Journal of Environmental Chemical Engineering 11 (2023) 109697

Contents lists available at ScienceDirect

Journal of Environmental Chemical Engineering

journal homepage: www.elsevier.com/locate/jece

Identification of microfibers in drinking water with Nile Red. Limitations and strengths

C.Bretas Alvim [a], M.A. Bes-Piá [a], J.A. Mendoza-Roca [a,*], J.L. Alonso-Molina [b]

[a] Instituto de Seguridad Industrial, Radiofísica y Medioambiental, Universitat Politècnica de València, Camino de Vera, s/n, Valencia 46022, Spain
[b] Instituto de Ingeniería del Agua y Medio Ambiente, Universitat Politècnica de Valencia, Valencia 46022, Spain

ARTICLE INFO

Keywords:
Drinking water
Textile microfibers
Microplastics
Nile Red

ABSTRACT

Traditional drinking water treatment plants (DWTP) are not specifically designed for removing microplastics (MPs) from drinking water. Therefore, this emergent contaminant has been identified in water sources and final drinking water. Microfibers from plastic-based materials are a relevant category of MPs that has attracted attention in recent studies due to their significant presence in environmental samples. This study investigated the presence of microfibers (> 10 μm) in the raw water and the final drinking water of a DWTP located in Spain. It was observed that the DWTP studied was capable of removing 86% of microfibers from raw water. Even with this high removal rate, an amount of 613 microfibers/L (including natural, artificial, and synthetic materials) was counted in drinking water after disinfection. The reactive Nile Red was tested to investigate its use for staining microfibers from the mentioned DWTP and also from tap and bottled water samples in order to identify them. It was observed that microfibers from environmental samples can have different manifestations of fluorescence compared to pristine polymers, and it could be related to degrees of degradation and/or adsorption of organic and inorganic material on the microfibers' surfaces. For this reason, the use of Nile Red could help understand the levels of degradation of these materials in the environment, and thus its use could go beyond the identification of MPs, which was initially proposed in previous studies.

1. Introduction

Textile microfibers are fibers less than 5 mm in length and approximately 10–20 μm thick [27]. Their origin can be related to the textile production processes and they can also be formed from the detachment of clothing items [12]. According to Browne et al. [5], more than 1900 polyester fibers (PET) can be released into the water after washing synthetic clothes. In other studies, Almroth et al. [1] described that a wool garment could shed approximately 110,000 fibers when washed and Sillanpää and Sainio [36] concluded that 2.5×10^5 and 1.3×10^7 PET and cotton microfibers, respectively, could be detached in the first washes. In another research, Athey et al. [2] studied the washing of denim garments (jeans) and found that 56,000 ± 4100 fibers can be shed per wash cycle. The number of fibers and microfibers that are shed from each garment will depend on various factors such as the properties of the fabric, temperature, time, and speed of washing, as well as the products used as detergents and softeners [8,13]. After being released from the fabrics, the microfibers will be present in the wastewater produced during laundry, which is discharged into wastewater treatment plants (WWTPs). Although WWTPs have been shown to have a high retention capacity (over 98%) of microparticles, including microfibers, due to the large volumes of effluent treated daily, the concentration of microfibers in the final treated water is not negligible [15,20]. For example, Pedrotti et al. [29] reported a concentration of 3.6×10^4 synthetic microfibers/m^3 in the final effluent of a WWTP in France. In this way, the final effluents from WWTPs are discharged into rivers, oceans, and seas, contributing significantly to the entry of microfibers into water resources. Finally, if drinking water treatment plants (DWTPs) collect natural waters contaminated with microfibers, these particles may end up in the final drinking water. In other words, microfibers are present in all water cycles.

Microfibers can be classified into natural, artificial and synthetic

Abbreviations: CA, cellulose acetate; DWTP, drinking water treatment plant; EPS, expanded polystyrene; HDPE, high-density polyethylene; LDPE, low-density polyethylene; MPs, microplastics; PAN, Polyacrylonitrile; PC, Polycarbonate; PE, Polyethylene; PET, polyethylene terephthalate; PP, Polypropylene; PS, Polystyrene; PUR, polyurethane; PVC, polyvinyl chloride; WWTP, wastewater treatment plant.

* Corresponding author.
E-mail address: jamendoz@iqn.upv.es (J.A. Mendoza-Roca).

https://doi.org/10.1016/j.jece.2023.109697
Received 2 January 2023; Received in revised form 24 February 2023; Accepted 13 March 2023
Available online 15 March 2023

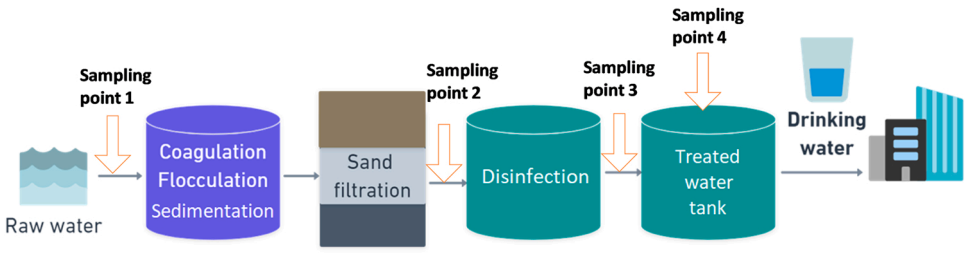

Fig. 1. Scheme of DWTP-A and sampling points.

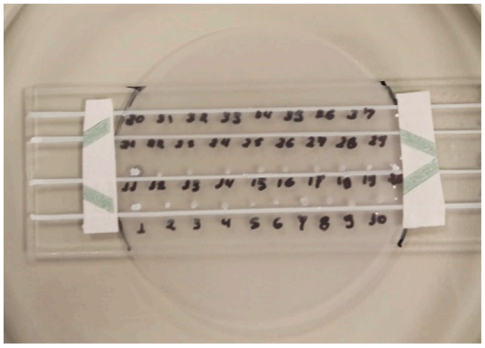

Fig. 2. PCTE filter positioned on a microscope slide to array microfibers stained with Nile Red.

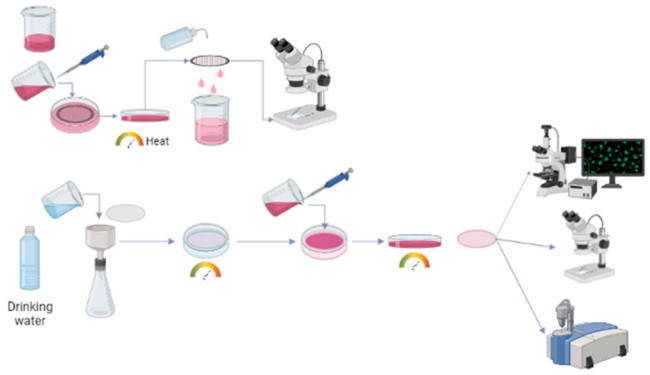

Fig. 3. Scheme of the methodology for the analysis of microfibers.

Table 1
Microfibers/L and turbidity values at the analyzed collection points.

Sample	Turbidity (NTU)	Microfibers/L
1	13	4450
2	0.515	833
3	0.617	613
4	0.297	549

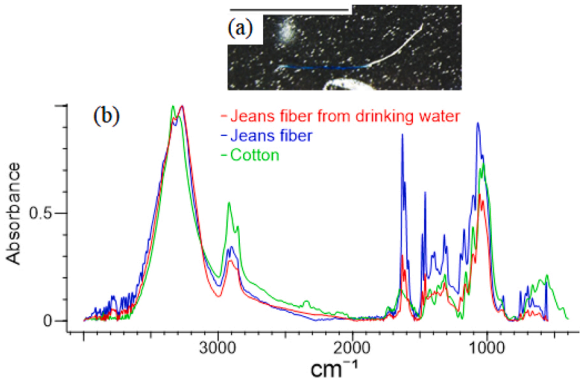

Fig. 4. Blue fiber found in drinking water (a); μ-ATR-FTIR spectra of blue fiber, jeans fiber, and cotton fiber (b).

