

Article

Fast Forward: Optimized Sample Preparation and Fluorescent Staining for Microplastic Detection

Michael Toni Sturm¹, Erika Myers¹, Anika Korzin^{1,2}, Sabrina Polierer^{1,2}, Dennis Schober^{1,2} and Katrin Schuhen^{1,*} 

¹ Wasser 3.0 GmbH, Neufeldstr. 17a-19a, 76187 Karlsruhe, Germany; myers@wasserdreinull.de (E.M.)

² abcr GmbH, Im Schlebert 10, 76187 Karlsruhe, Germany

* Correspondence: schuhen@wasserdreinull.de; Tel.: +49-721-1565-9593

Abstract: The fast, affordable, and standardized detection of microplastics (MP) remains one of the biggest challenges in MP research. Comparable data are essential for appropriate risk assessments and the implementation of laws and limit values. The fluorescent staining of MP in environmental samples is a possible solution to this problem. This study investigates the optimization of a sample preparation process (hydrogen peroxide digestion) and the staining process (temperature, concentration, time, surfactants as staining aids) for using a specifically developed fluorescent dye for MP detection. The optimization is performed by comparing the sample preparation process and staining of MP from different polymers and natural particles. Further, the suitability of the optimized process for the detection of fluoropolymers and tire abrasion was tested. The results show that the optimized method (increased temperature and optimized stain concentration) can detect microplastics reliably with a total sample preparation and measurement time of 2.5–3 h per sample, reaching recovery rates of 93.3% (polypropylene) to 101.7% (polyester). Moreover, two of the three tested fluoropolymers could be detected reliably. Tire abrasion could not be detected with the here presented method, as the black color leads to strong quenching. A long-term study measuring the MP pollution in the effluent of a municipal wastewater treatment plant compared the optimized and original processes and confirmed the stability of the improved method for routine measurements and contamination control.



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Keywords: microplastics; microplastic detection; fluorescent dye; Nile red; fluoropolymer; tire abrasion; fluorescent staining; process control; sustainable process design

1. Introduction

Microplastics (MP), plastic particles of a size smaller than 5 mm, are ubiquitous in the environment, from oceans, rivers, lakes, and soils, to the air we breathe and the food we eat [1,2]. They originate from various sources, such as the fragmentation of larger plastic debris, the shedding of synthetic fibers from textiles, tire abrasion, pellet loss, or microbeads from personal care products [3]. MP can pose risks to both wildlife and human health, as they interact with organisms in various ways and can interfere with physiological processes or carry and release harmful chemicals [4]. Thus, understanding the sources, fate, and effects of MP is critical for reliable risk assessments and the implementation of effective management strategies, and requires interdisciplinary and global collaboration.

To understand their sources and pathways, extensive and comparable data on MP pollution and emissions are necessary. This requires a comparable, precise, and affordable MP detection method, which remains one of the biggest challenges in MP research [5,6]. Separating and identifying MP in environmental samples, as well as in foods and beverages, is difficult due to their small size, varying morphology, and chemical diversity. Distinguishing MP from natural particles or other anthropogenic particles with similar appearances, properties, and chemical compositions is particularly challenging.

Thus, various methods for sampling, sample preparation, and detection are applied, and the resulting data are hardly comparable [5–7]. Many of the most commonly used

methods, such as Raman microscopy, FTIR microscopy, and Pyrolysis GC-MS, are technically challenging, time consuming, and cost intensive, as these measurement devices are expensive and trained staff are needed to operate them [5–7]. Further, extensive sample preparation, such as hydrogen peroxide digestion or density separation, is necessary to reduce the number of natural particles in the sample for more reliable MP identification. These sample preparation methods are not standardized, and add additional processing time, complexity, and variability to the process. Potential methods for continuous MP detection are also being investigated in many research projects, but there is currently no applicable method [8–11].

