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# Nylon 6 and nylon 6,6 micro- and nanoplastics: A first example of their accurate quantification, along with polyester (PET), in wastewater treatment plant sludges

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ABSTRACT

A novel procedure for nylon 6 and nylon 6,6 polyamide (PAs) microplastics (MPs) quantification is described for the first time. The overall procedure, including quantification of poly(ethylene terephthalate) (PET), was tested on wastewater treatment plant (WWTP) sludges. The three polymers account for the largest global share of synthetic textile microfibers, being possibly the most common MPs released upon laundering in urban wastewaters. Therefore, measuring their content in WWTP sludges may provide an accurate picture of the potential risks associated with both the inflow of these MPs in natural water bodies and the practice of using WWTP sludges as agricultural soil amendment. The novel procedure involves PAs depolymerization by acid hydrolysis followed by derivatization of the monomers 6-aminohexanoic acid (AHA) and hexamethylene diamine (HMDA) with a fluorophore. Reversed-phase HPLC analysis with fluorescence detection results in high sensitivities for both AHA (LOD = $8.85{\cdot}10^{-4}$ mg/L, LOQ = $3.73{\cdot}10^{-3}$ mg/L) and HMDA (LOD = $2.12{\cdot}10^{-4}$, LOQ = $7.04{\cdot}10^{-4}$ mg/L). PET quantification involves depolymerization, in this case by alkaline hydrolysis, followed by HPLC analysis of its comonomer terephthalic acid. Eight sludge samples from four WWTPs in Italy showed contamination in the 29.3–215.3 ppm and 10.6–134.6 ppm range for nylon 6 and nylon 6,6, respectively, and in the 520–1470 ppm range for PET.

## 1. Introduction

Synthetic microfibers (MFs) as a class of ubiquitous microplastics (MPs) polluting marine and freshwater environments are raising increasing global concern. Such MFs mainly originate from textile industry wastewaters, illegal dumping, abandoned fishing gears, and fabrics' release into household and industrial laundering wastewaters (Henry et al., 2019; Napper and Thompson, 2016; Belzagui et al., 2019; Welden and Cowie, 2017). Along with other types of MPs they eventually reach natural water bodies either untreated or, if present, after treatment in wastewater treatment plants (WWTPs). As a matter of fact, a large fraction of the MPs detected in the inflow and outflow of WWTPs was found to consists of textile MFs (Murphy et al., 2016; Long et al., 2019), in addition to polymeric microbeads from personal care products (Kalčíková et al., 2017). While the MPs not retained by the WWTP are released into the water outflow (Mason et al., 2016), the remaining fraction ends up in the WWTP sludge (Liu, et al., 2019; Edo, et al., 2020). MPs removal efficiency up to 99% in the treated wastewaters have been reported (Sun et al., 2019), although such removal rate has been questioned due to the limited accuracy of the detection methods (Okoffo et al., 2019). In any case, since the WWTP sludge polluted by the MPs removed from the wastewaters is often used in agricultural soil amendment after simple biological stabilization, it is likely to significantly contribute to the overall environmental pollution by MPs (Corradini et al., 2019).

Poly(ethylene terephthalate) (PET) polyester and polyamides (PAs, mainly nylon 6 and nylon 6,6) represent the largest fraction of synthetic MFs produced worldwide, roughly in a 10:1 ratio (Textile Exchange, 2019); it is to no surprise, then, that a significant fraction of the MPs in sludges from urban WWTPs was found to consist of PET MFs (Long et al., 2019; Wei et al., 2019; Magni et al., 2019). On the other hand, PAs have often gone undetected, an apparent incongruence suggesting that their detection is simply missed, possibly due to their spectroscopic features similar to those of proteinaceous material. In fact, the analytical protocols for the quantification of MPs in environmental matrices, including wastewaters and WWTPs sludges, is com-

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monly based on the physical separation of the MPs (Fuller and Gautam, 2016; Michielssen et al., 2016; Ziajahromi et al., 2017), followed by their detection by optical microscopy, and their identification by FT-IR (Mintenig et al., 2017; Yang et al., 2019) or Raman (Lares et al., 2019; Schwaferts et al., 2019) micro-spectroscopies. Hyphenated techniques combining thermal (Käppler et al., 2018; Dümichen et al., 2015) or hydrolytic (Zhang et al., 2019) polymer decomposition coupled with mass spectrometry have also been exploited to improve the accuracy of MPs quantification. However, most of the analytical protocols proposed so far involve lengthy operations of physical separation from the environmental matrix and particle counting. Besides, they are likely to result in either overestimation of the actual MPs content because of natural fibers being mistaken for synthetic MFs, or underestimation because of missed detection due to their small size and their further fragmentation into so-called nanoplastics (NPs) (Lomonaco et al., 2020) as a result of mechanical and chemical degradation processes.

While adequate accuracy and sensitivity have been recently achieved for PET through depolymerization and chromatographic separation and quantification of its building block terephthalic acid (TPA) by HPLC with spectroscopic or mass spectrometry detection (Zhang et al., 2019; Corti et al., 2020), quantification of PAs MFs is still a challenge. The procedure presented here is the first one, to the best of our knowledge, allowing the accurate and highly sensitive determination of the total content of nylon 6 and nylon 6,6 PAs in complex environmental matrices such as sediments, soils and sludges. The signature features are the acid-catalyzed hydrolytic depolymerization followed by attachment of a fluorescent tag to the monomers for their quantitative determination by reversed-phase HPLC. A two-step depolymerization protocol including our previous procedure for PET (Castelvetro et al., 2020), has been adopted for the analysis of PAs and PET in the sludge samples from four WWTPs in northern Tuscany, Italy.

## 2. Materials and methods

### 2.1. Chemicals

Dichloromethane (DCM, 99,9%, stabilized with amylene, Romil-SpS), hydrogen peroxide (30% w/v, Panreac), dansyl chloride (96%, Alfa Aesar, Kandel, Germany), and all other reagents and solvents were used as purchased. The 6 N hydrochloric acid (37%, Sigma-Aldrich) and 1.9 N sodium hydroxide (98.0%, Sigma-Aldrich) solutions for the hydrolytic depolymerizations were prepared with HPLC grade water. (Methanol (99.9%), and HPLC grade de-ionized water, sulfuric acid (95–98%), acetic acid (99,85%), hexadecyl-tributyl-phosphonium bromide (TBHDPB, 97%), isopropylamine (99.5%), n-butylamine (99.5%), and a set of amino acids (analytical standards, Table S1 in the Supplementary Material) were all products from Sigma-Aldrich.

