by *p*-vinyl chloride (PVC), *p*-butyl methacrylate (PBMA), *p*-ethylene (PE), *p*-styrene (PS), *p*-propylene (PP), *p*-vinyl alcohol (PVA), and *p*-vinyl butyral (PVB) (20%-80% frequency of detection). The mean concentration of MPs in five sampling

campaigns was 5.8 ± 0.6 particles/L in secondary WWTP effluents and 33.3 ± 8 particles/g in sludge. This study focused on monitoring campaign, in order to better understand the occurrence, impact, and risks associated with MPs on WWTPs.

Keywords: Microplastics, wastewater effluents, sewage sludge, micro-Raman spectroscopy, risk assessment

INTRODUCTION

Plastic particles ranging in size from 0.001 to 5 mm, so-called microplastics (MPs), have been used extensively during the last decades, while biotic and abiotic degradation of plastic debris also resulted in their formation in significant amounts. In compliance with the Commission Delegated Decision (EU, 2024/1441), "microplastic" is defined as a small unobtrusive particle that is solid, insoluble in water and composed partly or wholly of synthetic polymers or chemically modified natural polymers[1]. Based on the hazard model adopted from the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) by the United Nations, the majority of plastic polymer types are considered hazardous substances[2]. The comprehensive presence of microplastics on a global scale has raised worldwide concerns affecting any environmental parts[3]. In 2023, European plastics production reached 54 Mt., and by 2050, 12,000 Mt. of plastic waste will have been dumped or released into the environment[4].

Depending on their origin, MPs can be divided into two groups: primary MPs that are used in different products such as personal and cosmetic products and originate from the release of small particles in the environment during personal and industrial use, manufacture, and transportation[5]; secondary MPs result from fragmentation of larger particles due to various processes such as chemical/mechanical degradation, hydrolysis, photodegradation, biodegradation, and other mechanisms[6,7]. MPs are also classified according to the form, size, and composition of the polymer[8].

Wastewater treatment plants (WWTPs) represent the final stage of the man-made water system, receiving microplastic pollutants from a variety of sources, including household and industrial activities[9,10]. In this sense, WWTPs are recognized as one of the largest sinks of microplastics and are not always able to efficiently remove microplastics during treatment, resulting in their release into the environment[11]. Since then, MPs have been deemed emerging pollutants demonstrating significant effects on biota and humans. Conventional WWTPs have been evidenced to reduce 60%-80% of MPs from the water phase, resulting in their transfer to the sludge phase. This sludge can be landfilled or used as agricultural biosolids after treatment. For example, Talvitie *et al.*[12] reported that around 80% of both plastic and non-plastic microplastics are distributed in dried sludge, while Vollertsen and Hansen[13] estimated that around 75% of microplastics are present in the sludge phase.

Polyamide (PA) (~ 53%), polyethylene (PE) (~ 17%), polyester (PES) (~ 90%), polypropylene (PP), polyethylene terephthalate (PET), and polystyrene (PS) are among the most common polymer types present in wastewater and sludge[14,15]. These polymers are discharged into wastewater due to human activities and have applications in various products such as packaging, textiles, and consumer goods. Specifically, PET is a type of polyester originating from clothing and textiles, PA is a polymer used in textiles and fishing gears, and PE is used in facial or body cleansing products, wrapping film, and water bottles[16-18]. The concentration of MPs in wastewater has been studied widely. In contrast, research on sludge is relatively scarce[19]. For instance, high MP concentrations in sewage sludge are observed in Germany (Sleeze) from 74 to 495 particles/g and in China from 74 to 240 particles/g due to its large population size, with fragments being the most abundant form (55%)[20]. In one of Italy's largest WWTPs (Northern Italy, built in the 2000s), the

number of detected MPs in effluent wastewater was 0.4 ± 0.1 MPs/L, and 113 ± 57 MPs/g in sludge dry weight, mainly polyesters (35%) and polyamide (17%)[21]. Another study from the city of Cadiz (Spain) reported the presence of MPs from 16.40 ± 7.85 MPs/L to 131.35 ± 95.36 MPs/L in urban and industrial WWTP effluents, respectively[22]. Furthermore, the study carried out by Mason *et al.*[23] in 17 wastewater treatment plants in the United States presented an average of 0.05 MP/L in the final effluent, and the majority of them were determined as fibers (59%) followed by fragments (33%), while the size of 57% of MPs ranged between 0.125 mm and 0.355 mm. Finally, there is considerable variation in measuring MPs in sludge samples from other research works that have established the presence of MP in both primary and secondary sludge between 0.2 and 238 particles/g[7,18,24,25].

It has also recently been announced that MPs are considered "vectors" for absorbing and transferring toxic compounds in the environment, including pharmaceuticals, organochlorine pesticides, persistent organic pollutants (POPs), polybrominated diphenyl ethers, heavy metals, polychlorinated biphenyls, and polycyclic aromatic hydrocarbons[26-28]. This is because MPs have a lipophilic nature, small size, a large specific surface area, and surface charges, thus providing numerous sites for interactions with chemicals and micropollutants, such as Van-der Walls forces, hydrogen bonding, ionic, electrostatic, aromatic-aromatic and π-π interactions, leading to an additional risk for the environment[8,16,29].

Therefore, to better understand the contribution of MPs to the environment, there is an immediate need for further research into the potential risks associated with MPs in WWTP effluents and sludge. According to the Codex Alimentarius in 2011[30], exposure assessment, hazard characterization, and risk identification are the steps involved in the risk assessment of MPs. A hazard classification model for 55 thermoplastic and duroplastic polymers, based on the EU regulation (EC: European Commission) No. 1272/2008[31] and Labelling (CLP: Classification, Labelling and Packaging) regulation on hazard classes and categories of chemicals which pose a risk to the environment and human health, has been developed by Lithner *et al.* (2011)[2].

In literature, there are five main approaches for collecting microplastics based on their operating principles. These include static mechanical method through mesh filters (used in this study)[32], dynamic mechanical behavior[33], fluid dynamics[34], chemical approaches that involve the separation of microplastics with chemical agents, microorganisms, or solid adsorbents[35,36] and magnetic technology (e.g., magnets, electromagnets)[37]. As reported by Spreafico *et al.*[38], the static mechanical approach has the most numerous patents, as seen in the recent publication trends. This method can collect microplastics ranging from 10 μm to 1000 μm. Furthermore, various extraction procedures have been developed to effectively quantify and characterize MPs in complex environmental matrices. Based on the sample matrix, the main extraction methods are categorized as follows: density separation using hypersaline solutions (e.g., NaCl, NaI, $ZnCl_2$, NaBr, $CaCl_2$, KI)[39], elutriation using gas or liquid to distinguish particles[40], pressurized liquid extraction[41], magnetic separation (e.g., Fe-Nanoparticles)[42], electrostatic separation[43], and oil-extraction protocols (e.g., castor oil, canola oil, mineral oil)[44,45]. Several studies have also reported pre-extraction steps, including sieving to separate larger visible particles and filtration to isolate the solid phase from the liquid phase[46]. Finally, to improve the extraction of microplastics, it is crucial to apply methods for removing organic matter, either after or prior to the separation techniques, which include acidic/or alkaline digestion, as well as oxidative and enzymatic digestion[17].

Up next, methods used to determine MPs range from visual observation via a dissection microscope over pyrolysis-gas chromatography-mass spectrometry (pyr-GC-MS)[47] to Fourier-transform infrared spectroscopy (FT-IR) and microscopy-Raman spectroscopy[18,48,49]. The carbon-based polymers of MP can be

easily distinguished from other organic or inorganic particles using FTIR spectroscopy, and specific types of MP polymers can be identified using a library of spectra[50]. Compared with FTIR spectroscopy, micro-Raman spectroscopy serves as an alternative non-destructive technique for MP analysis and offers better spectral resolution (down to 1 μm compared to 10-20 μm for FTIR), broader spectral coverage, higher sensitivity to non-polar functionalities, less water intervention, and tighter spectral bandwidths[51-55]. Another advantage of using micro-Raman spectroscopy is that it can separate and give detailed information about the additive compounds (colorants, plasticizers, fillers, etc.) present in MP particles, compared to FTIR spectroscopy, which presents low identification percentages when MPs are covered with other materials[56]. However, sample pre-treatment, including sample digestion and separation from the contaminated matrices, is required prior to Raman and other spectroscopic detection methods for complex environmental or biological matrices where it is hard to recognize different shapes, sizes, and polymer types[52,57].

Therefore, the present study focuses on the application of methods to accurately identify and quantify microplastics in wastewater effluents and sewage sludge and provides a detailed investigation of microplastic concentration and characteristics (shape, size, and polymer type) across a typical WWTP in Ioannina, Greece. A simple and precise method including digestion of organic matter and density separation was validated for its efficacy in extracting microplastics from complex matrices. As far as we know, this study is the first of its kind to successfully apply micro-Raman spectroscopy combined with Particle Finder™ software based on mapping approaches for MP identification and visualization in complex environmental matrices such as sludge. The study finally targets the assessment of the associated risk of MPs occurrence in wastewater and sludge.

METHODS

Chemicals and materials

Hydrogen peroxide (H_2O_2) stock solution (30%), PS-NPs water suspension (2% solids content w/v), methanol, formic acid, phosphate-buffered saline (PBS, pH = 7), lipase obtained from *Candida rugosa,* protease from *Bacillus licheniformis*, and cellulase originated from *Aspergillus niger* were obtained from Sigma-Aldrich (Darmstadt, Germany). A purification system from Evoqua Water Technologies was used to provide ultrapure water. Iron (II) sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$) was from Merck (Darmstadt, Germany), while $ZnCl_2$ of high purity (> 98%) was provided from ChemLab (Zedelgem, Belgium). Macroporous silicon membranes of 5 and 2.5 μm pore diameter were obtained from smart Membranes [Halle (Saale), Germany].