As a standardized method for MP detection is the basis for the legislation and control of the limit values for MP emissions and pollution, the current lack of standardization and the high effort and costs connected with MP detection pose challenges for decision makers and stakeholders (Figure 1) [5]. Obtaining data on MP pollution is particularly difficult from industries, as they are often not willing to release their MP pollution status. In countries with little financial resources for research and environmental projects, there is almost no knowledge about MP pollution, neither from decision makers nor the public [12]. This shows how the associated lack of reliable data prevents effective management strategies from being developed.

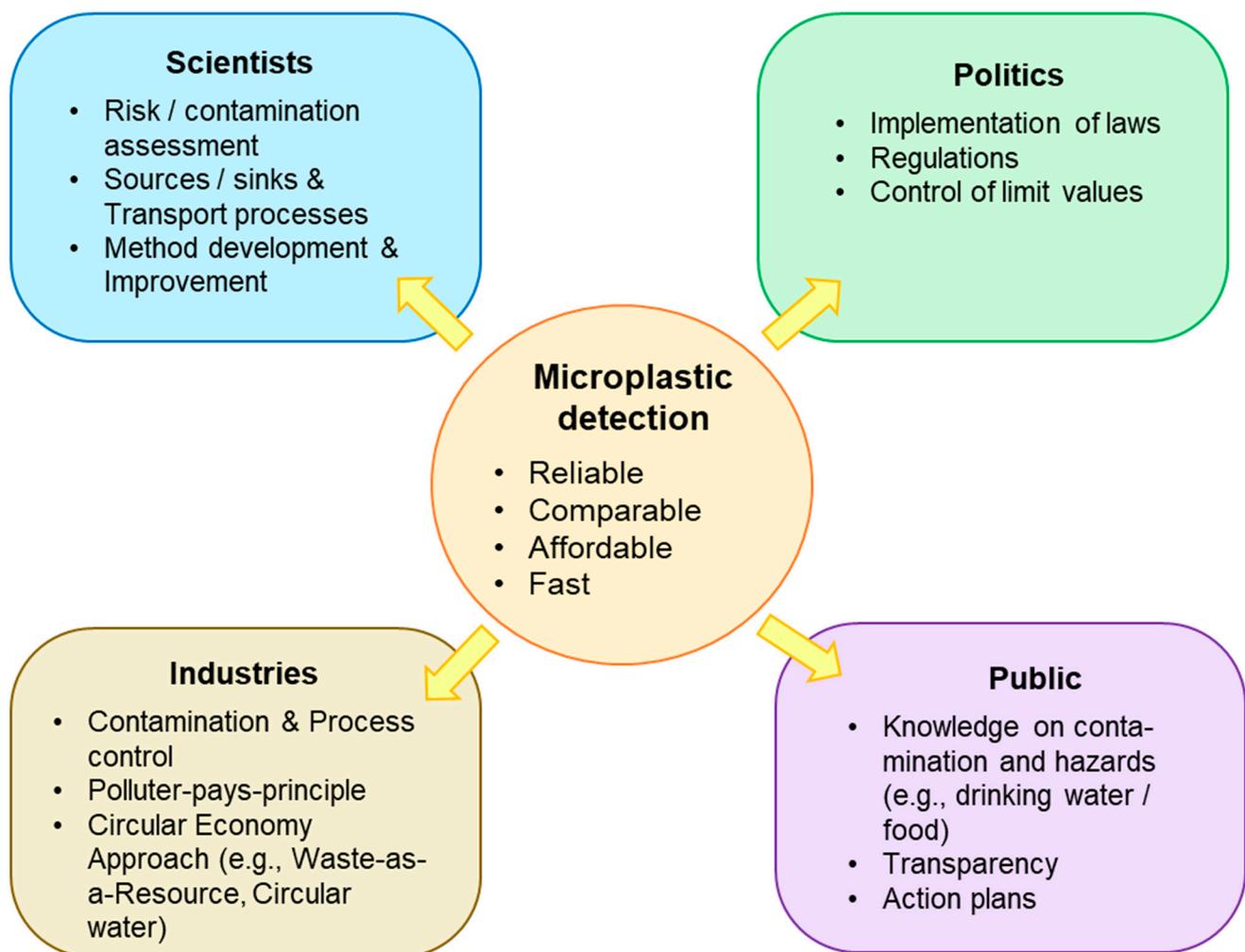


Figure 1. Importance of microplastics detection for scientists, politics, the public, and industries.