### 2.2. Polymeric reference materials

The following materials were used as reference polymers to setup the experimental conditions for their hydrolytic depolymerization: poly(hexamethylene adipate) (nylon 6,6) was a pure product from Sigma-Aldrich; poly(6-aminohexanoic acid) (nylon 6) was obtained from commercial fishing wire and swimsuit, submitted to extraction with DCM to remove any dyes and additives and checked for purity by FTIR (Fig. S1 and S2 in the Supplementary Material, SM); PET was a commercial micronized powder produced by cryogenic grinding of virgin PET (average size 509 μm; a gift from Poliplast S.p.A., Casnigo, BG, Italy).

### 2.3. Sludge samples

Sludge samples were collected from four WWTPs located in the northern coastal area of Tuscany (Italy), as listed in Table 1.

Each sample of approximately 1 kg of wet sludge was submitted to the following pre-treatments within 24 h from sampling: cellulose thimbles (0.8 μm pore size) loaded with three weighed aliquots of about 20 g for each wet sludge sample were extracted in a Soxhlet apparatus for 6 h with 200 mL methanol to remove the excess water and ensure stabilization against biological hazard. The thimbles with the solid residues were vacuum-dried and submitted to a second set of extractions with n-hexane (three subsequent 50 mL aliquots) performed in a sonication bath for 60 min at room temperature, to remove fats and other low MW environmental organic contaminants. The solid residues was finally separated by centrifugation and dried to constant weight in a vacuum. The first polymer extraction step was then carried out in refluxing DCM for 6 h using a Kumagawa apparatus, to obtain a DCM extractable fraction and a solid residue that was vacuum-dried to constant weight. All solvent extracts were collected separately. The following quantitative analyses of MPs are given as weight percent referred to the dry sludge residue from the methanol extraction as the starting sample.

### 2.4. Hydrolytic depolymerization procedures

For the depolymerization of nylon 6 and nylon 6,6 the same acid catalyzed hydrolysis procedure used for natural polypeptides, consisting in a 24 h treatment under refluxing aqueous 6 N HCl, was adopted (Hirs et al., 1954). The exhaustiveness of the hydrolytic depolymerization was preliminarily assessed by running a set of experiments on reference polymeric materials. Thus given amounts (about 100 mg each) of reference polymeric materials in the form of particles or fragments with size in the 0.1–0.5 mm range were heated at 105 °C in an excess (about 50 mL) aqueous 6 N HCl. A set of six identical experiments were run in parallel, each one in triplicate, and the six reactions were stopped at different times within 24 h. Then the hydrolyzate was filtered through a pre-weighed 0.22 μm PVDF membrane (PVDF is chemically resistant even in fuming 37 wt% HCl) and the polymeric residue was determined by weighing the dried filter on a 0.1 mg precision balance. Complete dissolution of the polyamides, suggesting completeness of the depolymerization, was typically achieved after about 16 h for both pure nylon 6,6 and the sample of nylon 6 obtained from the fishing wire. To ensure that the depolymerization would actually proceed to completeness, with conversion of nylon 6 and nylon 6,6 into the corresponding 6-aminohexanoic acid (AHA) and the 1:1 mixture of adipic acid and 1,6-hexamethylenediamine (HMDA), respectively, a 24 h reaction time was adopted in all the subsequent procedures. The absence of oligomeric species after 24 h treatment was further confirmed by HPLC analysis of AHA and HMDA, according to the protocol described in Section 2.5. Actually, a 6.9% solid residue was found even after 24 h depolymerization in the case of nylon 6 sampled from the swimsuit cloth; ATR FTIR analysis (Fig. S3 in SM) showed that such residue consisted of polyurethane, a common elastomeric filament used in stretchable mixed fiber fabrics.

The alkaline hydrolytic depolymerization of PET into its comonomers terephthalic acid (TPA) and ethylene glycol was performed according to the validated procedure reported elsewhere (Castelvetro et al., 2020). Briefly, the sample containing PET is stirred 6 h at 85 °C in a 1.9 N aqueous NaOH solution containing TBHDPB as a phase transfer catalyst. However, since in the complete analytical protocol described later the alkaline depolymerization step follows a previous acid catalyzed PAs depolymerization step, the stability of PET MPs under such acidic conditions was assessed by running a

**Table 1**
Sludge samples and characteristics of the relevant WWTP.

| Sample | Sampling date | Treatment Plant | Equivalent population | Wastewater input ($m^3$/month) | Sludge output (ton/month) | Industrial activities |
|---|---|---|---|---|---|---|
| LID-2 | 2019–2–02 | LID | 38,000 | 181,400 | 95 | Biomass-to-power plant, Gas station, Industrial laundry |
| QUE-2 | 2019–2–02 | QUE | 39,000 | 209,400 | 171 | Marble works, Gas & Carwash station |
| QUE-5 | 2019–5–22 | | | | | |
| FOS-5 | 2019–5–22 | FOSSA | 45,000 | 258,200 | 157 | Gas & Carwash station |
| LAV-5 | 2019–5–22 | LAV | 120,000 | 823,000 | 257 | Landfill, Marble works, Oil factory, Carwash, Plastics processing factory |
| LAV-5d [a] | 2019–5–22 | | | | | |
| LAV-5h [b] | 2019–5–22 | | | | | |
| LAV-1 | 2020–1–23 | | | | | |

[a] Sludge stabilized with lime.
[b] Sludge containing a polymeric flocculant additive.

polymer recovery experiment. Thus the micronized PET reference material was treated in 6 M HCl at 105 °C for 24 h; polymer recovery higher than 99.9% was obtained upon filtration on a 0.22 μm PVDF membrane.

