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Preparation and analysis of standards containing microfilaments/microplastic with fibre shape

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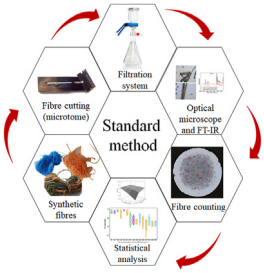
HIGHLIGHTS

- Testing and monitoring of microfilaments by using a standard method.
- Statistical relationship between theoretical microfilaments/detection probability.
- Detection probability >95% with high volume and N°filaments/liter less than 200.
- Determination of the factors which affect detection probability.

GRAPHICAL ABSTRACT



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ABSTRACT

Synthetic clothing represents a primary source of environmental pollution because of shedding of microfilaments during laundry washing or in textile processes. Although many approaches can be used for the evaluation of microplastic, there are no precise guideline to follow for the analysis labs. Here, an accurate method for the preparation of microfilaments standard suspensions to facilitate lab tests and the monitoring of microplastic in different matrices was developed. Different standard suspensions were prepared by using five different synthetic threads consisting of a different number of filaments cut with a predetermined length of 0.2 mm suspended in three different volumes of water. The suspensions were filtered and the microfilaments were counted. The number of microfilaments for each polymer solution were statistically elaborated with a logit model and the results showed that the probability of detecting them is higher than 95% when the concentration of microfilaments/L is lower than 200. Moreover, a relationship between the theoretical microfilaments contained in the samples and the detection probability of the single microfilament, for each suspension volume was highlighted.

1. Introduction

The presence of macroplastics in marine environment is unaesthetic and it can have several repercussions on tourism, shipping, fishing and aquaculture (Derraik, 2002). Furthermore, macroplastic damages marine life due to entrapment or ingestion by the local fauna. Recently, significant debris of minute fragments of plastics, called microplastics (MPs) has been found in oceans worldwide (Barnes et al., 2009) and their harmful effects on the marine ecosystem and biota are also supported by an increasing

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number of scientific papers, media and by companies that have been promoting processes and products with a lower environmental impact. At present, in accordance with the European Chemical Agency (ECHA) microplastic is defined as a material composed of solid polymeric-containing particles, to which additives or other substances may be added. The family of microplastics includes synthetic-based particles such as polyethylene (PE), polypropylene (PP), polystyrene (PS), polyamides (PA), polyethylene terephthalate (PET), polyvinyl chloride (PVC), polyacrylonitrile (PAN), polymethylacrylate (PMA), elastomers and silicone rubber with particles ranging from 1 nm to 5 mm and fibre lengths ranging from 3 nm to 15 mm and a length-to-diameter ratio of >3 (Miranville, 2020). In particular, microplastic contamination of the marine environment can be incorporated by the ecosystem (Cole et al., 2011). In microplastic debris, an important role is played by textile microfilamens, primary MP sources identified in water (Cai et al., 2017; Kanhai et al., 2018; Peeken et al., 2018; Sun et al., 2019). Synthetic clothing represents a primary source of pollution as shedding of microplastics with fibre shape during laundry washing or textile processes has several potential impact on the environment (Browne et al., 2011; Dris et al., 2015; Napper et al., 2015). This behaviour is due to their dimensions that enhance their partial pass through wastewater treatment plants (WWTPs) reaching environments such as oceans and seas. Hartline et al. (2016), assuming a WWTP microplastic removal rate of 98.4%, considering 0.35 m^3 of sewage per person per day, indicate that a city of 100,000 people would produce approximately 1.02 kg of microfilaments per day. Several works have already been published on this topic but, since all of them have used different methodologies, clear comparisons are difficult. Moreover, some authors have observed that data concerning WWTP vary considerably and this large variation is caused by wastewater plants together with the different sampling, preparation and identification methods used by scientific community. Several approaches have been performed. A visual sorting of the microplastics can be used, but it is not precise and it leads to an error higher than 70% in recognition (Hidalgo-Ruz et al., 2012). Considering the use of the microscope only, small fragments (<50 μm) are underestimated, whereas long fibres (<200 μm) create false-positive results (Ivleva et al., 2017). Schirinzi et al. (2019), used the chromatography coupled to high-resolution mass spectrometry (LCHRMS) for the quantification and chemical identification of PS microplastic in natural water. This technique does not give information on shape and size that can be obtained instead by optical and SEM microscopy as shown by different research groups (Belzagui et al., 2019; Schirinzi et al., 2019). Another rapid-screening approach to detect microplastics (PE, PS, PP, and nylon 6) is based on fluorescent tagging with Nile Red (Erni-Cassola et al., 2017; Maes et al., 2017). For the identification of polymer fragments thermal techniques such as differential scanning calorimetry (DSC) and thermo-gravimetric analysis (TGA), sometimes linked with chromatography-mass spectrometry (TDS-GC-MS) or pyrolysis (Py-GC-MS), are applied (Dümichen et al., 2015; Shim et al., 2017). Another common approach for sorting different-sized microplastic particles requires the use of sieves (Hildebrandt et al., 2019) using the gravimetric method to quantify the mass of microfilaments released during washing processes in real conditions. In addition, the same method was used in tests of washing machine wastewater (Dalla Fontana et al., 2020; De Falco et al., 2018; Hartline et al., 2016; Pirc et al., 2016) during washing cycles of different synthetic standard fabrics or clothes. Today an innovative approach is based on spectroscopic measurements. One of the novel techniques applied for the identification of microplastic is molecular spectroscopy (Micro-FTIR and Micro-Raman), which allows the identification of very small plastic particles and so it is appropriate for sorting and recognizing fibres. Micro-Raman technique would facilitate the detection of fibres on a filter with a spatial resolution of 1 μm (Kniggendorf et al., 2019; Löder et al., 2015). For particles, between 10 and 50 μm, collected on an IR filter (e.g. aluminum oxide or silicon) and directly analyzed, excellent results are obtained using the micro-FTIR, which is a combination of FTIR and optical microscopy (Ivleva et al., 2017). It is fast, non-destructive, and reproducible. Although many approaches can be applied for the quantification and identification of microplastic, there are no precise guidelines to follow for microfilaments preparation and their quantification in different samples. Unfortunately, microfilaments standard material fibres are rarely used in laboratory studies as they are unavailable for purchase. Existing methods for preparing microfilaments (MFs) are limited to cutting or cryogenically grinding synthetic cord, resulting in relatively large fibres (>500 μm in length) with a wide size distribution (Graham and Thompson, 2009; Murray and Cowie, 2011; Watts et al., 2015). Cole et al. have prepared MFs length between 40 and 100 μm of Nylon, polyethylene terephthalate and polypropylene fibres (10–28 μm diameter) used for toxicity testing in brine shrimp (Artemia sp.) (Cole et al., 2011).