An example of this is the directive 2020/2184 (on the quality of water intended for human consumption), which was introduced by the European Union (EU) and includes a watch list containing pollutants of concern [13]. This list includes MP and the states in which

they should be monitored in waters used for drinking water production. Additionally, the proposal for the new EU directive 2022/0345 (COD) on urban wastewater treatment requires a regular monitoring of the MP released from wastewater treatment plants [14].

To lay a foundation for legislation, the European Commission's Joint Research Center developed a project to determine a standardized method for MP detection, with 96 participating laboratories [15]. The scatter of measurement results was found to be very high, revealing a substantial lack of inter-laboratory reproducibility, largely independent of the analysis technique. Therefore, the European Commission could not recommend any of the methods as a reliable standard for MP detection.

One method of MP detection often discussed in the scientific community is fluorescent staining, as it is simple and fast to perform, and only a fluorescent imaging tool such as a fluorescent microscope is required [16–18]. Therefore, it may be a practicable and cost-effective method for routine MP detection. The process involves a fluorescent dye, typically the lipophilic dye Nile red, which is applied to a sample containing MP. In theory, the fluorescent dye selectively stains MP particles (which show a strong fluorescent signal), but not natural particles (which show no or a negligible fluorescent signal).

Applications of the method showed that in real environmental samples, certain natural particles, such as chitin or wood, can show fluorescent signals and cause false positives, which is the biggest drawback of the fluorescent staining method [16–19]. It is therefore necessary to perform a digestion step, such as hydrogen peroxide, acidic, or alkaline digestion, before the fluorescent staining, which reduces the amount of natural particles in the samples and also weakens their fluorescent signals [16–18]. Ongoing research is being conducted on the optimal staining concentration and procedures, as well as optimizing the fluorescent imaging process and implementing automated particle counting, but few studies have investigated Nile Red derivatives for optimized MP detection [16–18,20–22].

In a previous study by Sturm et al. (2023), a new fluorescent dye was developed and optimized for the fluorescent staining of MP in aqueous solutions [23]. The results show that the new dye leads to better fluorescent signals of different polymer types, leading to better recovery rates, while having a reduced risk for false positives by natural particles when compared to the standard Nile Red. The aim of the current study is to optimize the sample preparation and staining procedure using this new dye to obtain faster and more precise results. Therefore, the effects of staining time, temperature, dye concentration, and the use of surfactants as a staining aid were investigated. In addition, the ability of the staining method to detect fluoropolymers, polymers containing a strong C-F bond, is investigated, as they are of high environmental concern due to their longevity and harmful ingredients [24]. Tire abrasion is also evaluated, which has been identified as the primary source of MP into the environment.

To further reduce the time of the sample preparation, an accelerated hydrogen peroxide digestion at an increased temperature is tested. In a long-term experiment analyzing wastewater samples, the accelerated sample preparation method was compared to the previously developed slower method.

2. Material and Methods

2.1. Chemicals and Microplastics

The fluorescent dye abcr eco Wasser 3.0 detect mix MP-1 (AB930015), hydrogen peroxide (35% *w/w* aqueous solution, stab., AB171423), and Tween 20 (AB252047) were supplied by abcr GmbH, Karlsruhe, Germany. The suppliers for MP and natural particles can be found in Table 1:

Table 1. List of polymers, suppliers, and preparation methods for microplastics and natural particles.