Sample collection

Wastewater effluents and sludge samples were collected from the WWTP in Ioannina, Northwestern Greece. The WWTP serves a population of approximately 100,000 residents and handles mainly household and industrial effluents with an average flow of 17,600 m^3/day. The system consists of a primary treatment stage equipped with a ventilated tank for the removal of grit and a sedimentation tank, a biological treatment stage (secondary treatment) consisting of an anaerobic stage and an aerobic decomposition stage, and finally a disinfection stage (tertiary treatment) by chlorination. The treated water outflow is discharged into the Kalamas River. The activated sludge process is employed with approximately 60% sludge reuse and an average solids retention time (SRT) of 11 days. More information about the main activities in the facility zone, and the capacity and function of WWTP can be found in the latest publications[58,59]. Wastewater effluent samples of 40 L were collected in five different sampling periods in dry weather from March to July 2024 at the beginning of each month through short-time pump sampling, sieved through stainless steel screens with sizes of 1000, 500, 250, 125, 63, and 25 μm and then covered with aluminum foil for further analysis. Grab secondary sludge samples of 2 kg were also collected during the same period, stored in glass

bottles, and kept frozen in the dark at -20 °C. The sludge samples were then lyophilized and homogenized. They were stored at 4 °C until analysis.

Sample processing

Wastewater samples

The wastewater effluents were processed through oxidative digestion according to previously reported and validated methods[22,60-64]. Briefly, the contents of the mesh sieves (25-63, 63-125 and 250-500 μm) were washed into a 500 mL beaker using deionized water, while the particles in the mesh screens of 500-1000 and > 1000 μm were washed with water, collected by hand using metal tweezers and stored in Eppendorf tubes for analysis. 10 mL of a freshly prepared $FeSO_4$ solution (0.05 M) was added to the beakers containing the washed particles (150-200 mL water). Subsequently, 20 mL of hydrogen peroxide solution (30%) was added and mixed intensively to oxidize the organic matter. Next, the beakers were heated to 55 °C on a hotplate for 5 h, covered with aluminum foil while stirring continuously (600 rpm) and visually checked every hour. If necessary, a further 10 mL of hydrogen peroxide was added to the beakers until no solid particles could be seen. The solution obtained was cooled to room temperature, transferred to a 50 mL centrifuge tube and centrifuged at 4000 rpm for 10 min [Supplementary Figure 1]. Afterward, the supernatant proceeded to a density separation step using 30 mL of $ZnCl_2$ 2M solution (density 3.1 $g{\cdot}cm^{-3}$), stirred for 30 min and settled overnight. The buoyant particles were then filtered on a silicon membrane filter of 2.5 μm pore diameter and rinsed with ultrapure water. All tools and bottles were rinsed with water and methanol to prevent sample contamination throughout the treatment process.

Sewage sludge samples

The sludge samples were processed according to the methodology described elsewhere[25,65-69] with some modifications, including enzymatic digestion, oxidative digestion, floatation, and filtration. In brief, 5 g of the lyophilized sludge sample was transferred to 500 mL glass beakers, 10 mL of ultrapure water was added to each beaker and magnetic stirred for 10 minutes until the sample was well mixed with water. Enzymatic digestion was carried out using enzymes that have been reported to be effective in the removal of organic matter from sewage sludge for the extraction of MP[68]. So, a mixture of three enzymes (0.3 g lipase from *Candida rugosa*, 1.95 mL protease from *Bacillus licheniformis*, and 0.375 g cellulase from *Aspergillus niger*) was added to the sludge samples, incubated at 50 °C with continuously stirring for 96 h while a buffer solution (PBS) was added to achieve optimal pH for enzymes [Figure 1]. At the end of enzymatic digestion, an oxidative post-treatment followed using 20 mL H_2O_2 (30% v/v) under heating to 50 °C and stirring for 24 h. Digested samples in beakers were transferred to 50 mL centrifuge tubes, beakers rinsed with ultrapure water, and density separation proceeded as described above in the wastewater sample preparation section. Finally, the buoyant particles were then filtered on a silicon membrane filter of 5 μm pore diameter and rinsed with ultrapure water.

Method validation

PS has an intermediate density (1.05 g/mL) that covers a wide range of densities of the most known detected MPs in WWTPs while it is also one of the MPs commonly detected in WWTPs, and the availability of its standard suspension makes it appropriate for optimization experiments of the digestion process. In addition, previous studies[70] have also used PS for the optimization of different digestion methods in sludge. Thus, to validate the sample extraction methodology, an appropriate amount of PS-NPs suspension in water (2% solids content w/v) was spiked in 200 mL of tertiary treated wastewater effluent and 5 g dry sludge in a glass beaker, in order to generate an initial loading of 300 PS particles. Furthermore, 1 g of PVC particles [density (1.4 g/mL)] with a size range of 125-250 μm were also spiked in wastewater effluents and 0.1 g of PVC particles in 2 g of sludge. The samples were processed according to the methodology described above for wastewater and sludge, respectively. All MPs were counted using micro-Raman spectroscopy using the

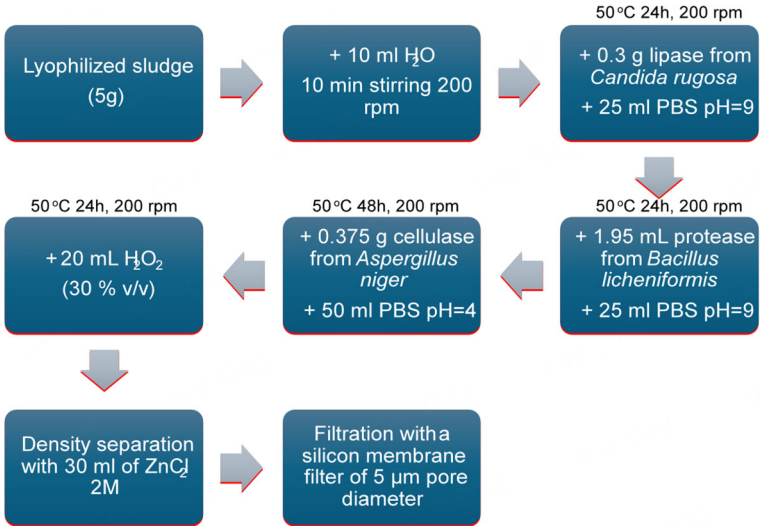

Figure 1. The optimized protocol used for the elimination of organic matter from sludge. PBS: phosphate-buffered saline.

software Particle Finder™. A total of 230 particles of the PS microbeads were recovered from wastewater samples, achieving 76.7% recovery, and 216 particles of PS were recovered from the sludge sample, achieving 72% recovery, which we deemed acceptable. PVC particles have been recovered in high yield, with extraction efficiencies ranging from 96% for effluents to 86.2% for sludge.

Identification, quantification and characterization of the extracted MPs

All suspect microplastics have been counted and classified as follows: shape (namely fiber, fragment, film, and sphere), size (perimeter and diameter), and volume. MP abundance was expressed as the median number of MP items per liter or per gram (a) at each sampling and (b) for all samplings and (c) as the frequency of detection of each microplastic in wastewater effluents and sludge samples.

The larger particles (500-1000 and > 1000 µm) were manually picked from the mesh screens, dried, weighted, and identified using attenuated total reflectance (ATR) - FTIR spectrophotometer (Shimadzu IR Spirit, Kyoto, Japan). FTIR spectra were recorded in transmittance mode, with 2 cm^{-1} resolution and 45 concurrent scans in the 4000 to 400 cm^{-1} region. The sample spectrum was compared to that of the library and the level of certainty was set up to > 70%.

For the determination of smaller MPs, a multi-modal Raman microscope, Labram Soleil™ (Horiba Scientific, Lyon, France), was used. The instrument is standardly equipped with two gratings (600 nm and 1800 nm) by default, a multiple channel detector and the LabSpec6 Spectroscopy suite software. Raman spectra were collected at variable magnifications of 5× and 50× using a laser with a wavelength of 532 nm and a power of 1.9-3 mW in the range of 1000-3800 cm^{-1}. The integrating time was 50-200 s (usually 200 s), and the number of cumulations was 3-10 times (usually 6). Then, the Particle Finder™ software was applied to wastewater and sludge samples, enabling automated particle location, analyzing important particle parameters, like size and volume, and later chemical characterization using Raman spectra. Recorded spectra were baseline corrected to improve spectra quality and match the recorded spectra with the KnowItAll™ library database reference with > 70% identification. Particle Finder™ is ideal for use in environmental applications, analyzing microplastics where the number of particles is large and tracking by hand and tagging each particle would be time-consuming.

The surface morphology of representative MPs was determined using a Field Emission Scanning Electron Microscopy (Thermo Fisher Pharos Phenom, Waltham, MA, USA), run under high vacuum (0.1 Pa) with the acceleration voltage set at 15 kV, using a 20:80 ratio of backscatter and secondary electron detection. Each sample was coated with a 5 nm layer of chromium before analysis, while the chemical composition of the surface of the MPs was investigated by EDS analysis.