As at the global level, intercalibration actions between laboratories have never been performed and there are no quality standards to refer to or standardized protocols for sampling, extraction, purification and characterization of microplastics.

The novelty of this work is the development of an *"easy to use"*, accurate method for the preparation of microfilaments standard suspensions to facilitate lab tests and the monitoring of microplastic in different matrices. The determination of microplastic in a real sample could be determined adding internal standards in order to verify the quality of all the operations, counting and identification.

2. Materials and methods

2.1. Materials

The different synthetic threads (PA 6, PA 6.6, PET, PP) used for the preparation of standard solutions were supplied by Aquafil S. p.A (Fig. 1) and are the following:

1. Multicolor PA 6 (128 filaments; 2300 dtex)
2. Orange PA 6 (180 filaments; 3450 dtex).
3. Blue PA 6.6 (68 filaments; 200 dtex).
4. Cream PET (256 filaments; 2970 dtex).
5. Orange PP (72 filaments; 70 dtex)

2.2. Chemicals and filters

Ethanol absolute anhydrous was purchased from Carlo Erba (Italy), while sodium hypochlorite with 15% of active chlorine was supplied by Acros Organics (Italy).

Macroporous silicon membranes were purchased from Smart MEMBRANE, Germany. They have the following characteristics: size 13 mm × 13 mm, pore size 5 μm, trigonal pore geometry, internal distance 12 μm, thickness 500 μm). The cleaning of silicon filter was checked using Leica DMLP polarizing microscope in reflection mode with magnifications of 50x-100x.