### 2.5. Analytical techniques

Optical microscopy observations of some of the larger MPs was carried out under visible and UV (Olympus U-RFL-T Mercury lamp) illumination using an OLYMPUS BX51M microscope equipped with an OLYMPUS XC50 high-resolution camera. Attenuated Total Reflectance infrared (ATR FTIR) spectra were recorded with a Perkin Elmer Spectrum GX spectrometer equipped with a MIRacle TM ATR accessory (angle of incidence 45°) and a Ge crystal. The spectra were recorded in the 650–4000 $cm^{-1}$ range at a 4 $cm^{-1}$ resolution.

Two instrumental setups were used for the High Performance Liquid Chromatography (HPLC) analyses. Quantitative determination of TPA was performed using a Jasco PU-1580 isocratic pump connected with a Jones-Genesis Aq 120 reversed-phase column (150 mm × 4.6 mm, 4 μm particle size) operating at room temperature and a Jasco 1575 UV–Vis detector set at 240 nm wavelength. Analyses were carried out on 20 μL sample solutions at 0.8 mL/min flow rate of an isocratic eluent mixture of 40/60 vol/vol methanol/HPLC-grade water (acidulated with 1 wt% acetic acid). For the analyses of the DNSCl derivatives of AHA and HMDA, an Agilent 1260 Infinity Binary LC instrument equipped with a pre-column, a reversed-phase Phenomenex-Aqua C18 column (250 mm × 4.6 mm, 5 μm particle size), and in series UV-Vis diode array (DAD VL+ 1260/G1315C) and fluorescence (FLD 1260/G1321B) detectors was used. Elution was performed at 1.0 mL/min flow rate both in isocratic mode, using a 60/38/1.5/0.5 vol% acetonitrile/water/acetic/triethylamine mixture, and in gradient mode. For the latter, a 2.5% acetic acid and 0.83% trimethylamine aqueous solution (phase A) was mixed with acetonitrile (phase B) according to the program reported in Table S2. The UV-DAD detector was set at $\lambda = 335$ nm, while the FLD detector was set at $\lambda = 335/522$ nm excitation/emission wavelengths.

### 2.6. General procedure for the quantitative determination of PAs and PET MFs

The total content of nylon 6, nylon 6,6, and PET contaminating MFs in the sludge samples was calculated from the content of the corresponding monomers, 6-aminocaproic acid (AHA), hexamethylenediamine (HMDA), and terephthalic acid (TPA), respectively, in the hydrolyzates obtained from the acid or alkaline depolymerization as previously described. In brief, 0.5–2.0 g of the dry solid residue from the DCM extraction of each sludge sample was transferred into a 100 mL round-bottomed flask equipped with a reflux condenser and magnetic stirring bar. After addition of 40 mL 6 N HCl the stirred mixture was heated to the reflux temperature of about at 105 °C for 24 h. At the end of the hydrolysis, the reaction mixture was vacuum-filtered on a 0.22 μm PVDF membrane to separate the solid residue from the acid solution. The filter membrane with the solid residue was carefully rinsed with small amounts of HPLC grade water for the subsequent treatments, while the acid solution was transferred in a 100 mL volumetric flask and taken to volume with 6 N HCl. A given volume (7–10 mL) of the obtained solution was weighed and taken to 6.5–7.5 pH with 5 N NaOH, then the final volume and weight of the obtained neutralized solution was recorded for the final calculation of monomer concentration. To enable a highly sensitive quantification of the amino monomers AHA and HMDA, their solutions were treated with 5-dimethylaminonaphtalene-1-sulfonyl chloride (dansyl chloride, DNSCl), a derivatizing fluorophore commonly used in protein sequencing (Fig. 1).

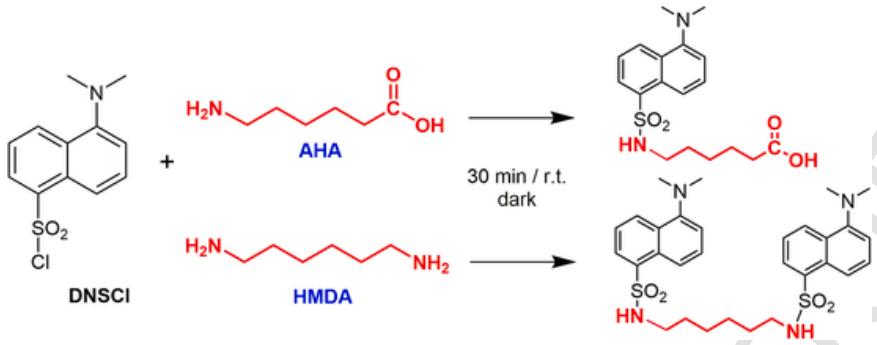


**Fig. 1.** Dansylation of the amino-monomers from the depolymerization of nylon 6 and nylon 6,6.

For this purpose, 0.1–0–5 mL of the neutralized product of acid hydrolysis was loaded in a 5 mL glass vial, followed by sequential addition of 1.0 mL aqueous $K_2CO_3$ solution (16.0 g/L) and 1.0 mL of a 5 g/L solution of DNSCl in acetone (18.5 μmol). After 30 min stirring at room temperature in the dark, an excess of n-butylamine (5.0 μL, 51·μmol) was added to quantitatively convert the unreacted DNSCl (Lau-Cam and Roos, 1993). The derivatized solution was then transferred into a 10 mL volumetric flask and taken to volume with a 1:1 (v/v) water/acetone mixture before HPLC analysis (see below). A set of amino acids (see Table S1 in SM), selected among those more likely to behave as interferents with the dansylated AHA and HMDA in the subsequent chromatographic analyses, were also dansylated and the prepared solutions (8.9–11.8 mmoL/L) were used to optimize the resolution of the chromatographic method. For best method accuracy, the solutions of amino acids were prepared in 5 M NaCl, a similar saline content as that of the neutralized acid hydrolyzates. Isopropylamine was used as an internal standard in the subsequent chromatographic analyses, its derivatization efficiency and quantitative detection having been assessed (similarly to other primary low molecular weight amines) as practically quantitative.