Risk assessment of microplastics

Microplastic risk assessment was calculated according to the methodologies used in literature[2,71-73]. Based on the abundance and polymer composition of MPs, the pollution loading index (PLI) method and polymer hazard index (HI) method were used to assess the MP pollution risk in the WWTP[73,74]. To make this assessment, the hazard scores of plastic polymers were adopted from Lithner *et al*[2]. The above protocol is beneficial for assessing both the combined effect of several pollutants and the environmental impact of particular contaminants in a specific location. The pollution load index for each sampling (PLIi) was used to quantify the overall pollution level of microplastics in wastewater and sludge. The following equations illustrate the assessment model:

$$CFi = Ci/Co \quad (1)$$

$$PLIi = \sqrt{CFi} \quad (2)$$

$$PLI = \sqrt[n]{PLI1 \times PLI2 \times PLI3 \times \dots \times PLIn} \quad (3)$$

where Ci is the abundance of microplastics at sampling i, Co is the minimum abundance value for all samplings, and CFi represents the pollution coefficient of MPs. The PLIi was calculated as the root of the CFi, while the pollution load index (PLI) denotes the nth root of the multiplication of all PLIi in samples. PLI < 10 indicates hazard category I, PLI 10-20 represents hazard category II, and PLI 20-30 and PLI > 30 show hazard category III and IV, respectively[75]. In order to assess a comprehensive indication of the risk posed by various microplastic polymers, Hakanson[76] suggested the idea of pollution risk index (PRI) that combines the PLI of microplastics and the hazard index (HI) of microplastic pollution according to the Equations (4-7).

$$HIi = \Sigma Pn \times Sn \quad (4)$$

$$HI = \sqrt[n]{HI1 \times HI2 \times HI3 \times \dots \times HIn} \quad (5)$$

$$PRIi = HIi \times PLIi \quad (6)$$

$$PRI = \sqrt[n]{PRI1 \times PRI2 \times PRI3 \times \dots \times PRIn} \quad (7)$$

where HIi is the hazard index of microplastic pollution at sampling i, Pn is the mass fraction of different microplastic polymer types at sampling i, Sn is the hazard score for microplastic polymers [Supplementary Table 1], HI is the hazard index of microplastic pollution of the WWTP for all samplings, PRIi is the pollution risk index of microplastics at sampling i, and PRI is the pollution risk index of the overall samplings of the WWTP. The risk categories of MPs hazard index and pollution risk index are represented in Supplementary Table 2.

RESULTS AND DISCUSSION

Occurrence of microplastics in wastewaters

The methodology used in this study successfully identified small MPs present in wastewater effluent samples. MPs were classified into six different particle sizes from 25 to > 1000 μm. The size distributions of MPs of wastewater effluent samples collected during different sampling periods are shown in Figure 2. The sizes of 25-63, 63-125, and 125-250 μm were the common sizes of MPs in all wastewater samplings, ranging from 5.8% to 37.3%, 18.6% to 55%, and 10.7% to 41.1%, respectively. It is noted that larger MPs (> 1000 μm) decreased during wastewater treatment steps and became smaller particles due to fragmentation[77]. Therefore, larger particles (> 1000 μm) in this study were between 5.5% and 10.4% in all wastewater samplings. The percentage variation of sizes 250-500 μm and 500-1000 μm are similar and varied from 5.5% to 12.6% and from 1.1% to 8.8%, respectively. The findings of this study were similar to those previously reported in other countries, i.e., MP particles at WWTPs were mainly in the range of 62.5-200 μm[64,78-80]. Furthermore, the size distribution in this study is also in agreement with a study that used micro-Raman spectroscopy for MP analysis in WWTP from Germany and found that 95% of all detected particles were in the $10 < d_p < 30$ μm and $30 < d_p < 100$ μm size classes[48].

The MP concentration (number of MPs per liter) according to the sieving sizes and across samplings is illustrated in Figure 3A and 3B, respectively. According to Figure 3A, the mesh screen sizes of 25-63, 63-125, and 125-250 μm presented the highest average MP concentration, i.e., 1.60 ± 1.3 particles/L, 1.76 ± 1.2 particles/L, and 1.31 ± 0.4 particles/L, respectively, while reduced average MP concentration from 0.19 to 0.50 particles/L was observed for larger particles from 250 to > 1000 μm size. The highest MP concentration count 9.05 ± 1.5 particles/L was found in the 4th sampling event and the lowest MP concentration 3.95 ± 0.56 particles/L in the 2nd sampling event. An average concentration of 5.8 ± 0.6 particles/L was detected in all samplings in the WWTP of Ioannina city. The reported MP concentrations in WWTP effluents in other studies ranged from 0.006 particles/L to 9 particles/L, which are in agreement with the concentration of MPs found in this study[10,12,61,81,82].

The MPs could be in the form of fiber, fragment, film, and sphere. The fragments occupied a significant proportion, representing 67 ± 12.9% of all the particles in all samplings, followed by fibers and spheres (15 ± 5.9% and 12.7 ± 5.3%, respectively) among all the wastewater samplings, while films occupied relatively the smallest proportion at 5.5 ± 5% [Figure 4].

A total of 1,575 particles were detected in this study in wastewater effluents, and 1,076 particles were confirmed to be MPs, representing 68.3% using micro-Raman spectroscopy combined with Particle Finder™ software. The Raman spectroscopy revealed 22 different polymers in all samplings. The dominant microplastics in wastewater effluents, as shown in Figure 5, were *p*-acrylic acid (PAA) (48.3 ± 2.1%), followed by *p*-acrylamide (PAM) (13 ± 6.5%), *p*-vinyl-chloride (9.9 ± 6.2%), *p*-acrylamide-co-*p*-acrylic acid (9.6 ± 8.1%), *p*-butyl methacrylate (8.6 ± 8.5%), and *p*-ethylene oxide (8 ± 0.1%). Other types of MPs, such as PE, PS, PP, EVA, PVA, p-vinyl stearate (PVS), p-vinyl acetate (PVAc), etc., contributed with little proportion ranging from 0.6% to 5.3%. Polyamide, *p*-acrylic acid, *p*-acetylene, and PAM-co-PAA were the most abundant polymers (100% frequency of detection), followed by PVC, PBMA, PE, PS, PP, PVA, and

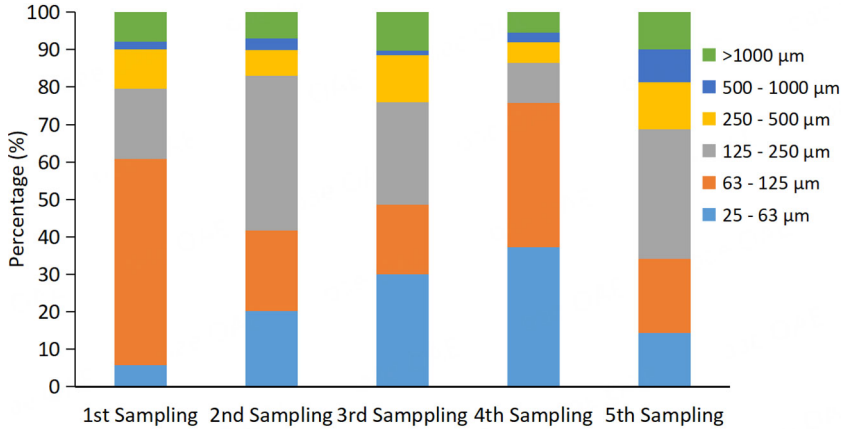

Figure 2. The distribution percentage (%) of the sizes of the MPs in each sampling campaign. MPs: Microplastics.

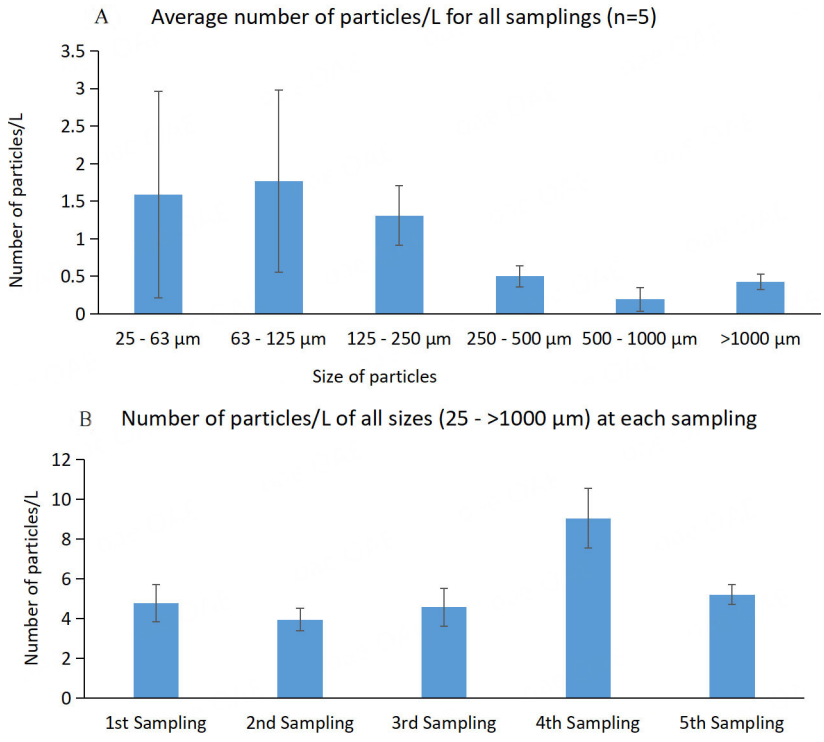

Figure 3. (A) MPs concentration (number of particles/liter) for all samplings according to their size and (B) MPs concentration (number of particles/liter) of all sieve sizes for each wastewater sampling. MPs: Microplastics.