2.3. Quality control of analysis (QA/QC)

Before preparing the standard suspensions, all glassware used was washed with ultrapure water/ethanol 1:1 in order to remove any contaminants. After washings, it was stored and protected with

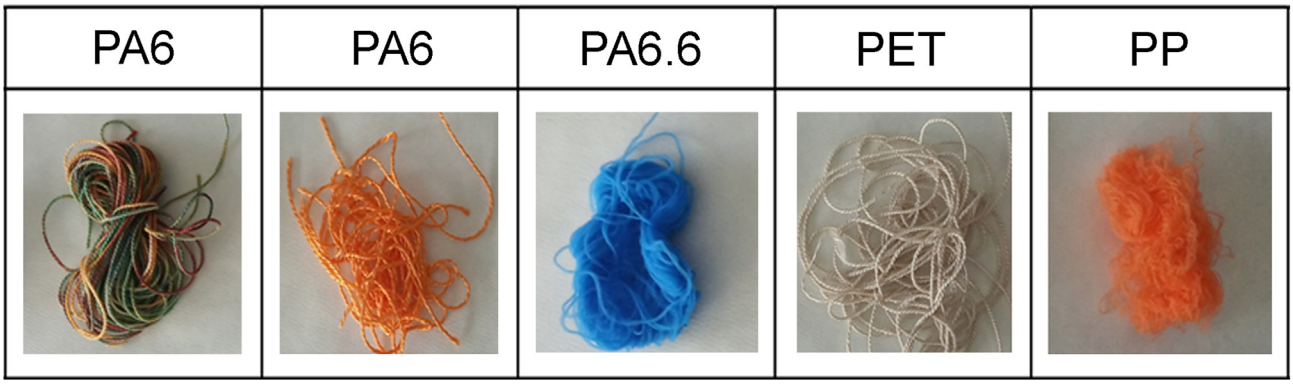

Fig. 1. Image of synthetic thread used for the preparation of standard solution. The standard thread was maintained in a room with controlled atmosphere as specified in IWTO-52, at 20 °C and 65% R.H.

the aid of suitable barriers (e.g. aluminium foil closing the inlets) to reduce any possible deposition of MFs present in the air. Sample containers made of glass were used. All tweezers and probes were cleaned before each procedure. Garments (included white coat) worn by analysts during sample handling were made of natural fibres instead synthetic ones. Silicon filters were finely cleaned before using to remove possible contaminations with the following procedure: (i) sonication for three times in ultrapure water, each of 10 min; (ii) cleaning in 10 ml of pure ethanol (99.5%); and (iii) check the absence of any residual fibre under a light microscope in reflection mode. However, the possible "environmental contamination" can be checked, by carrying out in "blank test" for each measurement.

2.4. Preparation of standard suspension

The samples for each standard suspension were cut following IWTO-8-97. Sample threads were mounted in a slide of a microtome and compressed firmly in the slot, using wool fibres. The surplus fibre from each side of the microtome plate was cut off using a sharp razor blade. A 0.2 mm pusher was inserted in the slot, and it was moved backwards and forwards to cause a fringe of fibres to project from the opposite side of the holder. Again, the protruding fringe of fibres flush was cut off using a razor blade. The pieces had a predetermined length equivalent to the length of the pusher (0.2 mm long) (Fig. 2). The blade was suitably cleaned by spraying ultrapure water on the surface to collect all the cut fibres in a 50 ml flask.

After being cut with a microtome, all the fibres were dispersed in 10 ml of distilled H_2O and 5–7 ml of sodium hypochlorite (prepared according to Regulation UE n. 1007/20,11[1]) was added to remove the wool, facilitating the counting of the synthetic fibres under the microscope. The suspension was shaken in a 50 ml flask with a mechanical stirrer at 130 r.p.m. for 40 min at room temperature.

After the treatment with sodium hypochlorite, the synthetic fibres were split into a large Erlenmeyer flask. The flask was washed with 50 ml aliquots of ultrapure water to reach the stated volumes (300 ml, 500 ml or 900 ml) in order to recover any fibres left attached to the walls and transfer them to the Erlenmeyer flask.

Finally, the flask was also rinsed with 10 ml of 1:1 solution of ultrapure water/ethanol. This phase further helped to recover the remaining fibres.

For each thread three different concentrations were obtained using the same number of filaments suspended in three volumes 300, 500, 900 ml. These are the concentrations for each sample: Multicolor PA6: (427; 256; 142) N° microfilaments/L; Orange PA6 (600; 360; 200) N° microfilaments/L; Blue PA 6.6 (227; 136; 76) N° microfilaments/L; Cream PET (853; 512; 284) N° microfilaments/L; Orange PP (240; 144; 80) N° microfilaments/L.

For quality control 5 replicates of synthetic polymer suspensions for each concentration were carried out. The concentrations of all the standard suspensions obtained spanned a wide range between 76 N° filaments/L and 853 N° filaments/L.