For the determination of the PET content, the solid residues collected at the end of the acid hydrolysis were treated under alkaline hydrolytic conditions to achieve the complete PET depolymerization as described in Section 2.4. According to the general procedure, the residue was transferred into a 100 mL round-bottomed flask equipped with reflux condenser and magnetic stirring bar, added with 40 mL of aqueous 1.9 N NaOH and TBHDPB as a phase transfer catalyst, then the mixture was heated under stirring at 85 °C for 6 h. The final solution was vacuum-filtered on a 0.22 μm PVDF membrane to remove any insoluble impurities, then transferred into a 50 mL volumetric flask and taken to volume with 1.9 N NaOH to prevent partial precipitation of the free acid TPA, much less water soluble than its disodium salt, sodium terephthalate. The obtained alkaline hydrolyzate was then further diluted with 1.9 N NaOH in a 1:600 vol ratio to lower the total organic matter concentration (residual biogenic fraction not removed from the sludge by the previous treatments) while maintaining sufficient sensitivity for the UV detection of TPA in the HPLC analysis. The actual dilution ratio required to obtain reproducible results was based on the evaluation of preliminary HPLC runs aimed at selecting the best compromise between the desired minimal reduction of sensitivity in the analyte quantification, and the required reproducibility of the analyses. In fact, the latter was found to be affected by column overloading by the above biogenic contaminants, causing peak broadening and peak shift and thus poorer chromatographic separation. Clearly, matrices with significantly different biogenic (in particular proteinaceous) content, such as e.g. marine sediments, soils, and wastewater sludges, may require a dedicated preliminary assessment to select the most appropriate dilution ratio, which will also affect the overall sensitivity of the method.

The amount of contaminating polymers in each sludge sample (given in ppm, or mg polymer/kg dry sludge) was calculated from the corresponding monomer concentrations $C_{AHA}$, $C_{HMDA}$, and $C_{TPA}$ (in ppm) determined by HPLC, based on the calibrated response of both UV and fluorescence detectors (see Figs. S4–S6 in SM), according to Eqs. (1)–(3):

$$\text{Nylon 6 (ppm)} = C_{AHA} \cdot \frac{MW_{PA6}}{MW_{AHA}} \quad (1)$$

$$\text{Nylon 6,6 (ppm)} = C_{HMDA} \cdot \frac{MW_{PA6,6}}{MW_{HMDA}} \quad (2)$$

$$\text{PET(ppm)} = C_{TPA} \cdot \frac{MW_{PET}}{MW_{TPA}} \quad (3)$$

where $MW_{PA6}$ = 113.16 g/mol, $MW_{PA6,6}$ = 226.32, and $MW_{PET}$ = 192.2 are the molecular weights of the repeating units in the corresponding polymers (Fig. S7 in SM), and $MW_{AHA}$ = 131.17 g/mol, $MW_{HMDA}$ = 116.21 g/mol, and $MW_{TPA}$ = 166.13 g/mol are those of the analytes.

### *2.7. Quantitative determination of AHA and HMDA in the acid hydrolyzates from reference nylon 6 and nylon 6,6 and from spiked sludge samples*

The sensitivity and accuracy of the quantitative determinations via HPLC was assessed for both UV and FLD detectors.

For the UV detector calibration 5 solutions of dansylated AHA in the 0.45–22.5·mg/L concentration range and 5 solutions of dansylated HMDA in the 0.642–5.13 mg/L concentration range, in each case with an additional blank sample (prepared by running the same dansylation reaction as in the solutions but without the aminated monomer) were analysed. The HPLC runs were performed in isocratic mode using a 60/38/1.5/0.5 vol% acetonitrile/water/acetic acid/triethylamine mixture as the eluent. Linear fits were obtained for both the dansylated AHA (Peak area = $1.22{\cdot}10^8{\cdot}C_{AHA} + 3828$, regression coefficient $r^2 > 0.999$) and the dansylated HMDA (Peak area = $2.91{\cdot}10^8{\cdot}C_{HMDA}$-2683, regression coefficient $r^2 > 0.999$), as shown in Fig. S4 of SM.

HPLC analysis of mixtures of AHA and HMDA with a set of amino acids and aliphatic amines selected among those structurally more similar to the two monomers, and thus more likely to co-elute in the HPLC runs, were also carried out after bulk dansylation to optimize the chromatographic conditions for peak resolution.

HPLC analyses were performed under gradient elution (see Section 2.5) for optimal peak resolution and shorter chromatographic runs (see Table S3 in SM). Thus solutions containing mixtures of the dansylated derivatives of AHA, HMDA, isopropylamine (iPA, used as internal standard), and the interfering amino acid Phe in the concentration range from 0.284 to 8.67 g/L in 5 M NaCl plus a blank sample were analyzed by FLD-HPLC for improved sensitivity (Biver et al., 2018). The ob-

tained linear fits of the 5-point measurements for each analyte (Figs. 2 and S5 in SM), were then used as calibration curves for the subsequent analyses of sludge samples.

Under the adopted FLD-HPLC conditions, the following values for the Limit of Detection (LOD) and the Limit of Quantification (LOQ) were calculated by dividing the concentration, or calibrated peak area, by the signal-to-noise ratio and multiplying by a factor 3 for LOD and 10 for LOQ (the blank samples gave no detectable signal): LOD = 0.885 μg/L and LOQ = 3.73 μg/L for the dansylated AHA; LOD = 0.212 μg/L and LOQ = 0.704 μg/L for the bis-dansylated HMDA (as expected, a higher sensitivity was found for the bis-dansylated HMDA).

The improvement in chromatographic resolution and signal to noise ratio resulting from the combination of gradient elution and FLD detection as compared to the use of isocratic elution and UV detection is apparent by inspection of Fig. S6 and Table S4 in the SM.