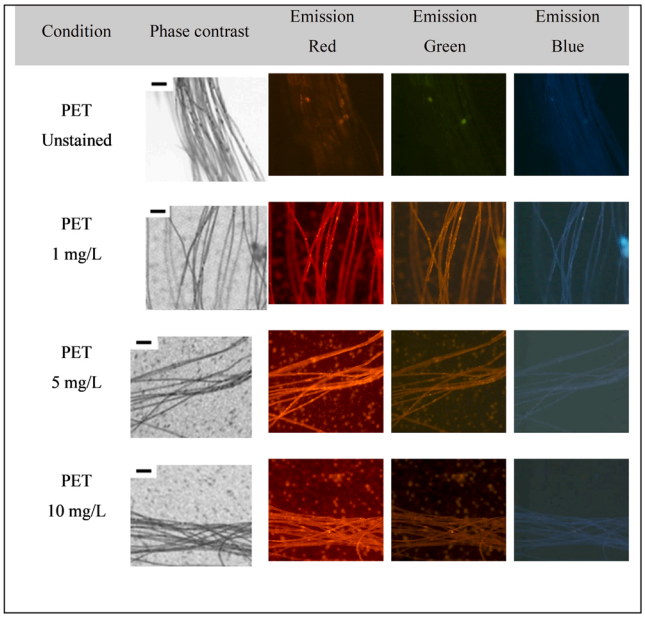

Fig. 5. PET fluorescence after staining with different concentrations of Nile Red solution (Scale bar 100 μm; magnification 100 X).

depending on their composition. Natural microfibres come from materials that are in fiber form in nature, like cotton or wool. The other two groups of microfibers come from fibers that have to be manufactured, either from chemical compounds (synthetic fibers like PET) or from natural polymers that are modified or transformed (artificial fibers) like rayon [33].

The presence of microfibers in drinking water has been described in recent studies ([14,18,26]). However, the different methodologies used by these authors make the direct comparison between the reported results difficult. In addition to the methodology used in each study, it is important to consider that DWTPs can catch water from different sources, such as rivers, reservoirs, and groundwater. These different water intake points will influence the greater or lesser presence of microfibers in drinking water [30,38]. When drinking water is collected for studying the presence of MPs (e.g., microfibers), spectrometric techniques such as Raman and FTIR are commonly used. These techniques allow the identification of the polymer chain and the classification between synthetic, natural, and artificial materials. However, the use of spectrometry is an expensive and time-consuming procedure, since each material must be identified individually, increasing the

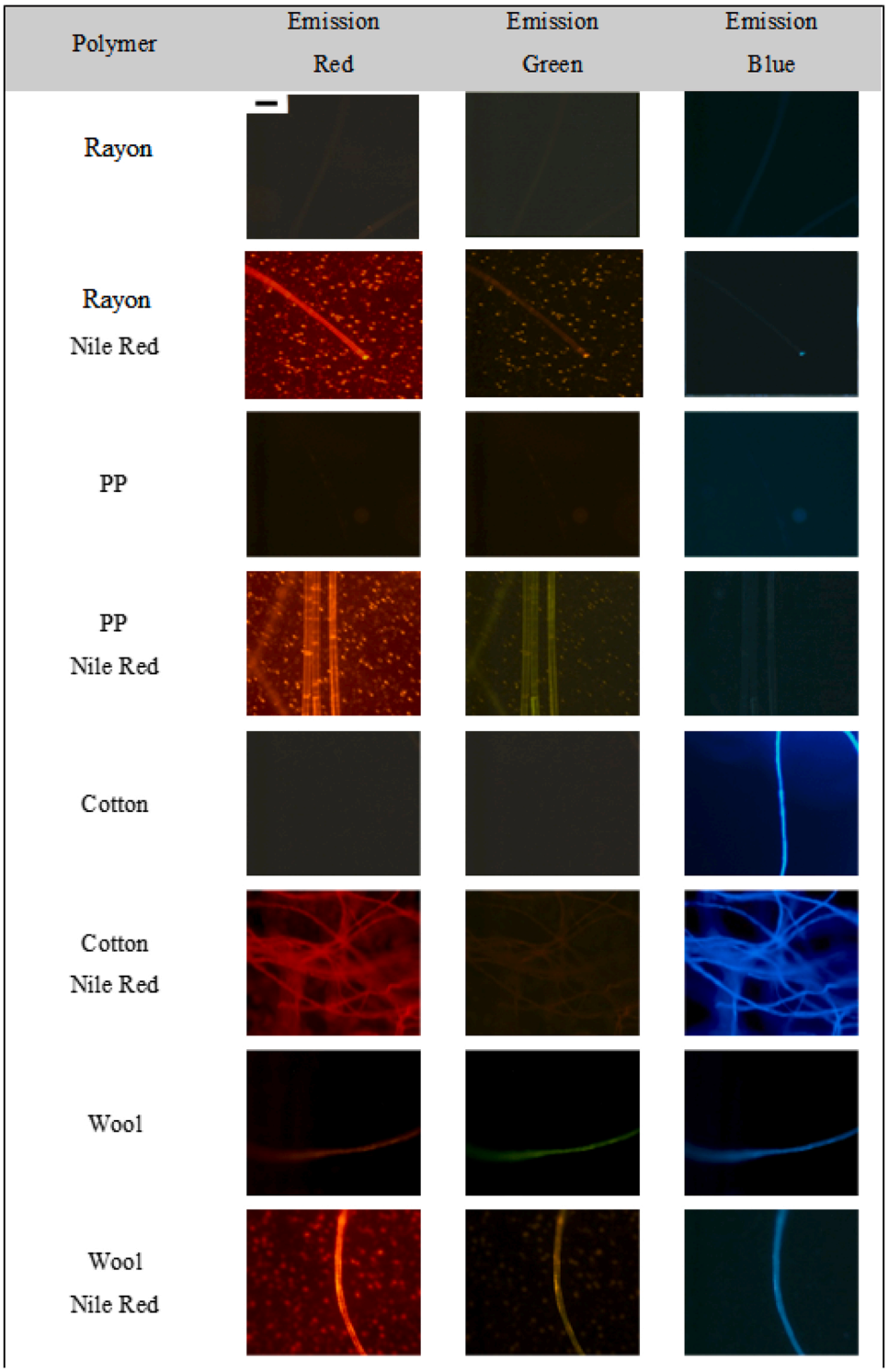

Fig. 6. Inherent fluorescence of textile microfibers and fluorescence after staining with 1 mg/L Nile Red (Scale bar 100 μm; magnification 100 X).

analysis time [32,35]. In addition, finding the best-operating conditions for the equipment for the correct generation of spectra (with high signal and low noise) is a task that requires a high knowledge of the equipment and the management of the samples, which adds more complexity to the process.

Besides the presence of MPs in drinking water, natural and artificial materials also deserve attention. Although cellulose-based microfibers are more rapidly biodegradable, the risk they pose to the environment cannot be ignored. As synthetic microfibers, natural and artificial ones contain chemical additives that can be leached into the environment and can result in harmful effects to vertebrates and invertebrates through the ingestion of these microparticles. Additionally, the greater biodegradability of cellulosic-based microfibers (natural and artificial) could be an aggravating factor by accelerating the release of additives in the environment or in the organism of living beings which have ingested them [19,33]. In a recent study by Kim et al. [16], it was reported that the ingestion of artificial lyocell microfibers led to intestinal damage in the *Artemia franciscana* shrimp species, as in the case of synthetic

Table 2
Studies related to the use of Nile Red for identifying MPs and experimental conditions applied.