2.5. Filtration procedure

50 ml of standard suspensions were gradually filtered on a silicon filter mounted on a glass-filter apparatus connected with a vacuum pump. The filters were placed with the mirror face upwards. After filtering the entire stock solution, the funnel walls were washed with a few ml of 1:1 ultrapure water/ethanol using a glass Pasteur pipette in order to recover the possible microfilaments adhering to the glass. Then, a final recovery wash (of the filtering system, the gasket and the flask containing the stock solution) was carried out with ultrapure water and a 1:1 solution of water/ethanol employing a different silicon filter. Since two filters were used, the final number of fibres was the sum of the fibres found on the two filters.

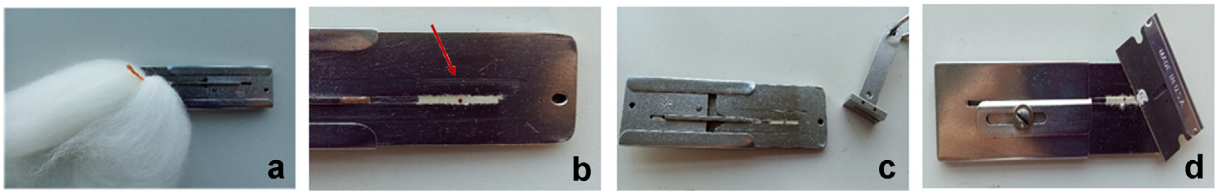

Fig. 2. Sample cutting preparation: a) standard fibres and wool in a slide of microtome, b) removing the protruding fringe by razor blade, c) choice of fibres length using a suitable pusher, d) fibre cutting (0.2 mm).

2.6. Micro and macro fourier transform infrared spectroscopy (micro FT-IR) analysis

The FT-IR spectra of synthetic fibres before and after NaClO treatment were acquired with Attenuated Total Reflection (ATR) technique in the range from 4000 to 650 cm^{-1} with 50 scansions and 4 cm^{-1} of spectral resolution by means of a Thermo Scientific™ Nicolet™ iN™10 Infrared Microscope, which couples an optical microscope with an infrared spectrophotometer. The counting of the fibre collected on the silicon filter was carried out with the microscope part of the Micro FT-IR and the imaging section of the Omnic ™ Picta ™ software. The filters were mounted on the stage with removable tape on the supporting of the filter.

The image analysis acquires the whole area of the filter, identifying the microfilaments that have to be analyzed and counted.

2.7. Statistical analysis

Logit regression analysis (Claessens et al., 2013) was used to investigate the relationship between concentration and single microfilament's detection probability as well as the impact of the type of material used (synthetic polymer). Logit model is widely used to investigate the relationship between a binary response variable and some other explanatory ones. In this study logit model was chosen due to of the binary nature of the data, in which a dependent variable has two possible values expressed as identification or non-identification for each individual microfilament in the suspension. Let Y_{ij}, $i = 1, \ldots, n$, $j = 1, \ldots m$, denote the response, that is the number of detected microfilaments for the i-th sample and j-th replication. Since for each sample, K is the theoretical number of microfilaments and it represents the number of independent trials that can be performed on it. Y_{ij} is distributed as a binomial random variable of size K and probability p_i. The logit model used explicit the relationship between the probability of detection of the single microfilaments, p_i, and the covariates by modelling:

$$\text{logit}\left(E\left(Z_{ijk}\middle|X_{1,ij},X_{2,ij}\right)\right)=\log(p_i/(1-p_i))=\beta_0+\beta_1 X_{1,ij}+\beta_{M(i)} \quad (1)$$

where Z_{ijk}, $k = 1, \ldots, K$ is a Bernoulli random variable representing the detection of the k-th microfilament in the i-th sample and j-th replication, $X_{1,ij}$ the concentration used and $\beta_{M(i)}$ the parameter representing the material's effect for the i-th sample.

3. Results and discussion

The protocol was optimized to produce standard suspensions with concentrations between 76 N° filaments/L and 853 N° filaments/L of synthetic microfilaments cut at pre-determined lengths of 200 μm.

Sample threads of 4 different polymers were mounted in the slide of a microtome and compressed firmly in the slot, using wool fibres, which were successfully removed by hypochlorite treatment.