Polymer Type	Abbreviation	Supplier	Preparation
Polyethylene	PE	LyondellBasell, Basell Polyolefine GmbH, Frankfurt, Germany	Granules for industrial use
Polypropylene	PP	LyondellBasell, Basell Polyolefine GmbH, Frankfurt, Germany	Granules for industrial use
Polyester	PES	EMS-Grilltech, Switzerland	Granules for industrial use
Polyamide	PA	EMS-Grilltech, Switzerland	Granules for industrial use
Polyvinylchloride	PVC	Sigma-Aldrich, Germany	Granules for industrial use
Fluorethylenpropylen	FEP	Ambofluor GmbH & Co. KG, Hamburg, Germany	Granules for industrial use
Ethylen-Tetrafluorethylen	ETFE	Ambofluor GmbH & Co. KG, Hamburg, Germany	Granules for industrial use
Polyvinylidene flouride	PVDF	Ambofluor GmbH & Co. KG, Hamburg, Germany	Granules for industrial use
Styrene-butadiene rubber	SBR	RCT Reichelt Chemietechnik GmbH + Co., Heidelberg, Germany	Shredding of the rubber sample (19746; SBR-Food-Platte—Shore 65°)
Natural rubber	NR	ARNOWA GmbH, Salzkotten, Germany	Cutting of a latex glove
Blend of SBR and NR	SBR-NR	WiCo Wichmann, Otto & Cie GmbH + Co. KG, Wenden, Germany	Shredding of the rubber sample (NR/SBR hell 65Sh FDA Rollen; G40160103010000)
Tire abrasion		Bike tire	Shredding of the tire
Tube abrasion		Bike tube	Shredding of the tube
Wood			Fine shavings of Quercus spec.
Chitin			Ground shell of Mytilidae
Chalk			Ground exoskeleton of Pandalus borealis

2.2. Hydrogen Peroxide Treatment

To digest and reduce the number of natural particles in the sample, a hydrogen peroxide treatment was performed based on Sturm et al. (2023) [23]. The samples are first filtered over a stainless-steel sieve (custom-made, 10 µm mesh, Ø 47 mm, Wolftechnik Filtersysteme GmbH & Co., KG, Weil, Germany) using vacuum filtration (DURAN® Filtering Apparatus, Cat. No. 257106304, DWK Life Sciences GmbH, Mainz, Germany).

The samples are then placed in a 250 mL beaker and covered with 20 mL of hydrogen peroxide. A total of 3 to 5 grains of iron (II)-sulfate are added, the beaker is covered with aluminum foil, and the hydrogen peroxide is heated up. The two treatment options, the original method from Sturm et al., 2023 and the new, optimized method for faster digestion, are summarized in Table 2. To avoid the particles melting onto the stainless-steel sieve or the over-boiling of the sample, the maximum temperature of the heating plate (C-MAG HP 10, IKA, Staufen, Germany) for the heating phases was set to 120 °C.

Table 2. Parameters for the fast (newly optimized) and slow (original) hydrogen peroxide digestion.

Version	Procedure
Slow (old method) Sturm et al. (2023) [23]	<ol style="list-style-type: none"> Heat up to boiling temperature for 1 min. Reduce temperature to 80 °C and stir for 4 h. Cool down to room temperature (RT) and stir for 20 h.
Fast (new method)	<ol style="list-style-type: none"> 100 °C for 1 h.

After the treatment, the stainless-steel filters are taken out of the beaker and rinsed into the beaker with water. Subsequently, the sample is filtered over the stainless steel filter again, to remove all digested residues smaller than 10 μm . The particles retained on the sieve are washed into a beaker with 100 mL of water, where they are subsequently stained.

2.3. Fluorescent Staining

To investigate the effects of the staining parameters on the fluorescent signal, 10 mg of MP (PE, PP, PES, PA, PVC) were dispersed in 100 mL of demineralized water and stained with the according parameters, varying the dye concentration, staining temperature, and staining time using abcr eco Wasser 3.0 detect mix MP-1. After the staining process, they were filtered over black disk filters (Metricel[®] Black PES Membrane Disk Filters, 0.80 μm pore size, Pall Cooperation, Dreieich, Germany) and stored in glass Petri dishes.