Concentration	Solvent	Conditions	Polymers and shape	Reference
1 mg/L	Methanol	60 min at 60 °C	Pristine microfibers (PP, PET, Cotton, wool, and rayon). Weathered microfibers from drinking water.	Current study
1 mg/L	Methanol	10 min at 60 °C	Pristine fragments (PE, PET, PVC, PS, PC, PUR, black tire rubber) and nylon-6 fibers. Weathered polymers collected from a beach in the UK.	Erni-Cassola et al. [9]
10 mg/L	Acetone	30 min	MPs from marine sediment samples. Classification as polar (probably Nylon and PET) and hydrophobic (probably PP, PE and PS) MPs according to staining results	Maes et al. [24]
10 mg/L	Ethanol	30 min	Pristine fragments (LDPE, HDPE, PP, PS, PET, EPS, CA, PVC, and pristine microfiber (Cotton, Linen, Polyester, cotton and polyester, nylon, and rayon). Weathered polymers collected from a beach in Portugal. Organic materials (algae, driftwood, feathers, bivalve shell, charcoal, fish muscle, shrimp chitin shell, shrimp muscle, palm fat, paraffin)	Prata et al. [31]

microfibers (PP and PET). It demonstrated that the possible toxic effects of the intake of natural and artificial microfibers should also be considered, in addition to those of synthetic microfibers. In another study, a mean of 1.38 ± 0.79 fibers (0.1–6 mm) per organism was found in the intestine of invertebrates belonging to the phylum Arthropoda, collected in the Bay of Calvi (Corsica). When carrying out the analysis of blue and red fibers, it was observed that, although the polymeric base of the fibers was of cellulosic origin, the dyes "Direct Blue 22" and "Direct Red 28" were also identified via Raman. According to the authors, isolated cellulose would not be an environmental problem. However, when it is treated with additives such as dyes, its toxic potential can be high. For example, the "Direct Red 28" dye identified in fibers ingested by invertebrates is classified as carcinogenic, mutagenic, or toxic. In the human intestine, there are bacteria capable of reducing chemical bonds of this additive, which gives rise to benzidine molecules, which are related to bladder and pancreatic cancer in humans [33].

Recent studies have suggested the use of staining techniques with Nile Red to aid the visual identification of MPs and natural materials [9, 17,24]. With staining methods, unlike spectrometry, it is not possible to identify the polymer chain, and therefore a precise classification of materials cannot be made. However, staining techniques allow natural fibers to be differentiated from synthetic ones.

Nile Red corresponds to a neutral aromatic molecular structure composed of two heteroatoms (nitrogen and oxygen) and with intense fluorescence properties in the apolar medium. Therefore, Nile Red is a reagent that is characterized by the effect of solvatochromism, which means that its fluorescence is linked to the polarity of the solvent used for its dissolution [24,25]. Since this dye has low solubility in water and does not show fluorescence in this solvent [25], different solvents, such as methanol, ethanol, and acetone, have been used for the preparation of Nile Red solutions. The choice of solvent must be based on its capacity of not damaging the microparticles. In this context, Konde et al. [17] suggested that acetone can result in the surface degradation of some plastics and therefore other solvents such as methanol or ethanol could be more recommended. In addition to the choice of solvent, the concentration of the solution, the temperature, and the reaction time are parameters that may govern the effectiveness of the staining. The adoption of higher reaction temperatures (60 °C – 70 °C) has shown more satisfactory staining results than staining at room temperature [17,38]. When the polymer chains are subjected to higher temperatures, the polymer chain is loose, and Nile Red can interact better with the polymer structure. Finally, when the polymer reaches room temperature its structure becomes dense, and Nile Red stays trapped in the polymer ([35]).

This study aims to evaluate the use of Nile Red to stain natural, artificial, and synthetic textile microfibers with the aim of identifying them through the emission of fluorescence. Limitations of this technique have been also brought out. For that, pristine microfibers of cotton, wool, rayon, polyester (PET), and polypropylene (PP) were used to scrutinize the experimental conditions for staining and to compare their identification by spectroscopic techniques with the eventual identification of microfibers of the same materials in a water sample by fluorescence. The differences of fluorescence of microfibers of the same materials (comparing pristine and found in water samples microfibers) led to relate fluorescence with polymer degradation, which is hardly described in the literature until now. In addition, samples from different sampling points of a DWTP have been taken for microfibers analysis, determining their removal efficiency.

2. Materials and methods

In this work, pristine microfibers from the most abundant materials in water samples (Section 2.2) have been stained with Nile Red to study their fluorescence after excitation at three different wavelengths (procedure detailed in Section 2.4). The number of microfibers in samples from different points of one drinking water treatment plant and in tap water (detailed in Section 2.3) has been determined and microfibers characterization has been performed comparing FTIR-ATR with the staining method applied.

2.1. Procedure to control and to assure the quality of measurements

To ensure the quality of the tests carried out, all the materials used (tweezers, Petri dishes, pipettes) were carefully cleaned with soap, deionized water, and ethanol. Polycarbonate filters (PCTE, 47 mm diameter, 10 μm pore size, Whatman) were randomly collected from the box and inspected directly with a stereomicroscope (LEICA MZ APO) to check the eventual presence of cross-contamination. In addition, to evaluate possible airborne contamination, two PCTE filters were inspected with the stereomicroscope and then were placed in an open Petri dish and left on the workbench for 24 h. After that, the filters were analyzed with the stereomicroscope for counting the particles deposited on them. To minimize the effects of airborne contamination on the samples, not only the materials were continuously washed with water, soap, and then ethanol, but also the Petri dishes. They were inspected with a magnifying glass for removing contaminants and all materials were covered with aluminum foil before being used.

2.2. Microfibers used to assess the efficiency of Nile Red

Pristine microfibers (natural, artificial, and synthetics) were used as patterns in this study. The natural and artificial fibers were cut from sewing threads (100% purity, Gütermann), while the synthetic

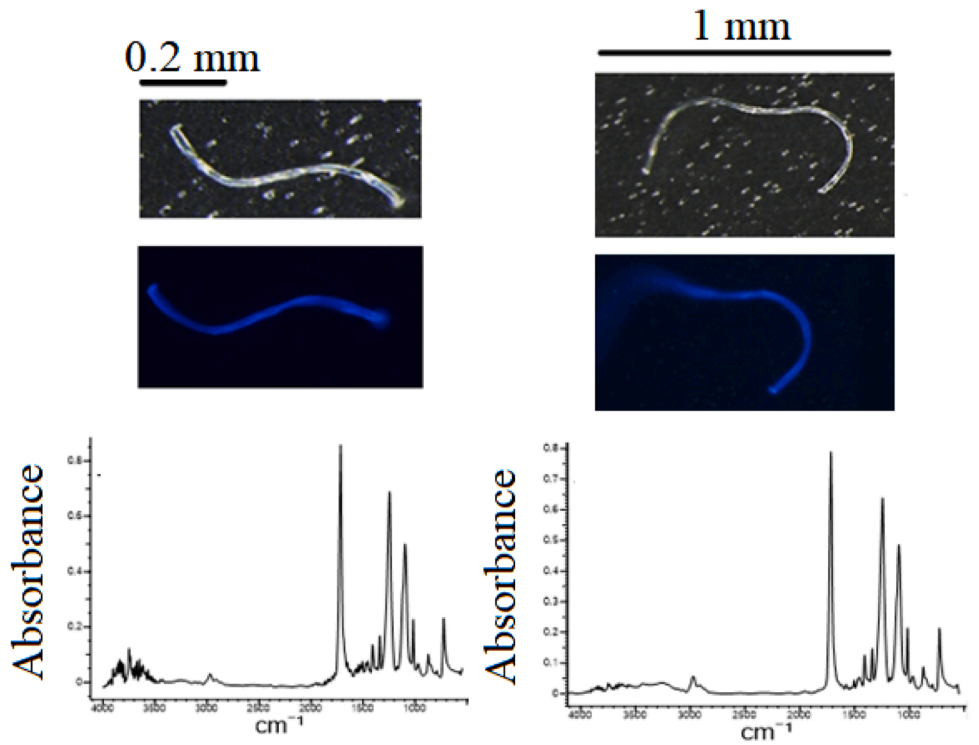

Fig. 7. Microfibers collected in drinking water with blue fluorescence identified as PET.