The wool fibres are well recognizable compared to the synthetic ones: they have an irregular diameter around 16/20 μm and a unique surface structure of overlapping scales, which are a characteristic of the morphology of animal hair fibres, easily observable

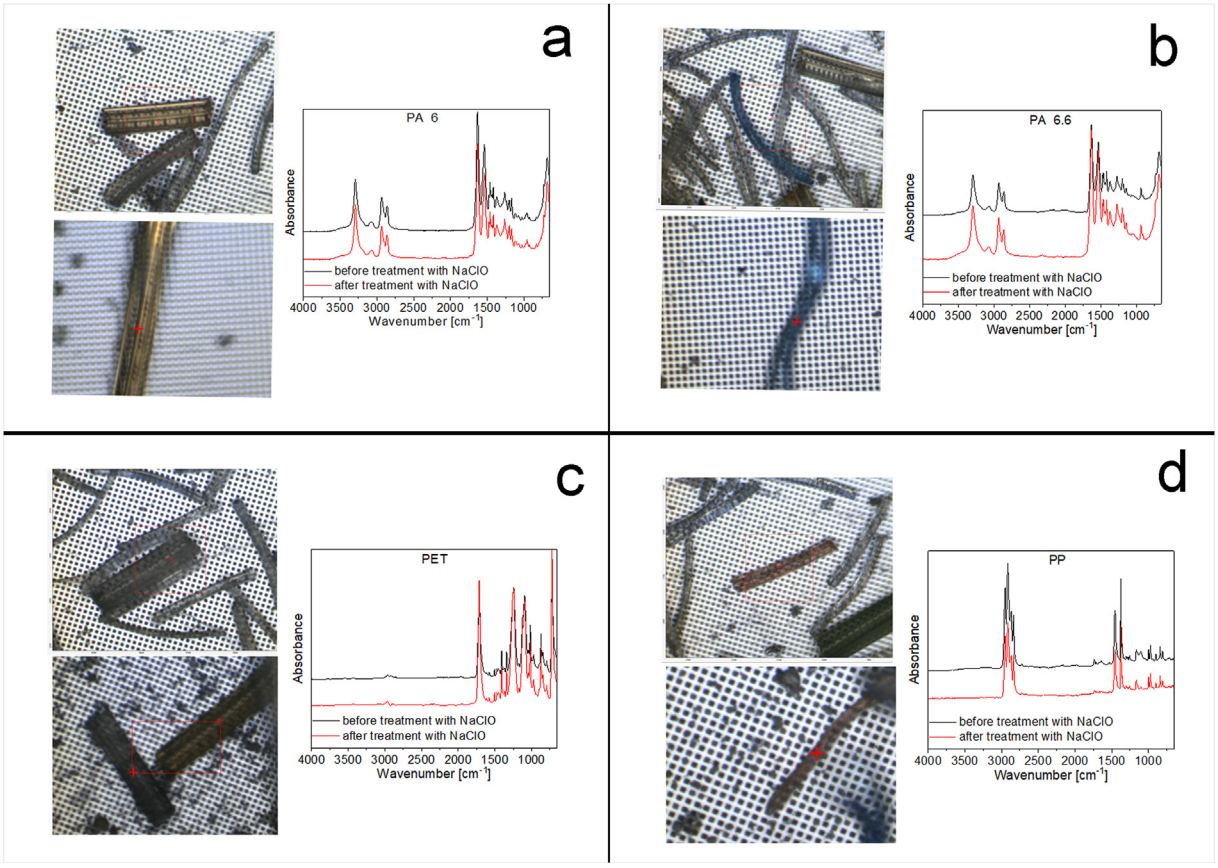

Fig. 3. Optical images of synthetic fibres, (a: orange PA 6, b: blue PA 6.6, c: orange PP, d: cream PET), before and after NaClO treatment and their respective FTIR spectra (data obtained using Micro-FTIR instrument). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