For the application of the staining for the recovery rates and wastewater samples, the following staining procedures were compared. The “fast” staining method developed in the current study was performed using 0.25 mg/L of abcr eco Wasser 3.0 detect mix MP-1 at 80 °C for one hour. The “slow” staining method, as presented in the previous study by Sturm et al., 2023, was performed using 0.5 mg/L of abcr eco Wasser 3.0 detect mix MP-1 at 5 °C for 24 h [23].

2.4. Fluorescent Imaging

Fluorescent imaging was performed according to Sturm et al., 2023 [23]. An optical microscope Leica DMS300 (Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany) was modified as a fluorescent microscope and used with the software LAS-X 3.0.1423224. For excitation, LED flashlights combined with bandpass filters were used. For the emission filters, bandpass filters were mounted in front of the lens of the microscope. The parameters for the fluorescent images can be found in Table 3.

Table 3. Parameters for fluorescent imaging of the microplastic samples.

Fluorescence	Excitation	Emission	Exposure	Gain
Blue	395 nm	430–480 nm	500 ms	4
Green	430–480 nm	500–570 nm	500 ms	4
Orange	500–570 nm.	570–640	500 ms	4

For the optimization of the staining procedure, the determination of the recovery rates, and the measurement of the environmental samples, green fluorescence is used [23].

2.5. Recovery Rates and Automated Microplastic Detection

To determine the recovery rates by particle numbers, 40 particles of either MP (PE, PP, PES, PA, PVC) or natural particles (wood, chalk, chitin) were added to deionized water. Subsequently, the filtration, hydrogen peroxide treatment, and staining process were performed. The experiments were performed in triplicates. The slow (original) and fast (new) hydrogen peroxide treatments were compared to determine their ability to reduce natural particles in the sample and preserve MP. The staining was performed with the fast-staining method using 0.25 mg/L of Wasser 3.0 detect mix MP-1 for 1 h at 80 °C.

Automated particle counting was performed using Leica LAS-X 3.0.1423224. The threshold for the particles was based on the brightness/fluorescence intensity of the stained particles. For automated particle detection, no image processing filters were applied. The brightness threshold was set to 25 (brightness range from 0 to 255) and determined by the brightness levels of the natural particles and microplastics from the recovery rates (further details in Figures S3 and S4). With a pixel size of $2.5 \times 2.5 \mu\text{m}$, the minimum size for the MP particles was set to 16 pixels, which is equivalent to $100 \mu\text{m}^2$ or a particle with a size of $10 \times 10 \mu\text{m}$.

2.6. Wastewater Sampling and Method Comparison

The sampling is performed according to Sturm et al., 2023 at the effluent of the wastewater treatment plant (WWTP) in Landau-Mörlheim, Germany [23].

For the sample collection, a stainless-steel filter cartridge with a mesh size of 10 µm (01WTGD, Wolftechnik Filtersysteme GmbH & Co., KG, Weil, Germany) and a 0.9 kW centrifugal pump (MG80B C-B-CMS1B, Grundfos, Erkrath, Germany) were used. Before each sampling process, the entire system was backflushed for 3 min with tap water. As the volume of tap water remaining in the system is 10 L, a total amount of 110 L water is filtered to receive a sample volume of 100 L.

After filtering 100 L of wastewater, the tubing is disconnected from the filter cartridge and the water remaining in the filter cartridge is poured into a stainless-steel bowl. The filter is removed from the cartridge and rinsed into the stainless-steel bowl with a pressurized spray bottle. The sample is transferred into a 2.5 L glass bottle for transport and storage.

For the method comparison, 2 × 500 mL subsamples were taken and processed using the slow processing method (Sturm et al., 2023) to the fast method (current study), from chapter 2.2 and 2.3 [23]. For sampling, the samples were shaken by hand for 30 s, then the subsample was taken by pouring the sample directly into a 500 mL volumetric cylinder.