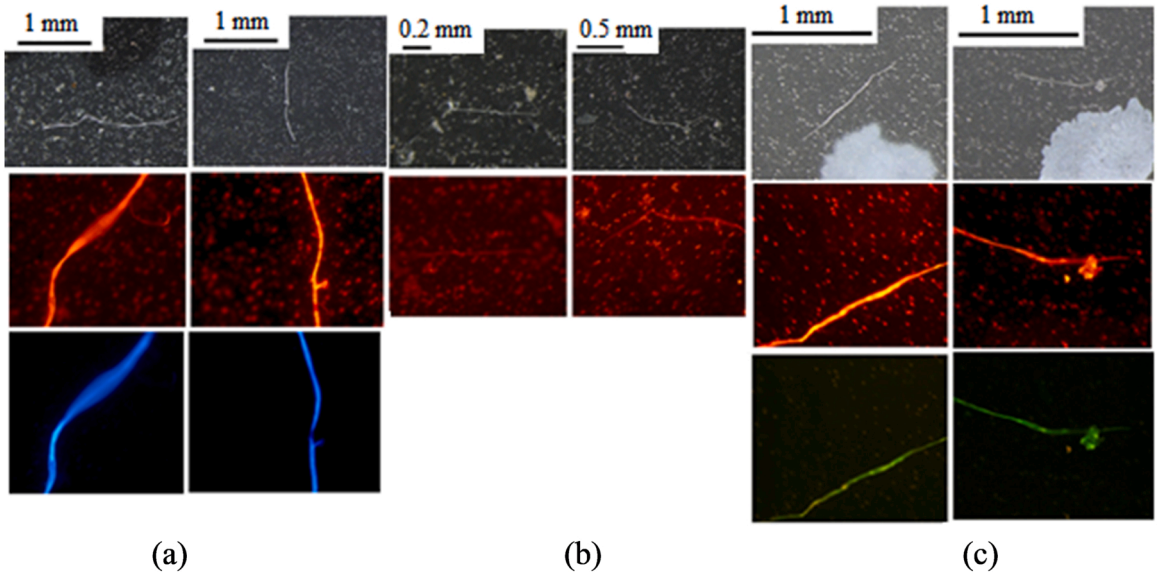

Fig. 8. Fluorescence Pattern 1 (a), Pattern 2 (b) and Pattern 3 (c) for cotton microfibers.

microfibers were obtained from bottles of the studied material (PET, PP). All of them were finally cut with lengths less than 5 mm and thickness around 10 – 20 μm. In this work, white/transparent and light-colored microfibers were used for two main reasons: firstly, the use of staining techniques for the identification of microfibers is more efficient for the recognition of white and transparent materials, since the presence of additives and dyes can worsen the staining process [28]. Secondly, the number of white/transparent microfibers can be underestimated due to the greater difficulty of their visual perception [4, 35,37] and the use of staining techniques could improve their identification. Natural (cotton and wool), artificial (rayon), and synthetic (PET - polyester, PP - polypropylene) microfibers were used in this study. The confirmation of the type of pristine microfibers was performed with a Fourier-Transformed-Infrared in mode Attenuated Total Reflectance (ATR-FTIR) with a germanium crystal (Vertex 80 Microscope Hyperion 1000 by Brucker). The equipment was operated with a spectral resolution of 6 cm^{-1}, 32 scans, and a range of wavelengths between 600 and 4000 cm^{-1}. In addition, a microscope is coupled with the equipment to allow the perfect alignment between the microfiber and the ATR-FTIR.

2.3. Drinking water samples

To examine the efficiency of a Drinking Water Treatment Plant (DWTP) located in Spain to remove microfibers, water samples were

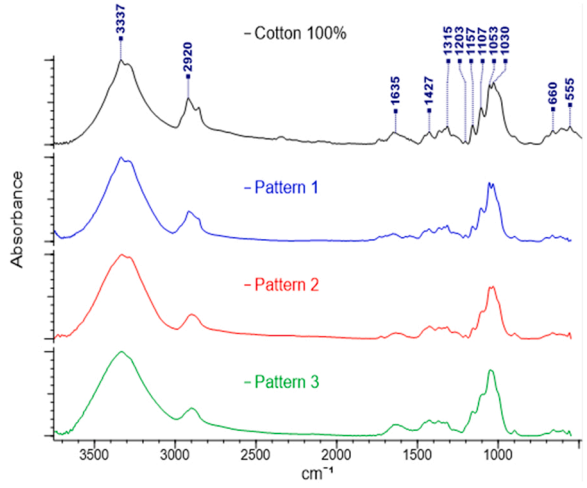

Fig. 9. μ-ATR-FTIR spectra corresponding to cotton fibers fluorescence patterns.

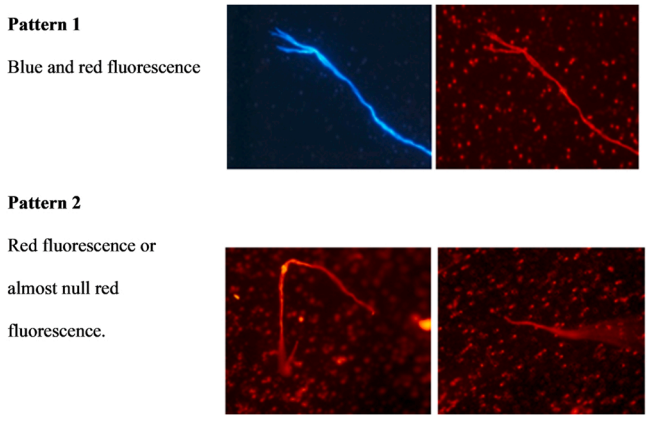

Fig. 10. Fluorescence patterns for rayon microfibers from drinking water.

collected in four stages of treatment. Fig. 1 shows a scheme with the processes of the DWTP and the sampling points. The treatment mainly consists of sedimentation (with previous coagulation and flocculation steps) and sand filtration, to remove suspended solids and turbidity, and a final disinfection stage with chlorine. After it, drinking water is stored and pumped to the water supply system. The samples analyzed were the raw water, collected from a reservoir, (labeled as sampling point 1), the effluent of sand filtration (labeled as sampling point 2), the effluent of disinfection stage (labeled as sampling point 3), and the water stored for supply (labeled as sampling point 4). Samples were taken in glass containers on the same day from this DWTP which was named DWTP-A. 100 mL of raw water (sampling point 1) were treated with a chemical digestion procedure to reduce organic matter. The chemical digestion was carried out with hydrogen peroxide (H_2O_2, 35%) at 60 ± 2 ºC for 2 h, using a volumetric ratio of 1:10 [H_2O_2:sample]. After chemical digestion, the sample was filtered on a translucent PCTE filter (47 mm diameter, 10 μm pore size, Whatman) and stored in a Petri dish. For the other samples (2, 3, and 4), 1 L of water was vacuum filtered through translucent PCTE filters (47 mm diameter, 10 μm pore size, Whatman) without chemical digestion. All PCTE filters were dried in an oven at 60 ºC for 1 h for completely removing the water. Finally, the filters were left in a desiccator (approximately 1 h) until reaching room temperature. The material retained on the filters was visually analyzed with the above-referred stereomicroscope with a magnification adjusted between 8 X and 80 X for counting microfibers > 10 μm. A black background was used for counting white/transparent microfibers and a white background for counting the light-colored ones. It was possible due to the fact that filters were translucent. Microfibers were quantified in all samples, and the sample taken from sampling point 3 was used for testing the viability of the staining method in order to identify the materials of the microfibers. Besides, the samples collected from sampling point 3 (DWTP-A) and microfibers from tap water (distributed by a DWTP-B)

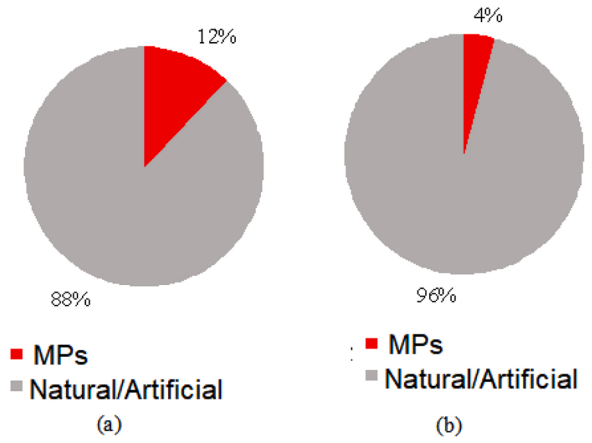

Fig. 11. Microfibers material identification with (a) μ-ATR-FTIR s and (b) fluorescence emission.