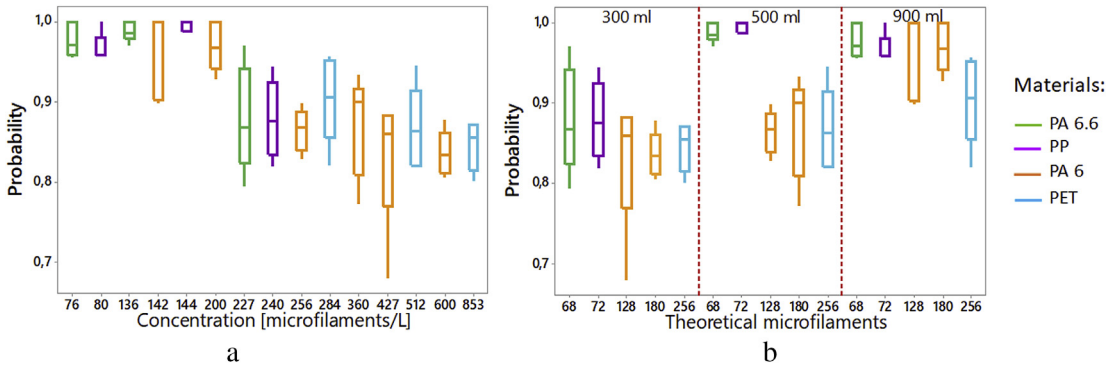

Fig. 4. a. Boxplots showing the relation between concentrations and the probability of detection of the single microfilament. b. Boxplots showing the relation between suspension volumes, theoretical microfilaments and the probability of detection of the single.

at around 100x by using light microscopy (Bradbury, 1976). Contrarily, synthetic fibres (e.g. PA) show a larger diameter, around 55–60 μm and a regular cylindrical shape for the whole fibre length with a regular and smooth surface. These fibres are called man-made fibres as they are obtained by extruding through the holes of a spinneret a polymer melt or polymer suspension and by combining the filaments to form a yarn. The size and shape of the spinneret holes determine the filament's cross-sectional shape. Mixing the synthetic filaments with wool is a necessary step to fill the microtome slot completely without altering the number of the filaments of the standard thread. After the hypochlorite treatment, the number of wool fibres significantly decreased and the counting of the standard fibres was easier, because no fibre overlapping was present. In order to check the preparation of the standards containing MFs in an accurate way, wool removal was observed by optical microscopy before and after the sodium hypochlorite treatment (Fig. 3). The effects of the hypochlorite aliquot and the bath ratio used during the treatment were also investigated with FTIR. The results showed that the spectra of all the synthetic polymers (PA 6.6, PA 6, PET and PP), after oxidative treatment, did not present any significant changes in characteristic band absorption when compared with reference samples (Fig. 3).

For each polymeric yarn, three suspensions at 300, 500, 900 ml were prepared to obtain different concentrations with the same number of filaments per yarn. The suspensions were filtered by using two silicon filters. The second filter was employed to collect microfilaments from the subsequent washes of the filtration system. This step ensures a complete collection of MFs from the standard suspension. After that, the sum of microfilaments collected on the filters for each thread was determined. The average value, standard deviation of 5 replicates of synthetic polymer suspensions for each concentration were carried out. Moreover, the standard recovery rate for each standard suspension was calculated. These all data are shown in Supplementary information (Table S1).

The MFs collected on each filter were counted with an optical microscope associated with a Micro-FTIR instrument. In this study, the entire area of the filter as opposed to a limited sector as shown in recent literature is acquired for the counting of the MFs (Corami et al., 2020). This difference is due to the ratio of MFs and volume of suspensions that determines a better uniform distribution of MFs on the whole area of the filter and a lower overlapping of the fibres (Figure S1 in supplementary information).

Moreover, a relationship between concentration obtained by counting and the probability of the detection of the single microfilaments was studied. The results show (Fig. 4a) that the probability of detecting the microfilaments is higher than 95% when the concentration of N° microfilaments/L is lower than 200 N° microfilaments/L, whereas, Fig. 4b shows the relationship between the theoretical microfilaments contained in the samples and the detection probability of the single microfilament, for each suspension volume.

Therefore, the higher the suspension volume is, the higher the detection probability is, while the more theoretical microfilaments are present, the lower the detection probability is. These relationships are also related to different kinds of materials represented by different colours.

The estimates of the parameters of the model and inference are obtained with the maximum likelihood method, the standard error and the p-values are reported in Table 1 (Young, 2019):

As shown in Table 1, the effect of the concentration, β_1, is highly significant: the dilution of the sample increases the detection probability. Moreover, the model highlights differences between the polymeric materials. Thus, this hypothesis was checked with a likelihood ratio test and proved that the influence of synthetic material is statistically significant (p-value <1e-5) PA6, PA66 PET, PP have a different detection probability. In particular, by looking at the estimate parameters in Table 1, PA66, PET and PP have a greater detection probability compared to PA6. The robustness of the conclusions was also proved by using the probit mode (Moodie, 2009), thus specifying a different link function between the conditional expectation and the linear predictor. However, further tests will be performed to confirm these preliminary data.