### 2.8. Quantitative determination of PET in spiked environmental samples

The total mass of PET micro- and nano- particles in the residues from the acid hydrolysis was determined according to the general procedure already described by some of the present authors for freshwater and marine sediments (Castelvetro et al., 2020). Briefly, the method is based on the alkaline depolymerization in the presence of phase transfer catalyst as described in Section 2.4, followed by reversed-phase HPLC analysis of the alkaline hydrolyzate that has previously undergone several purification steps. In particular, a first oxidation step of the alkaline hydrolyzate is carried out by using 30% $H_2O_2$ to remove the excess of organic contaminants. The solution is then acidified with

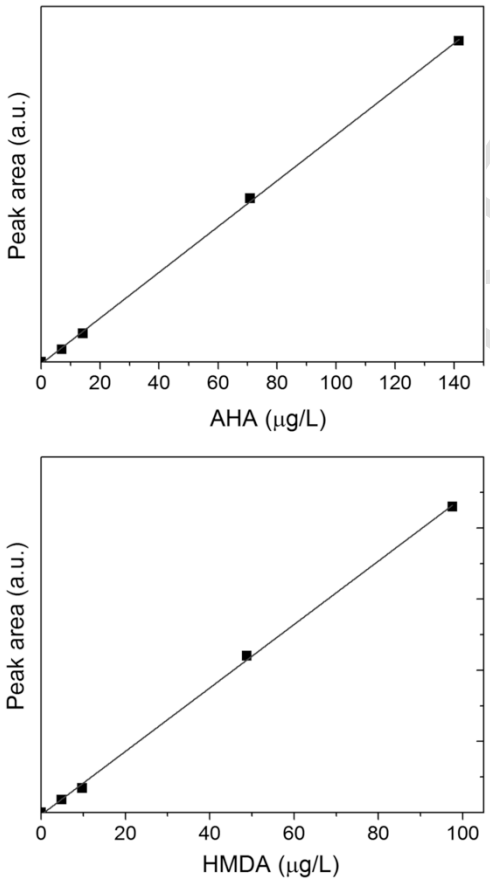


**Fig. 2.** HPLC calibration curves for dansylated AHA and HMDA (gradient elution) based on fluorescence detection. Linear fit parameters: Peak $Area_{AHA} = 9.66 \cdot 10^{-3} \cdot C_{AHA} - 8.06 \cdot 10^{-3}$ ($r^2 = 0.9996$); Peak $Area_{HMDA} = 4.46 \cdot 10^{-2} \cdot C_{HMDA} - 3.29 \cdot 10^{-2}$ ($r^2 = 0.9992$).

1.9 M $H_2SO_4$ and further purified and pre-concentrated by elution through a reversed-phase SPE cartridge (Macherey-Nagel Chromabond® C18ec, loaded with 500 mg stationary phase), followed by desorption of the TPA adsorbate with 0.8 mL MeOH. The recovered roughly 0.8 mL methanol solution is then weighed at 0.1 mg accuracy, and 0.5 mL of the is taken up with a micropipette, placed in a vial, weighed again at 0.1 mg accuracy, then added with 0.75 mL aqueous acetic acid (1 wt% in HPLC-grade water) to obtain a 40/60 vol% methanol/water mixture that is finally analyzed by reversed-phase HPLC with UV detection.

Quantification of TPA in the purified hydrolyzate was based on a calibration curve obtained by running four experiments in which 1 mL each of alkaline standard TPA solutions in 2 N NaOH at four different concentrations in the 0.21–1.7 mg/L range, plus a blank solution, were submitted to the above described purification procedure, and then analyzed by reversed-phase HPLC. A linear fit of the UV detector response vs TPA concentration ($C_{TPA}$) was obtained (fitting function: $Peak\ Area = 2.32 \cdot 10^{8} \cdot C_{TPA} - 7236$; regression coefficient $r^2 > 0.995$). As described in Section 2.4, recovery of PET in the residue from the acid hydrolysis step is quantitative; besides, TPA recovery from a sludge sample spiked with PET and submitted to alkaline hydrolysis and the subsequent purification steps was about 95% (see Section 2.9). Therefore, by considering the sensitivity of the HPLC method as in the case of AHA and HMDA, the values of LOD = 15.3 μg/L and LOQ = 51.1 μg/L were obtained for the quantification of PET under the adopted chromatographic conditions.

### 2.9. QA/QC

The effectiveness of the depolymerization of the reference PAs under the adopted acid hydrolysis conditions was assessed by measuring AHA and HMDA recovery. Conversions to monomer were found to be 96 ± 1.2% for pure nylon 6 and 98 ± 0.5% for pure nylon 6,6 based on the calibrations obtained under isocratic elution with UV detector (Fig. S4). Even higher monomer recovery rates of 98.0 ± 1.1% and 100.0 ± 0.8% for AHA and HMDA were actually determined when performing the same test on a sludge sample spiked with the reference nylon 6 and nylon 6,6, respectively, but using the high sensitivity FLD detector and FLD-HPLC based calibrations. For the latter test a sludge sample, previously submitted to the usual sequential extraction with methanol, hexane, and DCM, was divided in four portions of about 1.0 g each, then three of them were spiked with 5.0–7.0 mg nylon 6 and 13.0–15.0 mg nylon 6,6 (the range is related to the spiking experiments being performed in triplicates) with the fourth as a blank. All samples were submitted to the acid hydrolysis, neutralized with aqueous NaOH to about pH 6.5, and then submitted to DNSCl derivatization according to the previously described procedures.

Similarly, TPA recovery from PET hydrolytic depolymerization was validated in spiked environmental samples. Thus the DCM extraction residue from a sludge sample was split in four 10 g fractions and three of them were spiked with 9–19 mg PET micro-powder. The four samples were then individually subjected to acid hydrolysis according to the full procedure including the depolymerization of PAs contaminants, then the residues recovered by filtration were subjected to the basic hydrolysis for PET depolymerization and to the subsequent procedures for the quantitative determination of the TPA through UV-HPLC, as already described. The average TPA recovery over the three spiking experiments was 95.5 ± 0.7%.

Throughout the analytical protocol, from the initial extraction of the sludges with methanol to the final HPLC analyses, maximum care was taken to avoid contamination from airborne MFs and other particulate. Specifically, all vessels and containers were kept either covered with aluminum foil or closed with a glass or rubber stopper; all manipulations were performed by people wearing 100% cotton lab coats,

whenever possible in fume hoods located in a lab with fixtures ensuring 6 total filtered air changes per hour.

## 3. Results and discussion

The accurate quantification of MPs in complex environmental matrices such as sediments of natural water bodies, soil, and sludges from WWTPs is quite challenging, and generally approached with a combination of density separation (flotation) procedures (Xu et al., 2020) and spectroscopic or micro-spectroscopic analytical techniques that, with few notable exceptions (e.g. Zhang et al., 2019, sensitive determination but limited to PET and polycarbonate), do not ensure exhaustiveness, accuracy and/or sensitivity, particularly for less frequently found and polymers such as e.g. polyamides (Meixner et al., 2020; Zhou et al., 2020; Li et al., 2020). On the other hand, the widespread use of WWTP sludges in agricultural soil amendment suggests that not-biodegradable or poorly biodegradable material present in such sludges may accumulate over time in the treated soils, posing a potential hazard for the anthropized ecosystem and eventually for human health.