PVB (20%-80% frequency of detection). An average of 102 ± 44 particles were detected as PAA, 16.6 ± 13.5 as PVC, 22 ± 2.5 as PAM-co-PAA, 13 ± 14 as PBMA, 8.6 ± 12.4 as PA, 8.6 ± 11.9 as PE, and < 5 ± 1.9 as the other polymer types. As illustrated in Figure 6, at the smallest size range 25-63 µm, PAA was the dominant polymer type with 53.9%, followed by the co-polymer PAM-co-PAA (17.4%) and PVC (10%). A similar pattern was observed for the other sieve sizes ranging from 63 to 500 µm, while for larger particles 500-1000 µm and > 1000 µm, the most abundant polymer types were PA (21.2%, 38.5%), EVA (30.3%, 35.4%), and *p*-acetylene (9.1%, 6.2%), respectively. The other polymer types, such as PP, PE, PS, *p*-acetylene, etc., ranged between 0.3 and 9.4% on sieves with mesh widths of 25 - > 1000 µm. According to the size of MPs, PAA

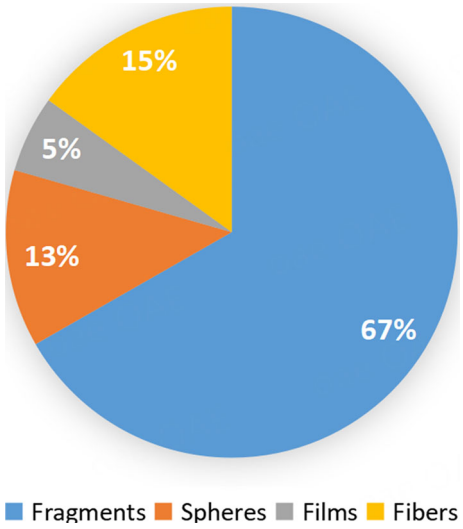

Figure 4. The distribution percentage (%) of the shapes of the MPs of all sizes (25 - > 1000 μm) in all samplings. MPs: Microplastics.

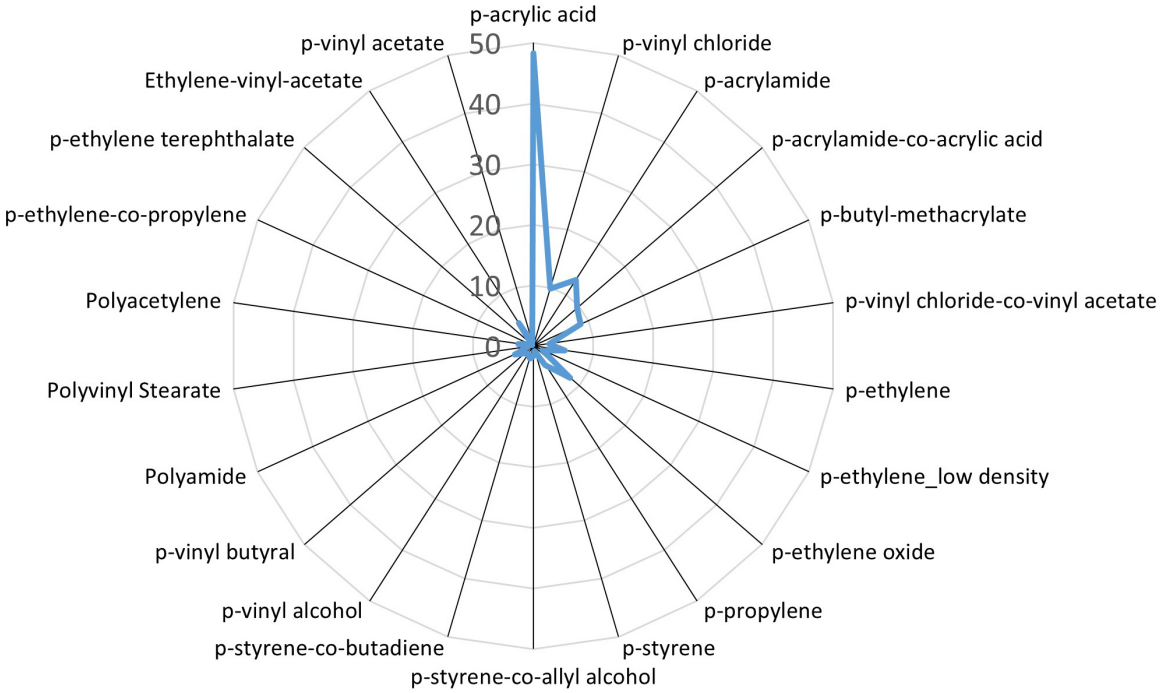

Figure 5. The average percentage of polymer type of MPs of all sizes (25 - > 1000 μm) in wastewater effluent samplings. MPs: Microplastics.

particles have a mean diameter of 94 μm and an average perimeter of 532 μm, PVC particles 375 μm and 1312 μm, respectively, PA 187.5 μm and 961 μm, respectively, while PAM-co-PAA particles 44 μm average diameter and 410 μm average perimeter. Finally, as shown in Figure 7, the majority of PAA detected particles are in the form of fragments (46.7%) and spheres (23.1%). They probably originated from the fragmentation of synthetic textile materials into small pieces in a variety of ways, such as the secondary MP[83]. This is quite different from recent studies, which found that fibers are the dominant form of microplastics, followed by fragments[23,64,84-86]. The differences can be related to random natural variations

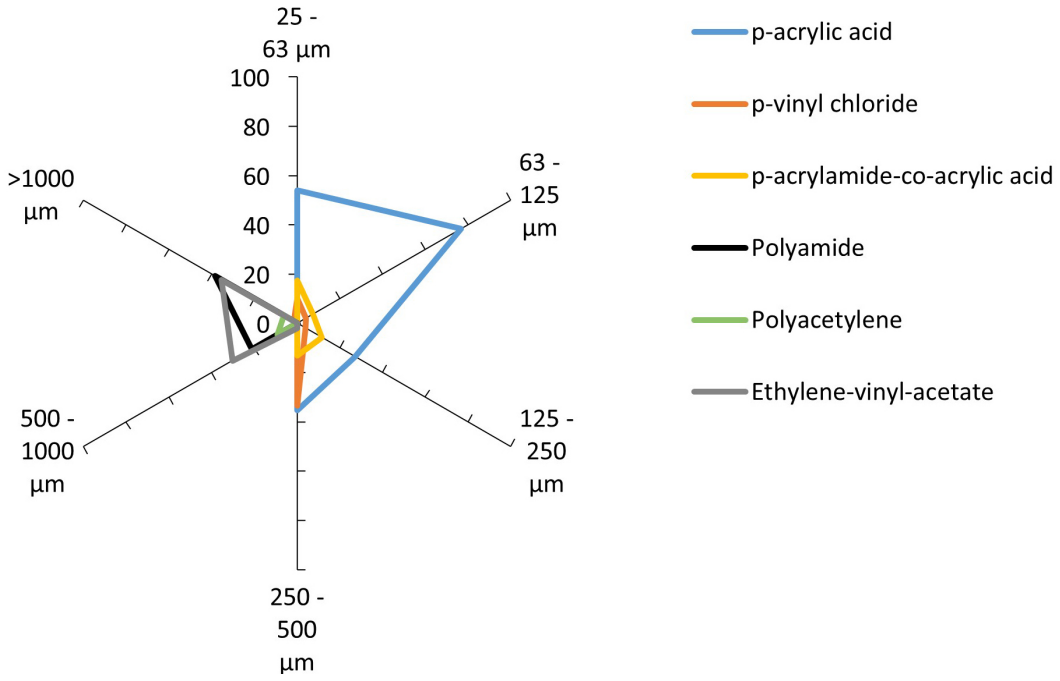

Figure 6. The percentage of polymer type of MPs according to their size in wastewater effluent samplings. MPs: Microplastics.

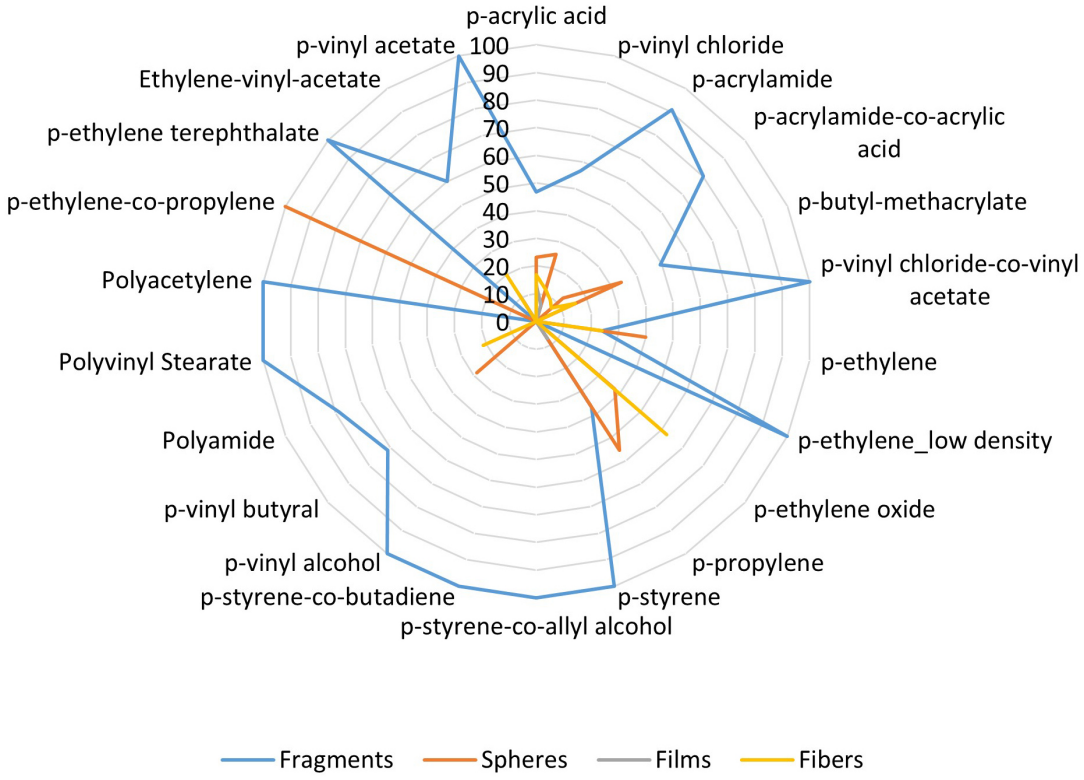

Figure 7. The percentage of polymer type of MPs according to their shape in wastewater effluent samplings. MPs: Microplastics.