To better visualize the model, Table 2 and Fig. 5 show the estimated probability surfaces for each material, calculated over a grid of values for the suspension volume and theoretical number of microfilaments. Due to the nature of the model, the material does not impact the shape of the 3 D surface but only its magnitude because there are no interactions in the model (equation (1)) with the concentration. According to the previous findings, higher the concentration is, lower is the probability of detection of the single microfilament.

Moreover, figure S2, reported in supplementary information, shows the single effect of the theoretical number of microfilaments

Table 1
Maximum likelihood estimates of the model parameters as specified in equation (1).

	Estimate	Std. Error	P-value
β_{0PA6}	2.69	0.08	<1e-5
β_1	−1.70	0.17	<1e-5
β_{PA66}	0.47	0.15	0.0024
β_{PET}	0.22	0.08	0.0055
β_{PP}	0.44	0.15	0.0025

Table 2
Estimated probability surfaces for different materials tested and their standard errors.

Theoretical Microfilaments	Suspension volume [mL]	Concentration [microfilaments/L]	PA6: detection probability	PA66: detection probability	PET: detection probability	PP:detection probability
68	300	227	0.909 (0.004)	0.941 (0.008)	0.926 (0.006)	0.940 (0.008)
68	500	136	0.921 (0.005)	0.949 (0.007)	0.936 (0.006)	0.948 (0.007)
68	900	76	0.928 (0.005)	0.954 (0.006)	0.942 (0.006)	0.953 (0.006)
72	300	240	0.908 (0.004)	0.940 (0.008)	0.924 (0.006)	0.939 (0.008)
72	500	144	0.920 (0.005)	0.949 (0.007)	0.935 (0.006)	0.948 (0.008)
72	900	80	0.928 (0.005)	0.954 (0.006)	0.941 (0.006)	0.953 (0.006)
128	300	427	0.877 (0.005)	0.919 (0.011)	0.899 (0.005)	0.918 (0.011)
128	500	256	0.905 (0.004)	0.938 (0.008)	0.923 (0.006)	0.937 (0.008)
128	900	142	0.921 (0.005)	0.949 (0.007)	0.935 (0.006)	0.948 (0.007)
180	300	600	0.842 (0.008)	0.894 (0.015)	0.869 (0.006)	0.893 (0.015)
180	500	360	0.889 (0.005)	0.927 (0.010)	0.909 (0.005)	0.926 (0.010)
180	900	200	0.913 (0.004)	0.944 (0.008)	0.929 (0.006)	0.943 (0.007)
256	300	853	0.776 (0.016)	0.846 (0.002)	0.812 (0.010)	0.844 (0.024)
256	500	512	0.861 (0.006)	0.908 (0.013)	0.885 (0.005)	0.906 (0.013)
256	900	284	0.901 (0.004)	0.935 (0.009)	0.919(0.006)	0.934 (0.008)