Table 3
Microfiber fluorescence patterns after staining with Nile red identified in drinking water (n = number of samples).

Microfiber		Fluorescence pattern			n
Cotton	1	Blue	Red		9
Rayon	1	Blue	Red		1
Cotton	2		Red/Null		6
Rayon	2		Red/Null		2
Cotton	3		Red	Green	3
PET ~~(polyester)~~		Blue			5
Total					**26**

were used to investigate the protocol with Nile Red. After being stained with Nile Red (without previous digestion due to the characteristics of the samples), the microfibers were randomly selected from the samples and positioned on the PCTE filter as described in the next section.

Turbidity was also measured for the samples of DWTP-A. These measurements were performed with a TL2310 laboratory turbidimeter (HACH).

2.4. Microfibers stain procedure with Nile Red

The staining method used in this study was based on the methodology reported by Erni-Cassola et al. [9]. A stock solution of 10 mg/L Nile Red (technical grade, N3013, Sigma-Aldrich) was prepared in methanol. The stock solution was covered with aluminum foil and kept in a fridge. Two more solutions of Nile Red with concentrations of 5 mg/L and 1 mg/L were prepared from the stock solution, covered with aluminum foil, and kept in a fridge until their use. The three concentrations of Nile Red (1 mg/L, 5 mg/L, and 10 mg/L) were tested to determine the best concentration to stain microfibers considering the fluorescence of microfibers and the lowest background signal. To set up the best concentration for staining microfibers, pristine polyester microfibers (PET) were used since polyester MPs have been commonly found in drinking water, and the Nile Red has been reported for dying MPs (Erni-Cassola et al.,201719; [31]). The PET microfibers were carefully positioned on the PCTE filter, and the filters were coated with Nile Red solution. The filters were kept in an oven for 30 min at 60 ºC. Finally, the filters were left in a desiccator (approximately 1 h) until reaching room temperature.

The fluorescence of the microfibers before and after staining was evaluated with an epifluorescence microscope (Olympus BX50, Paris) equipped with a 100 W mercury high-pressure lamp and a set of filters. The filters were the following: U-MWB (filter cube for blue excitation (456–490 nm) and green emission consisting of a set of mirrors DM500, BP450–480, and BA515), U-MWIB (filter cube for broadband green excitation (534–558 nm) and red emission consisting of a set of mirrors DM570, BP510–550, and BA590) and U-MWIG (filter cube for broadband ultraviolet excitation (365 nm) and blue emission consisting of a set of mirrors DM400, BP330–385, and BA420). The microscope was also equipped with an AxioCan ICc5 camera (Zeiss) and the software Zen 3.1 (Zeiss) was used to process the data. When the best concentration of Nile Red was selected, it was applied to stain the microfibers of the water samples of the DWTPs. Finally, a relation between the fluorescence emitted by the fibers and their polymer type was established.

Drinking water samples prepared as described in Section 2.3 were covered with the optimal concentration of Nile Red and kept in an oven for 30 min at 60 ºC. With the stereomicroscope, the microfibers retained on the PCTE filter were randomly positioned in the numbered positions written on a microscope slide placed under the PCTE filter (Fig. 2). The fluorescence of the positioned microfibers was obtained one by one with the epifluorescence microscope and finally, their polymeric composition was identified with the μ-ATR-FTIR equipment (Bruker). The spectral resolution was 6 cm^{-1} and the wavelength range was between 600 and 4000 cm^{-1}. Fig. 3 shows graphically the steps followed in the methodology.

3. Results and discussion

3.1. Quality assurance/control

Two PCTE filters were randomly collected from the box and analyzed with a stereomicroscope and no microfiber was identified, excluding the possibility of initial contamination by the filters. Regarding airborne contamination, after 24 h of exposure, the presence of 33 ± 12 microfibers per filter was observed. 97 ± 1% of the microfibers identified in the filters were white cotton fibers. According to these results, it is estimated that about 1.4 ± 0.5 microfibers/h could be deposited on the surfaces where the tests were carried out. This value indicates that it is very difficult for the sampling system to be free from the influence of air pollution.

3.2. Quantification and identification of microfibers in drinking water

Concerning the shape and color of microparticles in DWTP-A, microfibers corresponded to more than 75% of microparticles in all samples and white/transparent and light-colored microfibers were the main observed colors (more than 95%). Table 1 shows the number of microfibers counted in the four analyzed samples and their turbidity. It can be observed that the DWTP-A showed an efficiency of 86% for removing microfibers from raw water after disinfection (Table 1). However, the lower concentration of microfibers in the storage tank in comparison with those measured in the water after disinfection was unexpected since there is no treatment step between both sampling points. A recent study by Chu et al. [6] showed an 86% decrease in the concentration of microparticles in the tap water provided by a DWTP compared to the water at the exit of the DWTP. According to the authors, this result was caused by the adsorption of microparticles in the drinking water supply system. Therefore, it is hypothesized that the decrease in the concentration of microfibers between disinfection and the storage tank observed herein could be mainly linked to the adsorption of microparticles in the pumping and distribution system to the storage tank. Other mechanisms as sedimentation or flotation should be also considered.

Since microparticles – including microfibers - have a size between 5 mm and 1 μm (they are suspended particles), it can be inferred that water turbidity and microparticles number could be related. For turbidity values lower than 1 NTU, it is difficult to have a clear trend of the relationship between turbidity and microparticles number. In this way, the physicochemical characterization of the samples revealed a reduction in the turbidity value of the water in the storage tank (0.297 NTU) in comparison with water after disinfection (0.617 NTU). However, the turbidity of the sample from point 2 is slightly lower than the one of sample 3 in spite of the reduction of microparticles number. Li et al. [23] also point out that MPs contributed to samples turbidity. In addition to it, Sarkar et al. [34] reported a direct relation between turbidity and MPs abundance based on the machine learning algorithm applied to their analytical results in a DWTP in India.

It was also observed that most particles in tap water (distributed by DWTP-B) corresponded to microfibers (76 ± 7% of the total microparticles). The predominance of microfibers in drinking water has also been reported in previous studies [14,38]. The presence of microfibers in environmental samples has been related to their release during laundry. In our research, interesting evidence of the presence of textile microfibers in drinking water was the finding of blue fibers in all samples (Fig. 4a), which after their inspection showed the presence of the additive indigo blue, usually applied for dying jeans. Fig. 4b shows the spectra of a pristine microfiber of cotton (in green color), a microfiber obtained from blue jeans (blue color) and a microfiber separated from the drinking water sample which was identified as a microfiber coming from blue jeans (red color). The characteristic peaks in the cotton spectrum was also seen in the spectra of the blue jeans fibers (3280, 2850 and 1030 cm^{-1}). However, the spectra of these fibers also revealed peaks at 1623, 1609, 1584, 1481, and 1459 cm^{-1}, which could be related to the aromatic ring of the dye [3,21]. Another relevant observation was the appearance of degradation of the blue fibers in Fig. 4a since part of its color seemed to be leached from the fiber. The fact that the microfibers of environmental samples can undergo degradation processes is an important observation to understand the results of Nile Red. 67% and 80% of blue fibers analyzed from bottled and tap water, respectively, were identified as cotton fiber with indigo blue dye. These microfibers would be released from denim clothes in washing machines and ended in raw water taken by DWTPs, which were not able to retain 100% of these small fibers. Microfibers from other materials can derive

from contamination during transportation, storage, and water pipes. Even the indiscriminate disposal of face masks used in the current pandemic scenario (COVID-19) could be also a relevant source of fibers (such as cellulose, rayon, polyester, polypropylene, and others) to water pollution [10].

Regarding the polymer types in samples from DWTP-A after disinfection (sampling point 3), most of the fibers (74%) were natural (from cotton), 16% were identified as rayon MFs (artificial) an 10% were synthetic fibers (6.4% of PET and 3.6% of nylon. In tap water distributed by DWTP-B were identified 25 microfibers, being 76% natural fibers, 8% artificial (rayon) and 16% synthetic ones (PET and nylon). Although the presence of MPs in drinking water is evident, the harmfulness of this contaminant to human health is still not very clear due to the scarcity of research on this field.