The procedure presented here allows the accurate determination of the total content of poly(ethylene terephthalate) (PET) polyester and of the two most common polyamides (PAs) nylon 6, and nylon 6,6, MPs in complex matrices, as exemplified by the case study concerning a set of sludge samples from wastewater treatment plants (WWTPs) located in a urbanized area in north-west Tuscany, Italy.

To the best of our knowledge, this is the first report of an analytical procedure for the quantitative determination of the total content of PAs as microplastics (mainly MFs) in environmental samples.

WWTP sludges were chosen as exemplifying environmental samples because they combine the complexity of a matrix containing both inorganic and organic material, inclusive of biogenic low MW compounds and biopolymers, and a high probability to contain comparatively high concentrations of contaminating MFs compared to other environmental matrices. Indeed, WWTP sludges consist of the solids partly captured by, and partly generated during WWTPs treatment of a mix of urban and industrial wastewaters; they were thus expected to contain a sizable amount of MFs released upon textile laundering among other sources (Liu, et al., 2019; Edo, et al., 2020; Wong et al., 2020). Since the title PAs are commonly used as staple microfibers in textile yarn fabrication, they were also expected to be present in the WWTPs sludges, along with the nearly ubiquitous and much more abundant polyester MFs.

As reported in the flowchart of Fig. 3, the overall procedure for quantification of both PAs and PET MFs comprises:

- a pre-treatment stage based sequential extraction with organic solvents;
- two subsequent hydrolytic depolymerization steps, the first one under acid catalysis to depolymerize selectively all synthetic and biogenic polyamides, and the second one under alkaline catalysis to depolymerize PET;
- two dedicated purification/derivatization treatments for the two hydrolyzates, followed by HPLC separation and quantitative determination of the dansylated AHA and HMDA from the PAs and of terephthalic acid (TPA) from PET.

The acid-catalyzed hydrolytic depolymerization of the PAs is performed in the first step on WWTPs sludges previously extracted sequentially with methanol, hexane, and DCM. The subsequent HPLC quantification of the resulting AHA and HMDA amino building blocks requires a previous derivatization with a fluorophore. Indeed quantification of PAs presents several challenges: the first one is the depolymerization

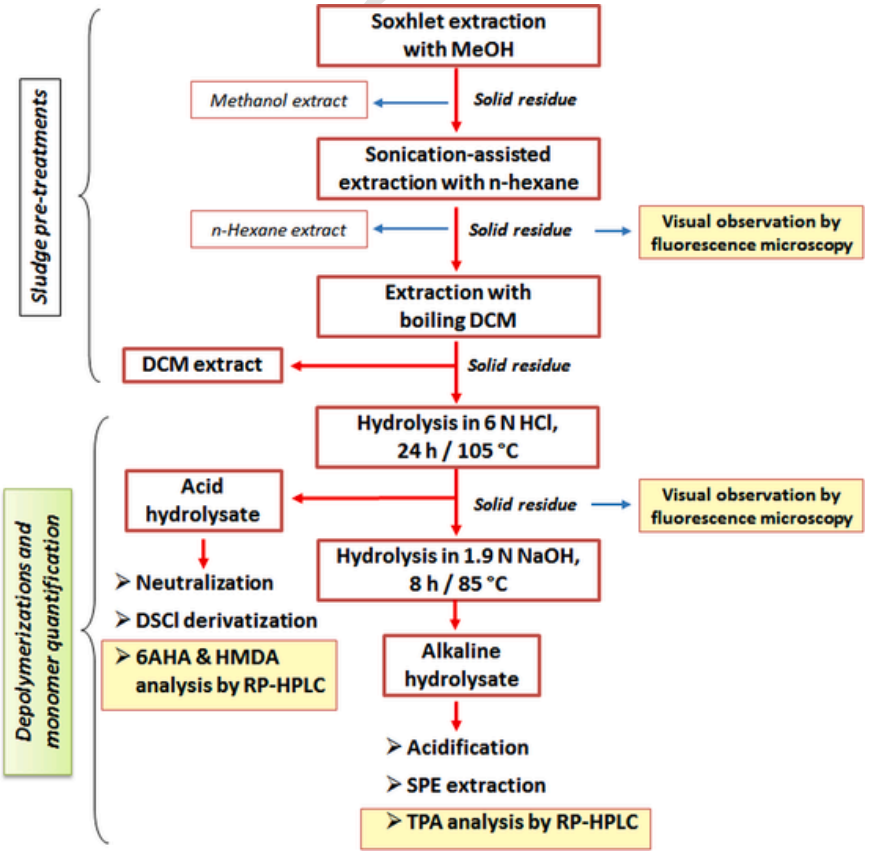


**Fig. 3.** Analytical protocol for the quantification of nylon 6 (from AHA), nylon 6,6 (from HMDA), and PET (from TPA) as contaminating MFs in sludge samples.

stage, that cannot be selective for PAs but will necessarily involve also naturally occurring polyamides, namely the polypeptide chains of proteins, and will thus require an efficient chromatographic separation of the aliphatic amines AHA and HMDA from the natural amino acids; the second one is the sensitivity of the detection, that cannot rely on the presence of UV absorbing moieties. The newly devised procedure is actually an adaptation of a procedure well established in proteomics for determination of total amino acids composition and sequencing of proteins (Walker, 1986). The procedure is based on the derivatization of all primary amines (amino acids as well as other biogenic amines) with a chromophore/fluorophore, and in the subsequent separation and quantification by HPLC. By using dansyl chloride (DNSCl) as the derivatizing agent, detection limits of the order of a few ppm and even down to the ppb level can be achieved.