among the samplings and the studied WWTP. Furthermore, the majority of observed PE and PP particles had spherical morphology (40% and 55.5%, respectively) matching microbeads from personal care products[87]. In the WWTP effluents examined by other researchers[48,82,88,89], PA, PAA, PVC, and polyesters predominated, which is also confirmed by the results of the present study. Comparable data for MP concentration, shape, size, and polymer type from other studies in different WWTPs across the world are presented in Table 1. The most abundant polymers in this study, *p*-acrylic acid, *p*-acrylamide, and *p*-acrylamide-co-acrylic acid, could originate from textiles, synthetic clothing, and medical devices[8], while the polymer type PVC has a wide range of uses and is usually employed in raincoats, seat covers, garden hoses, shoe soles, and shower curtains[18]. Detailed information about the uses of synthetic plastic particles detected in this study can be found in Supplementary Table 3.

Representative Raman spectra profiles for different polymer types are presented in Figure 8. For instance, in the Raman fingerprint range, PE had three main peaks, namely the peaks corresponding to the CH_2 twist at 1288 cm^{-1}, the C-H bending mode at about 1432-1450 cm^{-1}, and the C-H symmetric and antisymmetric stretching at 2870-2950 cm^{-1}[94]. The spectral regions associated with PP include CH_2 and CH_3 symmetric and antisymmetric vibrational modes corresponding to 2800-3100, 1460, and 1378 cm^{-1} bands, as well as the region of 1155 cm^{-1} for $[CH_2CH(CH_3)]_n$[54]. Furthermore, the band at 1300 cm^{-1} in *p*-acrylic acid is assigned to (-OH) bending vibrations, the sharp bands at 1694 and 1740 cm^{-1} correspond to C = O stretching, the peak at 1446 cm^{-1} at δ (CH_2) and the band at 2900 cm^{-1} arise from C-H stretching modes[95]. A peak at 1600 cm^{-1} is assigned to the NH_2 group of amides, the band at 1630 cm^{-1} corresponds to the stretching of the carbonyl (C = O) of acrylamide units in *p*-acrylic acid-co-acrylamide polymer, while the bands at 1433 and 1492 cm^{-1} are assigned to C-N stretching vibration of amides and the band at 1360 cm^{-1} is assigned to CH_2 wagging vibration[96]. Moreover, adsorption experiments in the aqueous phase were conducted with pristine PS particles with emerging contaminants, as well as weathering of pristine PS under sunlight. More details are provided with supplementary information, and the recorded Raman spectra are provided in Supplementary Figures 2 and 3. Neither pollutant adsorption nor weathering resulted in identification matching levels lower than 70%. The ATR-FTIR detection technique was used to determine the composition of the polymer particles in the size ranges of 500-1000 and > 1000 μm. As shown in Figure 9, spectral regions associated with PA include -C=O at 1640 and 1712 cm^{-1} bands and -C-N-H absorption peaks at 1250 and 1550 cm^{-1} bands[97]. The FTIR spectrum of PS shows characteristic bands at 3000-3100 cm^{-1} corresponding to the C-H stretches due to the aromatic ring, peaks at 2924 and 2846 cm^{-1} are attributed to the symmetric and asymmetric stretched vibrations of CH_2, while bands at 1602, 1490 and 1447 cm^{-1} correspond to the stretched vibration modes of the carbon-carbon bonds in the aromatic ring[98]. More examples of representative Raman and ATR-FTIR spectra for MPs detected in effluents and sludge are provided in the supplementary data [Supplementary Figures 4 and 5, respectively].

Scanning electron microscope (SEM) provides high-resolution images of a sample by directing an intense electron beam at the sample surface and can be used to analyze the weathering history of microplastics recovered from environmental compartments by examining the surface texture[99]. SEM combined with energy-dispersive X-ray spectroscopy (SEM-EDX) provides detailed information on microplastic elemental composition and inorganic additives[6]. Figure 10 shows the surface of the analyzed fibers and fragments, highlighting the effects of degradation processes. In fact, SEM micrographs of almost all the examined MPs presented surface cracking, fissures, pits, and roughness on the plastic particles. Furthermore, PA and PVC particles showed an irregular morphology with breaks and a rough surface with pores. De-la-Torre *et al.* (2022)[100], Hajji *et al.* (2021)[101] and Hajji *et al.* (2023)[9] reported similar surface characteristics upon the WWTP processes. In WWTPs, physical, chemical, and biological degradation processes took place in primary, secondary, and tertiary treatment steps. Hence, MPs are expected to be exposed to several

Table 1. Reported MP concentration, shape, size, and polymer type in wastewater effluents and sludge from various WWTPs in different countries comparable with this study

WWTP location	Predominant shapes	Predominant MP size	Predominant Polymer types	MP concentration Wastewater (MP L^{-1})	MP concentration Sludge (MP g^{-1})
Spain[22]	Fragments, fibers	100-355 μm	PE, PP, PS, acrylate	-	2.8-57.2 MP g^{-1}
Italy[21]	Fibers, films, fragments	100-500 μm	Polyesters, PA, PE	0.3 MP L^{-1}	59.5 MP g^{-1}
United Kingdom[90]	Fragments	25-178 μm	PE, PP, PET	2-54 MP L^{-1}	301-10,380 MP g^{-1}
Norway[24]	Beads, fragments	50-125 μm	PE, PP, PET, PA	-	6 MP g^{-1}
Australia[64]	Fragments, fibers	38-125 μm	PA, PE, PP, PET	2.8 MP L^{-1}	7.9 MP g^{-1}
Denmark[88]	Fragments	10-500 μm	Acrylates, PE, PP	54 MP L^{-1}	-
Australia[61]	Fragments, fibers	25 μm	PE, PP, PET	19.6-30.3 MP L^{-1} 0.18-0.96 MP L^{-1}	15.9-56.5 MP g^{-1}
Germany[82]	Fibers	50-100 μm	PE, PVA, PA, PS, PP	1-9 MP L^{-1}	1-24 MP g^{-1}
Germany[48]	Fibers	30-100 μm	PET, PE, PP, PS	3-5.9 MP L^{-1}	-
Changzhou, China[91]	Fragments, fibers	100-500 μm	PET, PE, PP, PS, PE-PP	3.6-13.6 MP L^{-1}	-
Finland[12]	Fibers, fragments	20-100 μm	PE, PP, Polyester	0.7-3.5 MP L^{-1}	76.3 ± 4.3 MP g^{-1}
France[92]	Fragments, fibers	20-80 μm, 80-200 μm	PS, PE, PAM	2.84 MP L^{-1}	16.13 ± 1.2 MP g^{-1}
Scotland[81]	Foams, fibers, films	> 500 μm	PAA, PA, PE, PS, Polyester	0.25-15.7 MP L^{-1}	7.8 ± 1.8 MP g^{-1}
Poland[93]	Fragments, fibers	109-300 μm	-	0.02-9.6 MP L^{-1}	6.7-62.6 MP g^{-1}
This study	Fragments, fibers	25-63 μm	PAA, PAM, PVC, PBMA, PE	5.8 ± 0.6 MP L^{-1}	33.3 ± 8 MP g^{-1}

MP: Microplastics; WWTPs: Wastewater treatment plants; PA: polyamide; PVC: p-vinyl chloride; PP: *p*-propylene; PS: p-styrene; PE: p-ethylene; PAA: *p*-acrylic acid; PAM: *p*-acrylamide; PET: Polyethylene terephthalate; PBMA: *p*-butyl methacrylate.

stressors, such as surface abrasion from the mechanical mixing during the grit and grease removal stage, as well as the aeration of the activated sludge tank[102]. In addition, microorganisms can form biofilms on the surface of MPs or ingest MPs, eradicating them and causing modifications on their surface[8]. Finally, tertiary treatment processes such as filtration and chlorination may play a major role in the surface alteration of MPs[101].