3.3. Determination of the optimal concentration of Nile Red and the fluorescence of pristine polymers

To determine the optimal concentration of Nile Red for staining microfibers, initially, the inherent fluorescence of the PET microfibers was analyzed. It was observed that unstained PET microfibers did not show any fluorescence (Fig. 5). Three different concentrations of Nile Red (1 mg/L, 5 mg/L, and 10 mg/L) were tested to determine the best concentration for staining the microfibers. Results are shown in Fig. 5. After staining, with 1 mg/L Nile Red, it was observed that the PET microfibers emitted a red fluorescence, which was not previously appreciated in the unstained microfiber. The increase in the concentration of Nile Red to 5 mg/ L and 10 mg/L caused, in both cases, the increase of the background fluorescence and therefore, it was decided to use a concentration of 1 mg/L of Nile Red. These results are coincident with the ones reported by Erni-Cassola et al. [9]. This concentration was finally used to evaluate the staining of the other microfibers (natural, artificial, and synthetic) used in this study.

As performed with PET microfibers, the microfibers of PP, rayon, cotton, and wool were analyzed in the epifluorescence microscope before the use of Nile Red and the inherent fluorescence of the microfibers was determined (Fig. 5). From the results of the inherent fluorescence of the microfibers, it was stated that cotton microfibers exhibited an intense blue fluorescence when excited at 365 nm. No fluorescence was observed when this natural polymer was excited at 456 – 490 nm and 534 – 558 nm. Wool showed slight fluorescence when excited. The rest of the microfibers (PET, PP, and Rayon) did not show any fluorescence before staining.

Fig. 6 also shows the images of the natural, synthetic, and artificial fibers after being stained with 1 mg/L of Nile Red. The fluorescence emitted by wool does not seem to be affected by the presence of Nile Red. The PP microfibers showed a slight red and strong green fluorescence. Cotton exhibited red fluorescence apart from the blue fluorescence naturally manifested without Nile Red. Finally, Rayon showed a slight red fluorescence. Based on these results, it can be stated that the identification of synthetic, natural, and artificial microfibers by fluorescence implies the evaluation of different excitation wavelengths. For instance, if just the red fluorescence was used to identify the presence of microfibers, PET and Rayon polymers would be bright, which could lead to a misinterpretation of results.

Although the material identification of MPs with spectrometric techniques is very precise, the use of these technologies can be expensive and very time-consuming. For this reason, Nile Red can be applied for staining MPs not only for distinguishing MPs from natural microfibers but also as an alternative technique for material identification. Table 2 shows the materials and staining conditions used in this work with those of other authors [9,24,31]. Some differences in results can be observed even when similar protocols were applied. For instance, Erni-Cassola et al. [9] found that all MPs fragments tested by them (except the black tire rubber) emitted in green after being stained with Nile Red. The authors stated that the use of green fluorescence was more suitable for the identification of MPs (compared to red fluorescence) because synthetic polymers fluoresced better under this condition. When red fluorescence was tested, weak or null fluorescence was emitted by MPs (PP, PET, and PS). In addition, Erni-Cassola et al. [9] stated that chitin (a natural polymer) also showed green fluorescence after staining with Nile Red, which could lead to a misinterpretation of results and overestimation of MPs. However, in this work, a very similar staining protocol has led to null green fluorescence of PET and a slight green fluorescence of PP microfibers. These differences in results may be related to microscope conditions (e.g., filter setup, wavelengths, light), or even could be related to the particles' shape. In another study, Prata et al. [31] tested several wavelengths to find the best conditions to identify MPs, observing that different materials had better fluorescence under different wavelengths. For instance, at 470 nm only pristine HDPE and PVC and weathered PE fibers did not show any fluorescence with Nile Red. However, under this wavelength, natural organic matter also fluoresced. At 530 nm almost all textile material fluoresced, except viscose and wool, CA and some fragments of natural organic matter also fluoresced at this wavelength. Unlike the results observed by Prata et al. [31], in this study wool microfibers showed a red fluorescence under 534 – 558 nm. Consequently, it is paramount to validate the applied protocol based on the experimental conditions. e.g., the microscopy used, solvent, and concentration of Nile Red among others. Once these considerations had been taken into account, a procedure consisting in varying the excitation wavelength could help to identify the materials of the microfibers saving time and costs.

3.4. Nile Red fluorescence of microfibers isolated from drinking water (DWTP-A) and determination of polymer type

Regarding the fluorescence of microfibers isolated from drinking water samples, it was observed that some microfibers showed an evident blue fluorescence pattern, whereas no green and red fluorescence were observed. The spectra obtained for all microfibers with this fluorescence pattern were identified as PET (Fig. 7). However, based on the fluorescence results for pristine polymers after staining (Fig. 6), the PET microfibers showed only red fluorescence. The discrepancy could be related to the fact that microfibers found in the environment can undergo numerous degradation processes or adsorb organic or inorganic materials, which can alter their surface chemical structure. Since the manifestation of fluorescence by Nile Red is linked to the polarity of the surface of the polymers [24], the modifications on the polymeric chain of microfibers due to degradation processes or adsorption of foreign compounds could influence the fluorescence generated by Nile Red. Similar results were also observed by Prata et al. [31]. The authors stated that PE pristine fluoresced after being stained with Nile Red, however, weathered PE did not. In addition to the degradation processes, the presence of additives in the fiber's structure could also result in different fluorescence. This initial observation indicates that the Nile Red staining process could be affected by specific external parameters such as the surface of the microfibers. Therefore, microfibers could present different manifestations of fluorescence for the same polymeric matrix. Other limitation of the technique arises when fibers are coloured. The original colour of the fiber implies a background colour that will influence on the emitted fluorescence. In drinking water, most of the microfibers found are transparent or white, but in wastewater the presence of a higher number of coloured microfibers would drive to difficulties to determine by fluorescence the microfiber material.

In addition to PET microfibers, natural cotton microfibers were also identified in drinking water. Unlike the PET microfibers, the cellulose microfibers did not show a single fluorescence pattern. According to the fluorescence results, cotton microfibers could have three different manifestations of fluorescence, which were called Pattern 1, Pattern 2, and Pattern 3. The fluorescence emission patterns (Fig. 8) correspond to blue and red fluorescence, non-existent or slight red fluorescence, and green and red fluorescence, respectively.

Pattern 1 was expected in agreement with the results presented in Fig. 5, in which the pristine cotton fiber emitted blue and red fluorescence after staining with Nile Red. However, Patterns 2 and 3 were novel. Fig. 9 shows the spectra of the microfiber material obtained by μ-ATR-FTIR.

According to the μ-ATR-FTIR spectra, the manifestation of different fluorescence patterns could be related to the degree of degradation of the fibers, which can alter the surface of the fibers. In Pattern 1 it was observed that the characteristic peaks of pristine cotton fibers were maintained. It could be assumed that cotton microfibers with this fluorescence pattern are approximate to pristine cotton fibers recently disposed of in the environment, with little degradation. However, in Pattern 2 it was observed that the peaks located at $1030 \pm 6\ cm^{-1}$, 1107 ± 6, and $1157 \pm 6\ cm^{-1}$ lost intensity compared to the pristine cotton structure and showed less pronounced picks. Finally, in Pattern 3 the peaks $1030 \pm 6\ cm^{-1}$, and $1203 \pm 6\ cm^{-1}$ were almost imperceptible, and the peaks $1107 \pm 6\ cm^{-1}$, $1157 \pm 6\ cm^{-1}$ were further reduced compared to Pattern 2. The progressive smoothing of the peaks in the cotton structure could be related to breaks in the cellulose chain due to degradation processes. Li et al. [22] reported similar results in their study on the biodegradability of cotton fibers. The authors also observed that in the spectra of cotton fibers after going through a degradation process via composting, the peaks at $1032\ cm^{-1}$ and $1061\ cm^{-1}$ suffered an expressive reduction. Furthermore, the decrease in the intensity of the peaks at $1169\ cm^{-1}$ and $1111\ cm^{-1}$ was also detected. The reduction of the peaks was related to the breaking of the main chains of the cellulose. Coletti et al. [7] investigated whether the degradation processes could influence the identification of fibers by ATR-FTIR and observed that, indeed, the degradation of the natural fibers (flax and hemp) studied affected their FTIR spectra in such a way that the two materials can become indistinguishable.