2.7. Contamination Control

To prevent contamination from airborne particles or plastic abrasion in the laboratory, only glass and metal equipment was used, and beakers and vessels were covered with aluminum foil. The laboratory was cleaned with lint free cotton rags before each use and a HEPA filter was operated to remove particles from the air. A low-lint protective suit (4510M, 3M Deutschland GmbH, Ness, Germany) was worn and cleaned with a lint brush before entering the laboratory to prevent contamination by clothing fibers. Blanks were measured for contamination control.

For the wastewater samples, all equipment was rinsed with tap water; the filters and tubes were also flushed with tap water before every use. In regular intervals (8–16 samples, depending on the particle load sampled water), the filter cartridge is cleaned with a high-pressure cleaner to clean the mesh of any residue and unclog the filter.

In addition, blanks were measured, and an average blank value of 5 MP/L was subtracted from the samples.

3. Results and Discussion

3.1. Optimization of the Staining Process

3.1.1. Staining Time

When comparing the effects of the staining times (Figure 2), there are no visible differences in the fluorescence intensities between the particles stained for 24 h, 5 h, and 1 h. Therefore, a staining time of 1 h is sufficient for the MP samples. PES shows the strongest fluorescent signal, followed by PA and PE. PP shows only a weak fluorescent signal and PVC had the weakest signal and was not detectable. A previous study investigated smaller timespans, ranging from 5 to 12 min [25]. The staining times showed clear differences in the fluorescence intensities, with longer staining times having a better effect. Within the current study, we found that after 1 h, the sorption of the dye to the plastic reached its equilibrium, and therefore no changes were observed after 1 h.

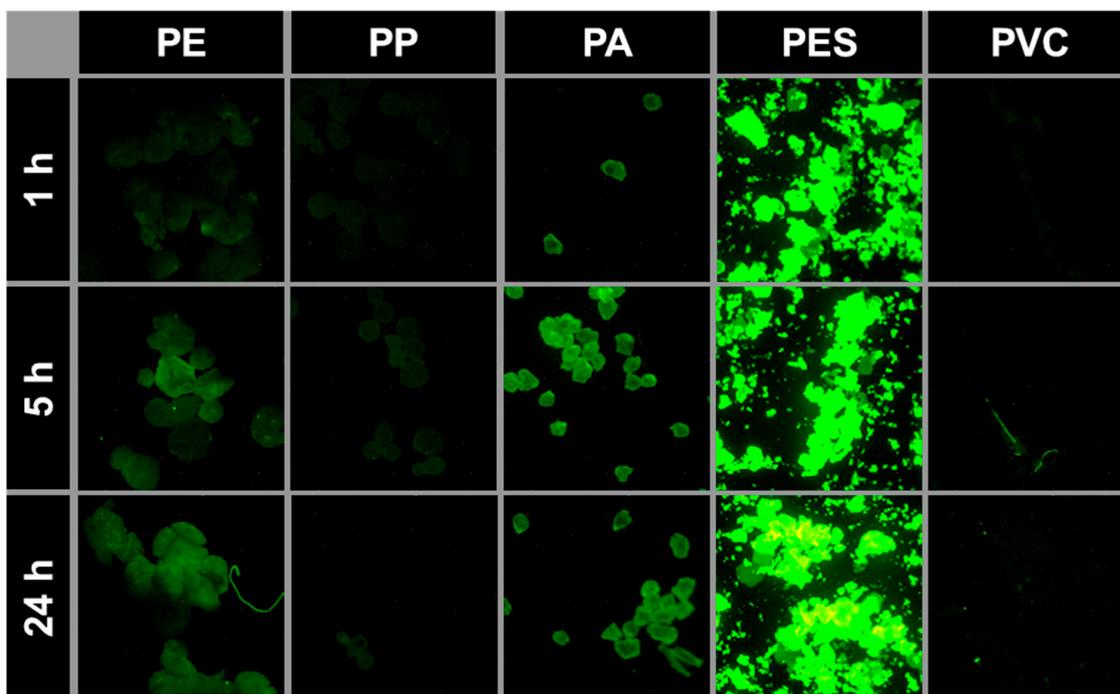


Figure 2. Influence of staining time—fluorescent images of microplastics from different polymers stained with 0.5 mg/L of abcr eco Wasser 3.0 detect mix MP-1 at 5 °C for different times (1 h; 5 h; 24 h).