It is revealed that carbon (C) and oxygen (O) are the most dominant constituents in the majority of the analyzed particles, according to the EDX elemental analysis of the selected MPs [Table 2]. In addition, small peaks of other elements, such as Na, N, Cl, K, Ca, P and S, are also present on the surface of analyzed MPs, originating from wastewater effluents and suspended solids in WWTPs. Moreover, these elements may originate either from additives, such as coloring agents and plasticizers used in plastics[103], or from the addition of various coagulants in the aeration tanks[104]. The O/C ratio is a significant index for the oxidation process on the surface of MPs in combination with the carbonyl (CI) and hydroxyl index (HI). The O/C ratio, CI, and HI indices were calculated according to literature[105,106] and are presented in Table 3. The results demonstrate a wide range of CI values, from 0.12 to 0.98, and O/C ratios ranging from 0.17 to 0.99, indicating moderate (CI values 0.15-0.3) and high levels of oxidation (CI > 0.31) and suggesting that non-aged and weathered MPs coexist in the samplings.

Microplastics in sludge samples

A total of 1,091 particles were detected in this study in sludge samples, and 832 particles were confirmed to be MPs particles, representing 76.3%, using micro-Raman spectroscopy combined with Particle Finder™

Table 2. W% and A% of the elements detected on the surface of representative MPs recovered from wastewater

Element	PA		PE		PS		PVC	
	(%W)	(%A)	(%W)	(%A)	(%W)	(%A)	(%W)	(%A)
Carbon	37.01	42.67	35.54	40.98	28.33	36.37	69.63	80.50
Nitrogen	28.78	28.45	28.03	27.71	17.72	19.50	n.d	n.d
Oxygen	32.6	28.21	35.94	31.10	37.54	36.18	15.88	13.80
Phosphorous	1.0	0.45	n.d	n.d	n.d	n.d	n.d	n.d
Potassium	0.60	0.21	n.d	n.d	1.20	0.47	n.d	n.d
Sulfur	n.d	n.d	0.50	0.22	2.40	1.15	n.d	n.d
Chlorine	n.d	n.d	n.d	n.d	3.50	1.52	14.09	5.52
Sodium	n.d	n.d	n.d	n.d	3.20	2.15	n.d	n.d
Calcium	n.d	n.d	n.d	n.d	4.40	1.70	n.d	n.d

PA: polyamide; PE: p-ethylene; PS: p-styrene; PVC: p-vinyl chloride; W%: Weight concentrations; A%: atomic concentrations.

Table 3. CI, HI, and O/C ratio of representative microplastics

Polymer	CI/Reference	HI/Reference	O/C ratio/Reference
PE (> 1000 μm)	0.96/0.016	1.02/0.007	0.76/0.007
PE (63-125 μm)	0.22/0.016	0.14/0.007	-
PP (125-250 μm)	0.24/0.018	0.21/0.015	-
PS (> 1000 μm)	0.98/0.026	0.95/0.017	0.99/0.025
PVC (> 1000 μm)	0.96/0.04	1.09/0.07	0.17/0.045
PVC (250-500 μm)	0.12/0.04	0.31/0.07	-
PA (> 1000 μm)	0.80/0.17	1.001/-	0.88/0.17

PE: p-ethylene; PS: p-styrene; PP: p-propylene; PVC: p-vinyl chloride; PA: polyamide; CI: Carbonyl index; HI: hydroxyl index.

software. The concentrations of microplastics detected in the sludge samples (n = 5) ranged from 20.4 to 40.2 particles/g dry weight with an average concentration of 33.3 ± 8 particles/g. The concentrations of microplastics detected are in a similar range to those found previously by other researchers[21,67,107-109], but lower than the reported average concentration from 37 to 286 particles/g dry weight in digested sludge from Finland[110], United States[111], United Kingdom[112,113], and Spain[86]. In sludge samples, the dominant MP types were *p*-styrene-co-butadiene (49.7%) and *p*-acrylic acid (39.8%), followed by ethylene-vinyl acetate (28.4%), *p*-acrylamide-co-acrylic acid (19.6%), and *p*-butyl methacrylate (19.6%) [Figure 11]. Polymer types such as PVA, PP, and PS were also determined in the samples, but at low percentages (6%-13%). *p*-acrylic acid was the most abundant polymer type (100% frequency of detection), followed by *p*-acrylamide-co-acrylic acid and *p*-butyl-methacrylate (60% frequency of detection) and PE, PS, PP, PVA, *p*-styrene-co-butadiene, PE-co-vinyl acetate, and PE-co-PAA (20%-40% frequency of detection). An average of 16.6 ± 15 particles/g detected as *p*-styrene-co-butadiene, 14.2 ± 11/g as PAA, 7.7 ± 5.7/g as PAM-co-PAA, 5.9 ± 1/g as PBMA, and < 5.8 ± 2/g as the other polymer types. The polymer composition of MPs in sludge presented some differences from that of effluent. More specifically, *p*-propylene occurrence increases in wastewater samples and decreases in sludge samples. This has occurred due to the fact that the density of PP (0.90 g/mL) is less than that of water. Furthermore, MPs that are denser than wastewater (PBMA: 1.07 g/mL, *p*-styrene-co-butadiene: 1.05 g/mL) are easily removed from wastewater by physical separation and settle to sludge[77,80]. This can be observed by the increased concentrations of the two polymer types in sludge compared to effluent samples. Moreover, it is also reported that one of the most common polymers found in sludge samples is rubber from car tires (such as butadiene rubber)[113]. This state is consistent with our study where the co-polymer, *p*-styrene-co-butadiene, was the most abundant polymer type found in sludge, also indicating the mixed character of the sewage system. Furthermore, inherent MPs were also classified

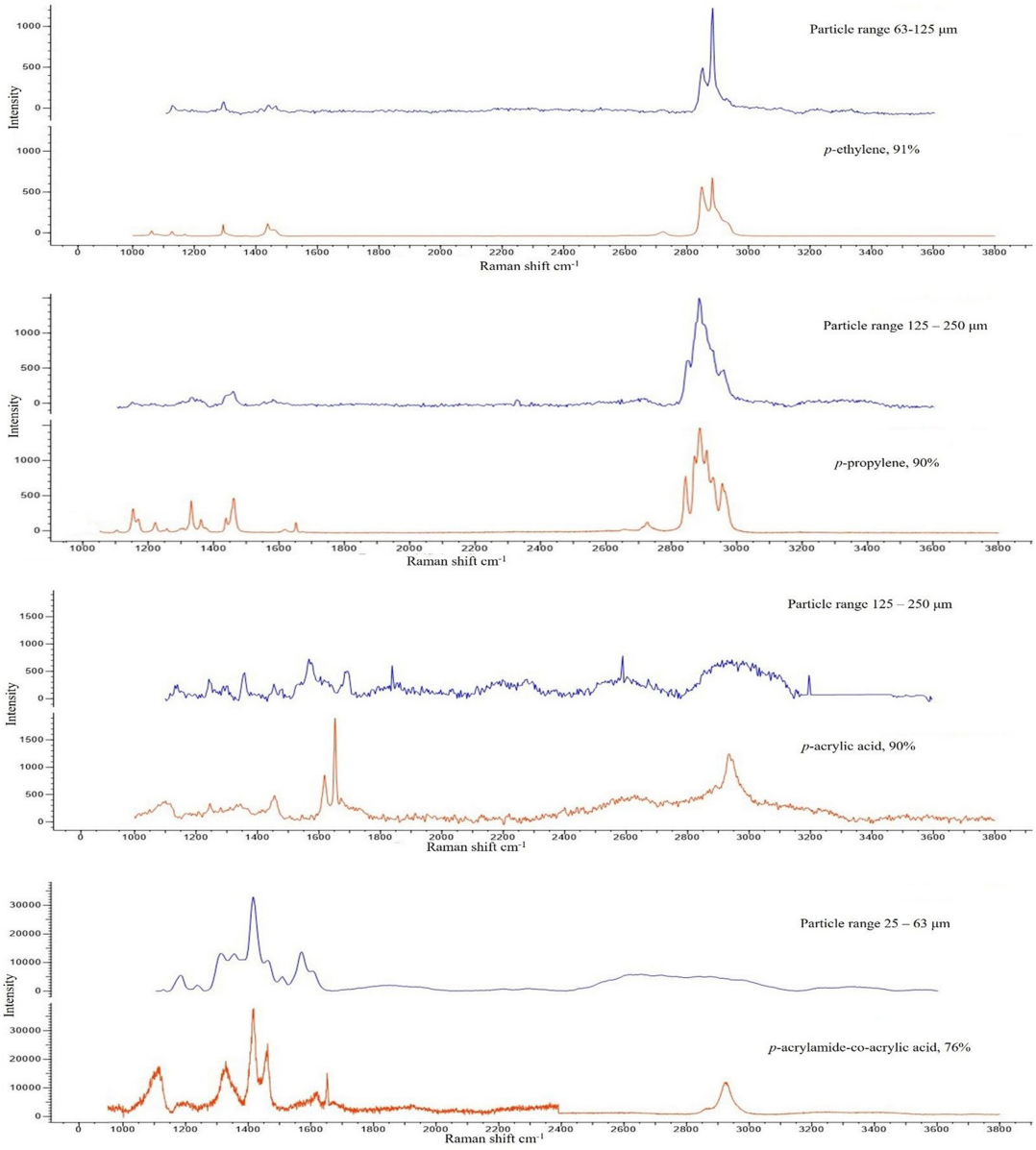

Figure 8. Representative Raman spectra of microplastics detected in wastewater effluents by micro-Raman spectroscopy.