Related to rayon, this artificial microfiber collected from drinking water also presented two fluorescence patterns. Fluorescence pattern 1 corresponds to the manifestation of blue and red fluorescence and pattern 2 corresponds to absence or slight red fluorescence (Fig. 10). However, in this case, it was not possible to link their relationship with the degradation processes. It would be necessary to analyze more microfibers of this material to improve the understanding of the phenomenon.

Table 3 summarizes the fluorescence patterns observed herein for each microfiber identified in drinking water. Due to the coincidence of fluorescence patterns 1 and 2 between cotton and rayon microfibers, these materials could not be differentiated after staining with Nile Red. However, the global presence of natural and artificial microfibers (cotton and rayon) and synthetic microfibers (polyester) could be estimated. In this study, just the fluorescence of cotton, rayon, and polyester from drinking water samples was determined since they were the predominant microfibers in the samples. Nevertheless, other natural, artificial, and synthetic polymers should be investigated for understanding their interactions with the Nile Red reagent.

3.5. Application of the Nile Red staining method to estimate microfibers in tap water (DWTP-B)

To determine the feasibility of using the Nile Red staining method in terms of the percentage estimation of synthetic, natural, and artificial microfibers in drinking water, a sampling of 29 randomly selected microfibers from the tap water sample was taken. The fluorescence of every microfiber was measured based on fluorescence patterns described in Table 3 (Fig. 11b). Results showed that 93% of microfibers followed a fluorescence pattern that would correspond to natural/artificial microfibers whereas only 7% of PET fibers. To check these results, the material identification of microfibers with μ-ATR-FTIR was carried out (Fig. 11a). It can be observed that the number of synthetic microfibers was very low in comparison with natural/artificial ones. This is in agreement with Feld et al. [11]. These authors reported only a 3% of synthetic microfibers in tap water samples in Denmark (though a 17% of the microfibers could not be identified). These authors identified 1 PET microfiber, 1 PS microfiber and 2 PP microfibers. Although these authors identified microfibers from 3 materials, it is confirmed that there is not a great variety of materials in tap water microfibers, being PET (the synthetic microfibers material found in this work) one of the most relevant one.

Comparing the fluorescence after the staining, it can be stated that Nile Red could help not only in the estimation of MPs (synthetic microfibers) and other microfibers (natural/artificial) present in drinking water but also in their identification. Further analysis should be carried out in order to confirm the feasibility of the method.

4. Conclusions

This study investigated the occurrence of microfibers in the water of two DWTPs. In DWTP-A, microfibers were eliminated in a percentage of 86% (comparing raw water and water after disinfection). Microfibers were identified in all the samples as most of the microparticles (75% of microparticles identified).

FTIR results showed that synthetic, natural, and artificial polymers were present in all the samples. Concerning the use of Nile Red to identify microfibers material in drinking water, it was observed that its use for the visual sorting of microfibers - between natural, artificial, and synthetic -is not an obvious procedure since the manifestation of fluorescence can be influenced by the level of degradation of the material. Moreover, since the fluorescence of the materials could be affected by the applied experimental conditions such as the microscopy (lamp and a set of filters), solvent, and concentration of Nile Red, it is important to validate the method proposed by staining pristine polymers and compare its efficiency when the method is applied to microfibers from drinking water samples. Taking into account these considerations, the use of Nile Red could help to identify the material of the microfibers. For it, the fluorescence of the microfibers after staining with Nile Red using different excitation wavelengths is proposed from the results of this work. In the analyzed samples of tap water (DWTP-B) with this methodology, it was identified 4% of synthetic microfibers. In addition, the staining with Nile Red could also be an interesting tool for helping in understanding the levels of degradation of these materials in the environment, and even in estimating the time they have been in nature.

CRediT authorship contribution statement

C. Bretas Alvim: Investigation, Formal analysis, Writing investigation. **M.A. Bes-Piá:** Funding acquisition, Supervision, Conceptualization. **J.A. Mendoza-Roca:** Funding acquisition, Supervision, Conceptualization. **J.L. Alonso-Molina:** Investigation, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

Authors thank the Spanish Ministry of Science, Innovation, and Universities for the financial support (Reference of the project: RTI2018–096916-B-I00).

References

[1] C.B.M. Almroth, L. Åström, S. Roslund, H. Petersson, M. Johansson, N.K. Persson, Quantifying shedding of synthetic fibers from textiles; a source of microplastics released into the environment, Environ. Sci. Pollut. Res. 25 (2018) 1191–1199, https://doi.org/10.1007/s11356-017-0528-7.

[2] S.N. Athey, J.K. Adams, L.M. Erdle, L.M. Jantunen, P.A. Helm, S.A. Finkelstein, M. L. Diamond, The widespread environmental footprint of indigo denim microfibers from blue jeans, Environ. Sci. Technol. Lett. 7 (2020) 840–847, https://doi.org/10.1021/acs.estlett.0c00498.

[3] A. Baran, A. Fiedler, H. Schulz, M. Baranska, In situ Raman and IR spectroscopic analysis of indigo dye, Anal. Methods 2 (2010) 1372–1376, https://doi.org/10.1039/c0ay00311e.

[4] O.Ó. Briain, A.R. Marques Mendes, S. McCarron, M.G. Healy, L. Morrison, The role of wet wipes and sanitary towels as a source of white microplastic fibres in the marine environment, Water Res. 182 (2020), 116021, https://doi.org/10.1016/j.watres.2020.116021.

[5] M.A. Browne, P. Crump, S.J. Niven, E. Teuten, A. Tonkin, T. Galloway, R. Thompson, Accumulation of microplastic on shorelines woldwide: sources and sinks, Environ. Sci. Technol. 45 (2011) 9175–9179, https://doi.org/10.1021/es201811s.

[6] X. Chu, B. Zheng, Z. Li, C. Cai, Z. Peng, P. Zhao, Y. Tian, Occurrence and distribution of microplastics in water supply systems: in water and pipe scales, Sci. Total Environ. 803 (2022), 150004, https://doi.org/10.1016/j.scitotenv.2021.150004.

[7] F. Coletti, M. Romani, G. Ceres, U. Zammit, M.C. Guidi, Evaluation of microscopy techniques and ATR-FTIR spectroscopy on textile fibers from the Vesuvian area: a pilot study on degradation processes that prevent the characterization of bast fibers, J. Archaeol. Sci. Rep. 36 (2021), 102794, https://doi.org/10.1016/j.jasrep.2021.102794.

[8] F.De De Falco, M. Pia, G. Gentile, E. Di, R. Escudero, R. Villalba, R. Mossotti, A. Montarsolo, S. Gavignano, C. Tonin, M. Avella, Evaluation of microplastic release caused by textile washing processes of synthetic fabrics, Environ. Pollut. 236 (2018) 916–925, https://doi.org/10.1016/j.envpol.2017.10.057.

[9] G. Erni-Cassola, M.I. Gibson, R.C. Thompson, J.A. Christie-Oleza, Lost, but found with nile red: a novel method for detecting and quantifying small microplastics (1 mm to 20 μm) in environmental samples, Environ. Sci. Technol. 51 (2017) 13641–13648, https://doi.org/10.1021/acs.est.7b04512.

[10] O.O. Fadare, E.D. Okoffo, Covid-19 face masks: a potential source of microplastic fibers in the environment, Sci. Total Environ. 737 (2020), 140279, https://doi.org/10.1016/j.scitotenv.2020.140279.

[11] L. Feld, V.H. da Silva, F. Murphy, N.B. Hartmann, J. Strand, A study of microplastic particles in danish tap water, Water 2021 (13) (2021) 2097, https://doi.org/10.3390/w13152097.