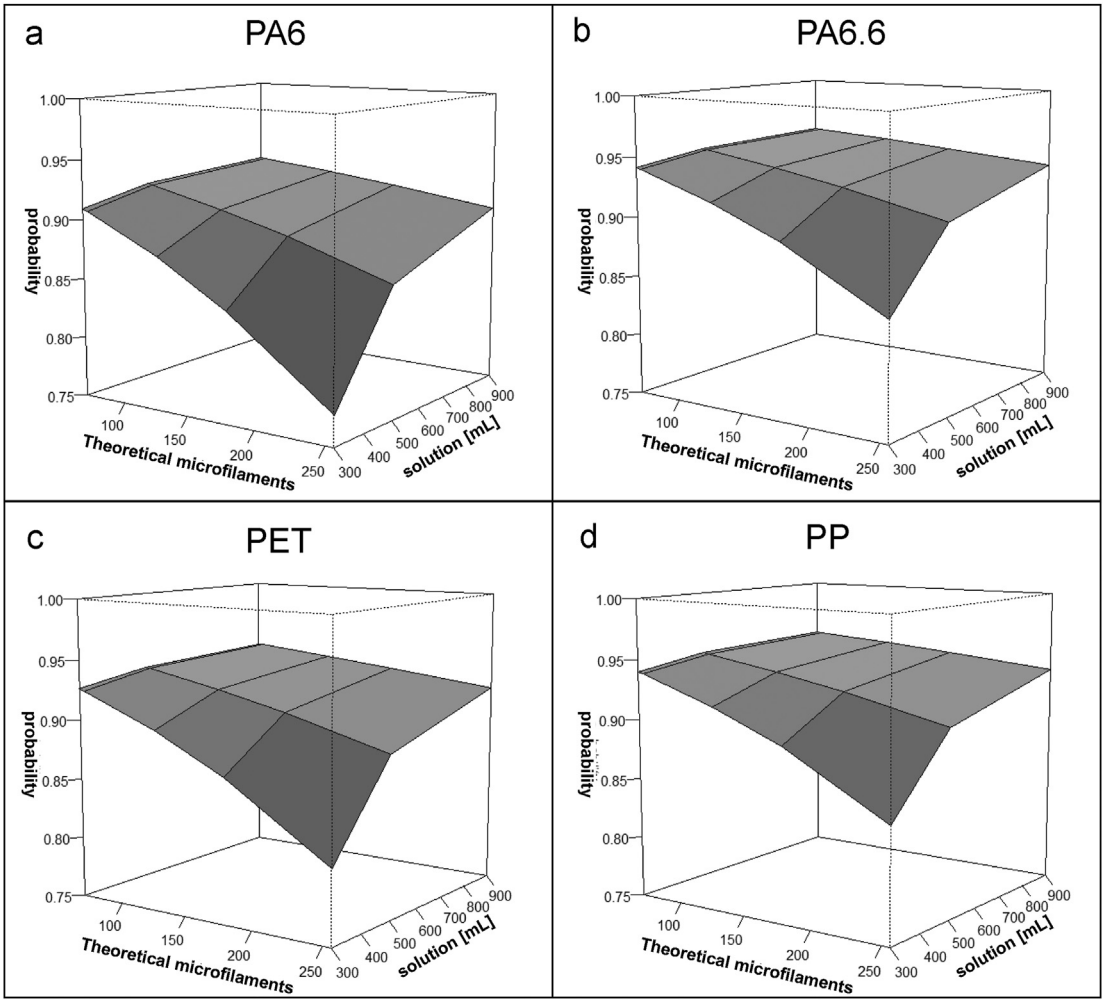

Fig. 5. Estimated probability surfaces for different synthetic materials used.

and the suspension volume and concentration on the detection probability are highlighted. These estimated relationships are obtained by averaging the different surfaces in order to elicit the effect of a specific variable by marginalizing the others. The 95% confidence intervals are percentile-based ones obtained via stratified bootstrap method, in which each sample is a stratum (Johnson, 2001). Thus, an increase in the number of microfilaments in the sample reduces the detection probability; conversely, an increase in the suspension volume increases the detection probability.

4. Conclusions

In this work a protocol was optimized to produce standard suspensions with concentrations between 76 N° filaments/L and 853 N° filaments/L of synthetic microfilaments using four different polymer threads were cut at pre-determined lengths of 200 μm following IWTO-8-97 and dispersed in three solutions of 300, 500, 900 ml to obtain three different concentrations. The solutions were filtered through a silicon filter and the microfilaments were counted with optical microscopy associated with a Micro-FTIR instrument. Five replicates per type of synthetic polymers were carried out for each sample and the data were statistically analyzed by using a logit method. The results highlighted the relationship between concentration and probability of the detection of the single microfilaments. The probability of detecting the microfilaments is higher than 95% when the concentration of microfilaments/L is lower than 200. Moreover, the statistical analysis shows that an increase in the number of microfilaments in the sample suspension reduces the detection probability, while an increase in the suspension volume increases the detection probability. These results seem to confirm that it is possible to use an appropriate concentration of microfilaments as an internal standard and to evaluate the recovery rate in microplastics analysis in real sample.

The work is in progress and it is part of a method presented to ISO (International Organization for Standardization) and CEN (European Committee for Standardization) for the evaluation of microplastics in textile sector, which could prove suitable for the analysis of microplastics of different origins.

In the end, standard microfilaments suspensions will be coupled by an ecotoxicological study able to point out also their impacts on the aquatic biocoenosis.

Author statement

Raffaella Mossotti: Writing – review & editing, Conceptualization, Methodology, Writing – original draft, Project administration, Funding acquisition. Tiziano Battistini: Project administration, Funding acquisition, Supervision, Review. Anastasia Anceschi: Writing – review & editing, Supervision Enrico Gasparin: Writing, Review, Statistical validation. Giulia Dalla Fontana: Writing – review & editing, Conceptualization, Methodology Writing – original draft.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2020.129410.

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