according to their diameter and perimeter [Figure 12A and 12B], with an average size of 123.7 ± 58 µm diameter and 889.3 ± 651 µm perimeter. As shown in Figure 12, particles identified as *p*-styrene-co-butadiene and *p*-ethylene had the largest sizes, with average diameters of 166.7 ± 77.5 µm and 144.7 ± 0.5 µm, respectively. Xu and Bai (2022)[114] reported that more than 85% of MPs in sewage sludge had particle sizes below 500 µm, with 44.73% of MPs in the 100-200 µm range and 29.45% in the 200-500 µm range. This is also confirmed in the present study. *P*-styrene-co-butadiene particles have the largest average size of 166.7 ± 77.5 µm among all the polymer particles detected; therefore, they can be more easily precipitated in sludge. Finally, the fragments occupied again a significant proportion, representing 75 ± 5.6% of all the particles in all sludge samples, followed by films, fibers, and spheres (12.3 ± 4.7%, 6.9 ± 4.5%, and 5.9 ± 4.5%, respectively) [Figure 13A]. Most of the particles detected as PAA are in the form of fragments (41.5%) and spheres (24%), along with *p*-styrene-co-butadiene particles (fragments, 18.6% and spheres,14.7%)

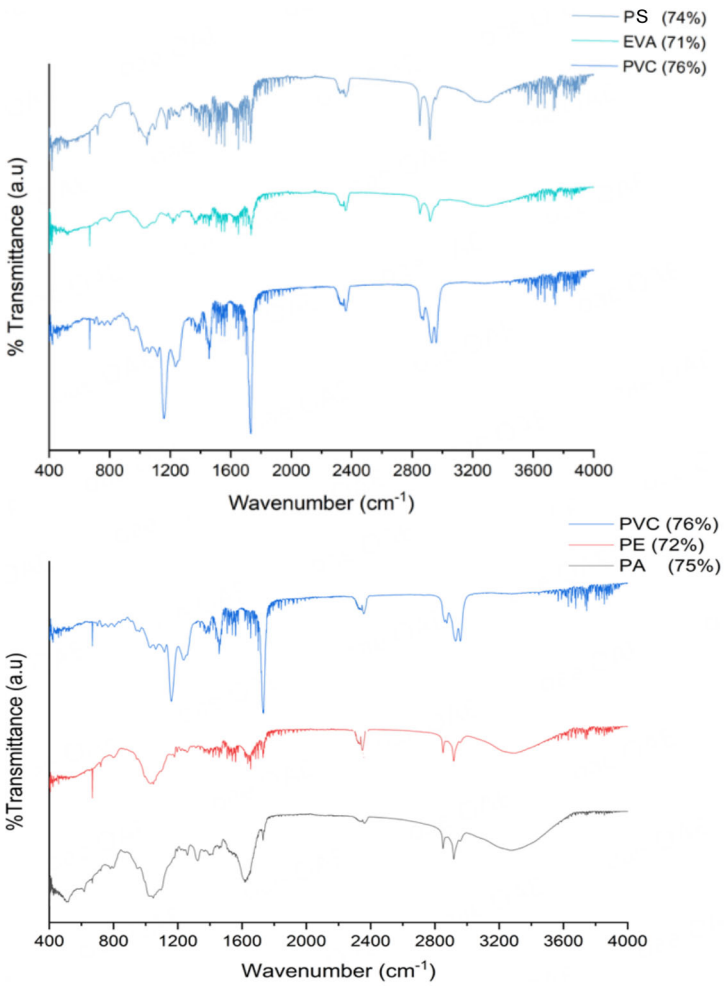

Figure 9. Representative ATR-FTIR spectra of microplastics detected in wastewater samples (size range 500-1000 and > 1000 μm).

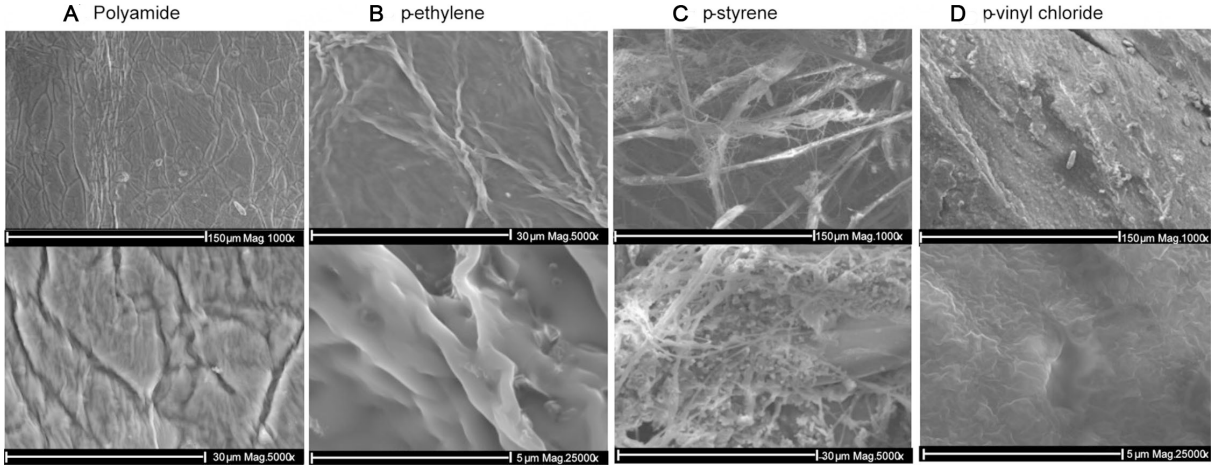

Figure 10. SEM images of representative MPs recovered from wastewater, which were previously identified as (A) Polyamide (PA), (B) p-ethylene (PE), (C) p-styrene (PS), and (D) p-vinyl chloride (PVC). MPs: Microplastics; SEM: Scanning electron microscope.

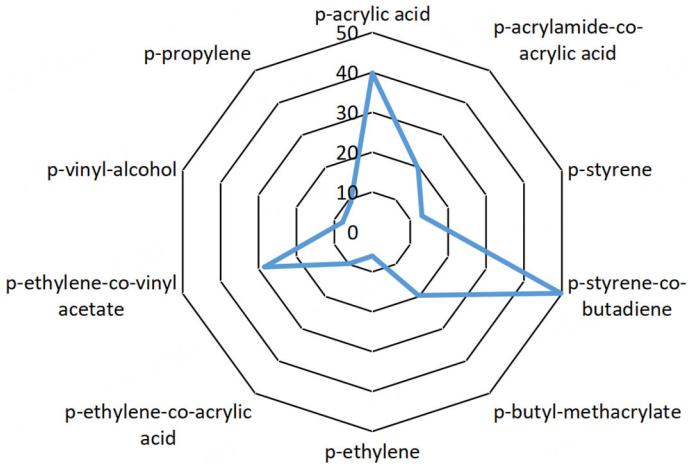

Figure 11. The average percentage of MPs of all sizes in sludge samplings according to the polymer type. MPs: Microplastics.

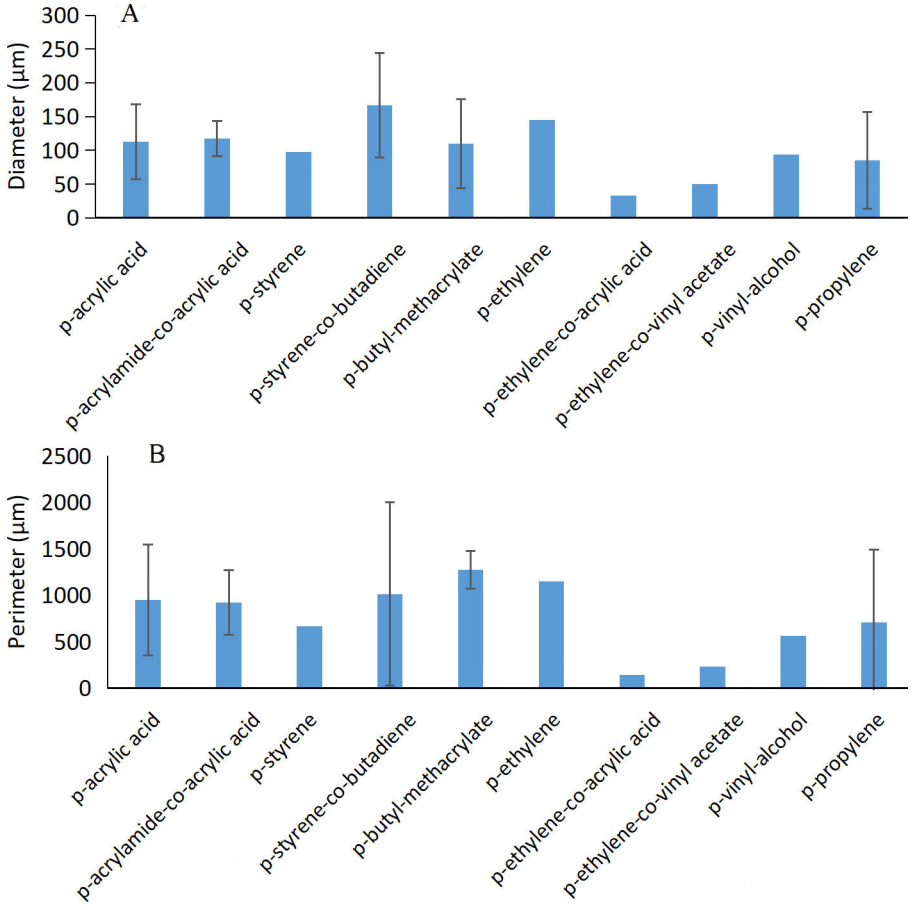

Figure 12. (A) average contribution of each polymer type to the diameter of MPs and (B) average contribution of each polymer type to the perimeter of MPs extracted from sludge samples (n = 5). MPs: Microplastics.