Dansylation of AHA and HMDA was performed by adapting a previously proposed procedure for the accurate quantification of AHA in pharmaceutical preparations (Lau-Cam and Roos, 1993); other reported functionalization procedures turned out to be either not as efficient (low conversions) or too lengthy in the case of complex matrices such as the WWTP sludges. Under the adopted hydrolytic depolymerization and derivatization conditions most of the proteins not removed from the sample by the previous solvent extraction procedures will result in the presence of dansylated amino acids potentially interfering with the reversed-phase HPLC separation and quantification of AHA and HMDA. Therefore, an efficient HPLC separation by gradient solvent elution is required to avoid overlapping between the peaks from the two PAs and those from natural amino acids, and to shorten the elution time for the bis-dansylated HMDA, while FLD detection ensures maximum sensitivity.

The base-catalyzed hydrolytic depolymerization of PET and its quantification through HPLC analysis of the TPA co-monomer is performed according to a procedure previously developed for PET in marine and lake sediments (Corti et al., 2020), as summarized in Section 2.8.

Individual polymer concentrations are then calculated from the measured concentration of each parent monomer, based on the assumption that the latter would only originate from the corresponding homopolymer (this is the case for AHA from nylon 6) or binary copolymer (HMDA from nylon 6,6 and TPA from PET). This means that the possible presence of parent ter-polymers such as e.g. in amorphized PET, obtained industrially by introducing small amounts of a different diol (e.g. 1,4-cyclohexanediol or 1,4-butanediol) or diacid (e.g. orthophthalic acid or naphthalene dicarboxylic acid) would lead to a moderate underestimation of the actual polymer content when converting the detected monomer mass into the parent copolymer mass.

Once the overall procedure had been validated for repeatability and consistency, it was applied on the set of eight sludge samples from four different WWTPs located in north-western Tuscany, Italy, as listed in Table 1.

The as-received wet sludges were preliminarily extracted with methanol to remove the excess water and ensure stabilization against biological hazard, then with n-hexane to remove fats and other low MW environmental organic contaminants (e.g. phthalates, natural aliphatic amines, etc.) which could have interfered with the subsequent HPLC analyses of the depolymerization products TPA, AHA, and HMDA. The extracted solid residues from the methanol extraction were vacuum dried until constant weight (Table 2) before the second extraction step, and the dry weight was considered in the subsequent calculation of the content of contaminating polymers in the sludges.

Examination of the hexane extraction residues by optical microscopy revealed a massive presence of synthetic fibers and polymeric fragments (see representative examples from LID-2 in Fig. 4a–f).

In the last extraction step with DCM most residual low MW organic compounds, both natural and synthetic, are co-extracted with DCM-soluble MPs, that is, those consisting of vinyl polymers (polystyrene, most acrylics and vinyl esters, PVC, etc.) and highly degraded (oxidized and oligomeric) polyolefins (Ceccarini et al., 2018). From the DCM extracts a modest overall 0.3–0.6 wt% solids were obtained, which were not further analyzed for the actual MPs content since the main object of this study was the evaluation of the contamination by PAs and PET that are insoluble the above solvents.

### 3.1. Quantification of nylon 6 and nylon 6,6

The solvent-extracted solid residues of the sludge samples were submitted to acid hydrolysis in boiling HCl 6 M as detailed in the Section 2.4. After vacuum filtration of the acid hydrolyzate solution, the weight loss determined as the difference from the initial (methanol extracted) dry weight of the sample and the dry weight of the solid residues was recorded (Table 2) for a first estimation of the maximum content of aminated molecules (including amino acids and monomers from PAs) in the hydrolyzate solution. The latter was taken into account for ensuring that the subsequent derivatization reaction be run in the presence of an excess DNSCl, and for selecting a suitable dilution of the hydrolyzate to preserve the HPLC column from overloading that may cause retention time shifts and peak broadening. Dilution results in reduction of the method sensitivity, but it may be necessary for complex matrices such as WWTP sludges.

The concentration of soluble matter in the acid hydrolyzates from the sludge samples, including the inorganic fraction solubilized by the

**Table 2**
Analytical data concerning (i) the starting amount of dried sludges after methanol extraction, (ii) the partial solubilization upon acid hydrolysis, and (iii) the concentrations of the PAs in the dried sludges as calculated from the measured concentrations of AHA and HMDA.

| Sample | Sludge preparation | | | Acid hydrolysis | | | AHA (mg/kg) | HMDA (mg/kg) | Nylon 6 (ppm) | Nylon 6,6 (ppm) |
|---|---|---|---|---|---|---|---|---|---|---|
| | Wet sludge (g) | MeOH Residue (g) | Dry residue (%) | Sample weight (g) | Final residue (g) | Weight loss (%) | | | | |
| LID-2 | 16.828 | 2.424 | 14.4 | 1.65 | 1.09 | 33.9 | 244.6 | 14.4 | 211.0 | 28.1 |
| QUE-2 | 18.502 | 2.195 | 11.7 | 1.51 | 0.97 | 35.8 | 77.7 | 18.6 | 67.0 | 36.3 |
| QUE-5 | 25.932 | 4.666 | 18.3 | 2.36 | 1.56 | 34.1 | 33.9 | 5.4 | 29.3 | 10.6 |
| FOSSA-5 | 22.828 | 3.378 | 14.8 | 1.98 | 1.32 | 33.2 | 40.2 | 69.1 | 34.7 | 134.6 |
| LAV-5 | 11.547 | 1.732 | 15.0 | 1.44 | 0.93 | 35.1 | 249.5 | 66.0 | 215.3 | 128.5 |
| LAV-5d | 13.615 | 1.947 | 14.3 | 1.37 | 0.89 | 35.4 | 127.5 | 29.4 | 110.0 | 57.3 |
| LAV-5h | 12.565 | 1.935 | 15.4 | 1.33 | 0.89 | 32.8 | 158.2 | 26.6 | 136.5 | 51.7 |
| LAV-1 | 10.473 | 1.550 | 14.8 | 1.41 | 0.34 | 75.9 | n.d. | 42.5 | n.d. | 82.8 |