[12] B. Henry, K. Laitala, I.G. Klepp, Microfibres from apparel and home textiles: prospects for including microplastics in environmental sustainability assessment, Sci. Total Environ. 652 (2019) 483–494, https://doi.org/10.1016/j.scitotenv.2018.10.166.

[13] E. Hernandez, B. Nowack, D.M. Mitrano, Polyester textiles as a source of microplastics from households: a mechanistic study to understand microfiber release during washing, Environ. Sci. Technol. 51 (2017) 7036–7046, https://doi.org/10.1021/acs.est.7b01750.

[14] D. Kankanige, S. Babel, Smaller-sized micro-plastics (MPs) contamination in single-use PET-bottled water in Thailand, Sci. Total Environ. 717 (2020), 137232, https://doi.org/10.1016/j.scitotenv.2020.137232.

[15] M. Kazour, S. Terki, K. Rabhi, S. Jemaa, G. Khalaf, R. Amara, Sources of microplastics pollution in the marine environment: importance of wastewater treatment plant and coastal landfill, Mar. Pollut. Bull. 146 (2019) 608–618, https://doi.org/10.1016/j.marpolbul.2019.06.066.

[16] L. Kim, S.A. Kim, T.H. Kim, J. Kim, Y.-J. An, Synthetic and natural microfibers induce gut damage in the brine shrimp Artemia franciscana, Aquat. Toxicol. 232 (2021), 105748, https://doi.org/10.1016/j.aquatox.2021.105748.

[17] S. Konde, J. Ornik, J.A. Prume, J. Taiber, M. Koch, Exploring the potential of photoluminescence spectroscopy in combination with Nile Red staining for microplastic detection, Mar. Pollut. Bull. 159 (2020), 111475, https://doi.org/10.1016/j.marpolbul.2020.111475.

[18] M. Kosuth, S.A. Mason, E.V. Wattenberg, Anthropogenic contamination of tap water, beer, and sea salt, PLoS One 13 (2018) 1–18, https://doi.org/10.1371/journal.pone.0194970.

[19] S.M. Ladewig, S. Bao, A.T. Chow, Natural fibers: a missing link to chemical pollution dispersion in aquatic environments, Environ. Sci. Technol. 49 (2015) 12609–12610, https://doi.org/10.1021/acs.est.5b04754.

[20] M. Lares, M.C. Ncibi, Sillanpää Markus, Sillanpää Mika, Occurrence, identification and removal of microplastic particles and fibers in conventional activated sludge process and advanced MBR technology, Water Res 133 (2018) 236–246, https://doi.org/10.1016/j.watres.2018.01.049.

[21] J. Lee, M.H. Kang, K.B. Lee, Y. Lee, Characterization of natural dyes and traditional korean silk fabric by surface analytical techniques, Mater. (Basel) 6 (2013) 2007–2025, https://doi.org/10.3390/ma6052007.

[22] L. Li, M. Frey, K.J. Browning, Biodegradability study on cotton and polyester fabrics, J. Eng. Fiber Fabr. 5 (2010) 42–53, https://doi.org/10.1177/155892501000500406.

[23] C.R. Li, R. Busquets, R.B. Moruzzi, L.C. Campos, Preliminary study on low-density polystyrene microplastics bead removal from drinking water by coagulation-flocculation and sedimentation, J. Water Process. Eng. 44 (2021), 102346, https://doi.org/10.1016/j.jwpe.2021.102346.

[24] T. Maes, R. Jessop, N. Wellner, K. Haupt, A.G. Mayes, A rapid-screening approach to detect and quantify microplastics based on fluorescent tagging with Nile Red, Nat. Publ. Gr. (2017) 1–10, https://doi.org/10.1038/srep44501.

[25] V. Martinez, M. Henary, Nile Red and Nile Blue: applications and syntheses of structural analogues, Chem. Eur. J. 22 (39) (2016) 13764–13782, https://doi.org/10.1002/chem.201601570.

[26] S.A. Mason, V.G. Welch, J. Neratko, Synthetic polymer contamination in bottled water, Front. Chem. 6 (2018) 1–17, https://doi.org/10.3389/fchem.2018.00407.

[27] I.E. Napper, R.C. Thompson, Release of synthetic microplastic plastic fibres from domestic washing machines: effects of fabric type and washing conditions, Mar. Pollut. Bull. 112 (2016) 39–45, https://doi.org/10.1016/j.marpolbul.2016.09.025.

[28] H.A. Nel, A.J. Chetwynd, L. Kelleher, I. Lynch, I. Mansfield, H. Margenat, S. Onoja, P. Goldberg Oppenheimer, G.H. Sambrook Smith, S. Krause, Detection limits are central to improve reporting standards when using Nile red for microplastic quantification, Chemosphere (2021) 263, https://doi.org/10.1016/j.chemosphere.2020.127953.

[29] M.L. Pedrotti, S. Petit, B. Eyheraguibel, M.E. Kerros, A. Elineau, J.F. Ghiglione, J. F. Loret, A. Rostan, G. Gorsky, Pollution by anthropogenic microfibers in North-West Mediterranean Sea and efficiency of microfiber removal by a wastewater treatment plant, Sci. Total Environ. 758 (2021), 144195, https://doi.org/10.1016/j.scitotenv.2020.144195.

[30] M. Pivokonský, L. Cermakova, K. Novotna, P. Peer, T. Cajthaml, V. Janda, Occurrence of microplastics in raw and treated drinking water, Sci. Total Environ. 643 (2018) 1644–1651, https://doi.org/10.1016/j.scitotenv.2018.08.102.

[31] J.C. Prata, V. Reis, J.T.V. Matos, J.P. da Costa, A.C. Duarte, T. Rocha-Santos, A new approach for routine quantification of microplastics using Nile Red and automated software (MP-VAT, Sci. Total Environ. 690 (2019) 1277–1283, https://doi.org/10.1016/j.scitotenv.2019.07.060.

[32] J.C. Prata, J.P. da Costa, A.J.S. Fernandes, F.M. da Costa, A.C. Duarte, T. Rocha-Santos, Selection of microplastics by Nile Red staining increases environmental sample throughput by micro-Raman spectroscopy, Sci. Total Environ. 783 (2021), 146979, https://doi.org/10.1016/j.scitotenv.2021.146979.

[33] F. Remy, F. Collard, B. Gilbert, P. Compère, G. Eppe, G. Lepoint, When microplastic is not plastic: the ingestion of artificial cellulose fibers by macrofauna living in seagrass macrophytodetritus, Environ. Sci. Technol. 49 (2015) 11158–11166, https://doi.org/10.1021/acs.est.5b02005.

[34] D.J. Sarkar, S.D. Sarkar, B.K. Das, J.K. Praharaj, D.K. Mahajan, B. Purokait, T. R. Mohanty, D. Mohanty, P. Gogoi, Y.S. Kumar, B.K. Bebera, R.K. Manna, S. Samanta, Microplastics removal efficiency of drinking water treatment plant with pulse clarifier, J. Hazard. Mater. 413 (2021), 125347, https://doi.org/10.1016/j.jhazmat.2021.125347.

[35] V.C. Shruti, F. Pérez-Guevara, P.D. Roy, G. Kutralam-Muniasamy, Analyzing microplastics with Nile Red: emerging trends, challenges, and prospects, J. Hazard. Mater. 423 (2022), 127171, https://doi.org/10.1016/j.jhazmat.2021.127171.

[36] M. Sillanpää, P. Sainio, Release of polyester and cotton fibers from textiles in machine washings, Environ. Sci. Pollut. Res. 24 (2017) 19313–19321, https://doi.org/10.1007/s11356-017-9621-1.

[37] A. Stolte, S. Forster, G. Gerdts, H. Schubert, Microplastic concentrations in beach sediments along the German Baltic coast, Mar. Pollut. Bull. 99 (2015) 216–229, https://doi.org/10.1016/j.marpolbul.2015.07.022.

[38] Z. Wang, T. Lin, W. Chen, Occurrence and removal of microplastics in an advanced drinking water treatment plant (ADWTP, Sci. Total Environ. 700 (2020), 134520, https://doi.org/10.1016/j.scitotenv.2019.134520.