[Figure 13B]. The same conclusion was reached by previous research[27,62,78,79], and comparable data on MP shape, size, and polymer type can be found in Table 1.

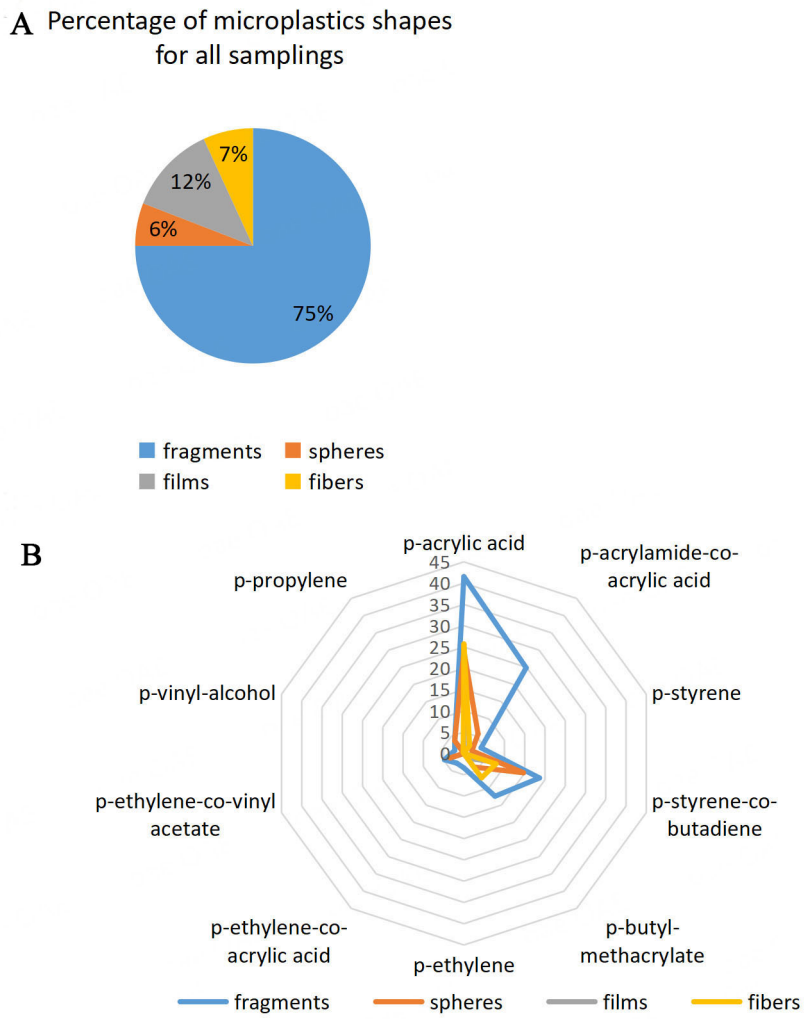

Figure 13. (A) the distribution percentage (%) of the shapes of the MPs of all sizes in all samplings and (B) the percentage of polymer type of MPs according to their shape in sludge samples. MPs: Microplastics.

Risk assessment of microplastics at the WWTP

The PLI, HI, and PRI indices for wastewater and sludge samples were calculated and illustrated in Tables 4 and 5, respectively. All samplings (n = 5) had a PLI > 1, which indicates extended pollution by MPs in wastewater effluents as well as in sludge. For wastewater, the highest MP abundance was observed in the 4th sampling, as mentioned before, which correlated directly with the highest PLI value (1.51) for this sampling. In sludge samples, the PLI index for MPs ranged between 1.0 and 1.40. The overall pollution load index of all samplings was 1.14 for wastewater effluents and 1.47 for sludge, representing hazard category I, less than 10. For polymer hazard index, the 2nd sampling in wastewater effluents and the 4th sampling in sludge presented the greatest HI value (81.02 and 2441.1, respectively). Based on the various MP polymer types identified in different samplings, all wastewater effluent samples exhibited a low-medium hazard index (hazard category II) (HI = 44.5). Among the sludge samples, each five samplings exhibited hazard indices ranging from low to medium and to high (hazard category I, II, and III), but the overall polymeric hazard index of all samplings was 86.6, representing hazard category II (10-100, low-medium). The higher polymeric hazard scores of the toxic polymers detected in sludge samples, such as *p*-acrylic acid and *p*-styrene, are responsible for the high-risk characteristics even though their proportions were lower than those of other polymers. In contrast, a higher abundance of low-hazard polymers contributes to lower

Table 4. PLI, HI, and PRI indices of MPs in WW

Samplings (Wastewater)	PLI	HI	PRI	Risk assessment of HI	Risk assessment of PRI
WW-1	1.09	51.8	57.0	Low medium, (II)	Low, (I)
WW-2	1.0	81.0	81.0	Low medium, (II)	Low, (I)
WW-3	1.07	43.5	46.8	Low medium, (II)	Low, (I)
WW-4	1.51	13.9	21.2	Low medium, (II)	Low, (I)
WW-5	1.07	68.3	73.4	Low medium, (II)	Low, (I)

PLI: pollution load index; HI: hydroxyl index ; PRI: pollution risk index; MPs: Microplastics; WW: wastewater.

Table 5. PLI, HI, and PRI indices of MPs in SS

Samplings (sewage sludge)	PLI	HI	PRI	Risk assessment of HI	Risk assessment of PRI
SS-1	1.28	35.8	45.9	Low medium, (II)	Low, (I)
SS-2	1.40	1306.8	1834.5	High, (III)	Very high, (IV)
SS-3	1.39	75.1	104.9	Low medium, (II)	Low, (I)
SS-4	1.26	2441.1	3076.4	High, (III)	Very high, (IV)
SS-5	1.0	0.60	0.60	Low, (I)	Low, (I)

PLI: pollution load index; HI: hydroxyl index ; PRI: pollution risk index; MPs: Microplastics; SS: sewage sludge.

polymeric risk values[2,115]. Finally, the PRI index of the WWTP was calculated by combining PLI and HI values. For wastewater samples, the PRI for each sampling ranged from 21.2 to 81.0, representing the minor risk category, while the overall PRI of all sampling was 50.7, less than 150, representing hazard category I. For sludge substrate, PRI values showed a significant variability across the five sampling periods. The 2nd sludge sampling and 4th sampling showed PRI values of 1834.5 and 3076.4, respectively, and are in the "danger" risk category (IV), while the rest of the sludge samplings present minor risk category (0.6 to 104.9, hazard category I). Nevertheless, the overall pollution risk index for sludge in the WWTP presented a value of 109.1, less than < 150, and fell into the low risk category (hazard level I). As a result, the high MP abundance, HI values, and the presence of hazardous polymers were all correlated with elevated PRI values. Finally, the ecological risk associated with the presence of microplastics in the Kalamas River water body, where treated water effluents are discharged, was estimated based on dilution factors. The Kalamas River has an annual average flow of 54.2 m^3/s in winter and 32 m^3/s in summer[116]. The PLI values were greater than 1 in the two cases, indicating that the section of the river receiving the WWTP was polluted with microplastics. However, based on the abundance, polymer type, and toxicity of microplastics, HI and PRI values in both cases for all samplings were smaller than 10 and 150, respectively, indicating low risk (category I). To the best of our knowledge, this is the first study in Greece that provides important data and assesses the environmental risk of MPs in the WWTPs. Up to now, MP contamination in WWTP systems has not been part of the conventional environmental impact assessment. As the problem of MPs increases seriously, MP risk assessment will help to fully understand the status of MP pollutants, their migration pathways, and the potential harm to human health in order to improve both the aquatic and the terrestrial environment.

CONCLUSIONS

The current study investigated MP pollution, a long-lasting problem, in the municipal WWTP of Ioannina, Greece. This study provides a comprehensive assessment of MP abundance and characteristics (morphotype, size, and polymer composition) in wastewater effluents as well as sewage sludge by utilizing an efficacious oxidative-enzymatic treatment protocol that reduces organic matter and extracts MPs

efficiently from sludge and wastewater effluents. The mean concentration of MPs was 5.8 ± 0.6 particles/L in effluents and 33.3 ± 8 particles/g in sludge. Raman spectroscopy showed that the predominant shapes of MPs in sewage treatment wastewater and sludge were fragments, followed by fibers, spheres, and films. The microplastics mainly consisted of *p*-acrylic acid, polyamide, *p*-ethylene, and *p*-styrene-co-butadiene. Furthermore, this study provides data about the risk assessment of MPs in effluents and sludge from the WWTP. The assessment of the pollution load index of MPs in all samplings (WW and SS) indicated a low risk, categorized as hazard category I. The overall pollution risk index for all samplings (WW and SS) also showed a low risk, hazard level I, with the exception of high risk in some cases in sludge samplings 2 and 4. In conclusion, the present research work provides an in-depth knowledge of the occurrence and characteristics of MPs detected in the typical WWTP in Greece and also gives an assessment of the potential risks associated with the discharge of MPs into the environment.

DECLARATIONS

Acknowledgments

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Authors' contributions

Conception and design of the study and data analysis and interpretation: Miserli K, Konstantinou I
Data acquisition, validation, technical and material support: Miserli K
Writing - review and editing: Miserli K, Konstantinou I
Supervision, project administration: Konstantinou I

Availability of data and materials

The data presented in this study are available in this article and the associated Supplementary Material.

Conflicts of interest

Konstantinou I is an Editorial Board member of the journal *Water Emerging Contaminants & Nanoplastics*. Konstantinou I was not involved in any steps of editorial processing, notably including reviewer selection, manuscript handling, and decision making. The other authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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