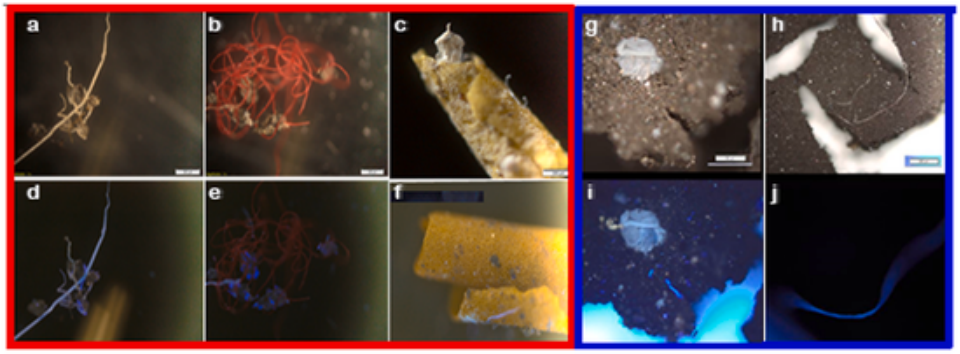


**Fig. 4.** Optical micrographs of fibers and fragments taken in reflected light (above, micrographs a–c and g–h) and fluorescence mode (below, micrographs d–f and i–j). The red-framed micrographs on the left (a–f) show fibers and fragments in the residue from sample LID-2 after hexane extraction; the blue-framed micrographs on the right (g–j) show fibers and fragments still present in the residue after the acid hydrolysis step for sample LAV-1 (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

acid attack, ranged from 4.4 to 8.0 mg/mL. After neutralization with 2 N aqueous NaOH the solutions were diluted in a 1:100 ratio with HPLC grade water and then reacted with a fivefold DNSCl excess with respect to the maximum estimated content of aminated compounds. The presence of the excess DNSCl at the end of the reaction was confirmed by the rapid discoloration of the solutions upon addition of a stoichiometric excess n-butylamine that nearly instantaneously converts all residual DNSCl into the corresponding N-butyl derivative. The nylon 6 and nylon 6,6 contents in the sludge samples, determined by FLD-HPLC analysis performed as described in the Section 2.7, are listed in Table 2.

All samples were found to contain relatively high amounts of the two polyamides, in the 29–215 ppm range for nylon 6 and in the 10–134 range for nylon 6,6 vs. dry sludge, the larger relative amount of nylon 6 being consistent with the larger global market share of nylon 6 fibers (Wesołowski and Płachta, 2016). Given the high accuracy and precision of polymer quantification based on monomer recovery in the triplicate spiking experiments (see Section 2.9), the variability in the AHA and HMDA concentrations measured for the various samples can be ascribed to the intrinsic compositional variability of the different sludges samples.

### 3.2. Quantification of PET

Microfibers and plastic fragments were still detectable in the solid residues from the acid hydrolysis, as shown in Fig. 4g–j. The residues were then submitted to alkaline hydrolysis in NaOH 1.9 M as described in the Section 2.4. After vacuum filtration of the obtained alkaline solution, the dry weight of the solid residues was recorded for an estimation of the amount of hydrolysable material (Table 3).

**Table 3**
PET concentration in the dried sludges calculated from the measured TPA concentration after alkaline depolymerization.

| Sample | Acid hydrolysis Residue (g) | Weight loss on alkaline hydrolysis (%) | TPA (ppm) | PET (ppm) |
|---|---|---|---|---|
| LID-2 | 0.64 | 41.3 | 1118 | 1293 |
| QUE-2 | 0.61 | 37.1 | 1012 | 1171 |
| QUE-5 | 0.96 | 38.3 | 449 | 520 |
| FOSSA-5 | 0.79 | 40.5 | 1271 | 1470 |
| LAV-5 | 0.57 | 38.4 | 837 | 968 |
| LAV-5d | 0.54 | 39.9 | 750 | 868 |
| LAV-5h | 0.52 | 41.1 | 788 | 912 |

As in the case of the analysis of PAs, the amount of hydrolysable material was taken into account for selecting, if appropriate, the dilution ratio aimed at preventing overloading of the HPLC column. Preliminary measurements indicated as appropriate a 1:600 dilution in NaOH 1.9 M, based on the starting concentrations of 3.5–6 mg/mL of soluble matter for the different samples. The results of the HPLC analyses with UV detection are reported in Table 3.

## 4. Conclusions

The described analytical protocol allows the quantification of the total mass of each one of the three most common textile MFs that are likely to represent a significant source of micro- and nano-plastics polluting natural water bodies, namely PET, nylon 6 and nylon 6,6. The overall procedure includes the first description of a method allowing quantification of the total mass of each one of the two above PAs in complex matrices with high selectivity, accuracy and sensitivity (from the ppm down to the ppb range, depending on the required dilutions during the purification steps) as compared to the conventional quantification by mechanical separation by flotation and identification by micro-spectroscopies. Since the new procedure for PAs was developed and tested only in the present case study of WWTP sludges, it should be considered as a proof of concept requiring further validation with a broader range of environmental matrices.

Approximately a tenfold concentration of PET with respect to the polyamides was detected in the sludge samples, a result that might not incidentally be in agreement with the global production (and consumption) of PET and polyamides textile fibers, representing approximately 50% and 5%, respectively, of about 90 Mton/year in 2015 (Textile Exchange, 2019).

The high sensitivity and high resolution of the HPLC analysis, particularly in the case of the polyamide monomers with fluorescent tags, allows easy extension of the protocol to a broad range of other environmental matrices spanning from agricultural soil to mineral waters, with highly variable expected contamination levels. The much more accurate evaluation of the extent of pollution by PAs MFs achieved with the new analytical procedure will also allow to better understand the actual impact of PAs MPs and NPs polluting natural water bodies, which may originate not only from textiles but possibly also from degraded orphan fishing lines, fishing nets and other PA-based fishing gears.

## CRediT authorship contribution statement

**Valter Castelvetro:** Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Validation, Writing - review & editing. **Andrea Corti:** Conceptualization, Investigation, Methodology, Data curation, Validation, Writing - original draft. **Alessio Cecca-**

**rini:** Resources. **Antonella Petri:** Methodology, Resources. **Virginia Vinciguerra:** Investigation, Data curation, 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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## Author contributions

Valter Castelvetro: Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Validation, Writing - review & editing. Andrea Corti: Conceptualization, Investigation, Methodology, Data curation, Validation, Writing - original draft. Alessio Ceccarini: Resources. Antonella Petri: Methodology, Resources. Virginia Vinciguerra: Investigation, Data curation, Writing - original draft.

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2020.124364.

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