3.1.2. Staining Temperature

Investigations on the staining temperature (Figure 3) showed that higher temperatures positively impact the staining efficiency and resulting fluorescent signal. This is especially visible for PE, PP, and PVC. PE and PP only showed very weak fluorescent signals at room temperature while PVC displayed no fluorescent signal. In studies using Nile red as a fluorescent stain, PVC shows no or only a very weak fluorescent signal for green fluorescence, making it undetectable [19]. Staining at 80 °C increases the fluorescent signals. This makes PVC detectable and PE and PP easier to detect.

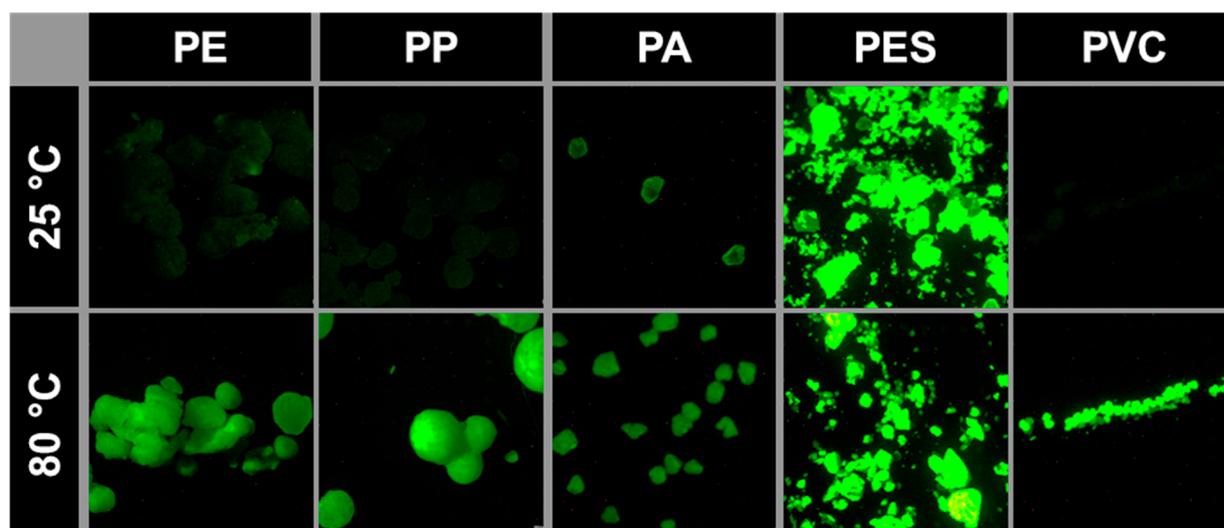


Figure 3. Influence of staining temperature—fluorescent images of microplastics from different polymers stained with 0.5 mg/L of abcr eco Wasser 3.0 detect mix MP-1 for 1 h at different temperatures (25 °C; 80 °C).

Other studies have also revealed that higher temperatures have a positive impact on the staining procedure [25–28]. Higher temperatures can yield a better adsorption of the fluorescent dye onto the MP surface and into the polymeric network. Nevertheless, some polymer types can be sensitive to high temperatures [29–31].

3.1.3. Dye Concentration

Looking at the different dye concentrations used for staining, the fluorescence signals at 0.25 mg/L for PE, PA, PES, and PVC are stronger than for 0.5 mg/L. For 0.05 mg/L, a decrease in the fluorescence intensity is observed, especially for PVC and PES (Figure 4). Therefore 0.25 mg/L is determined to be the optimal concentration for staining at 80 °C for 1 h.

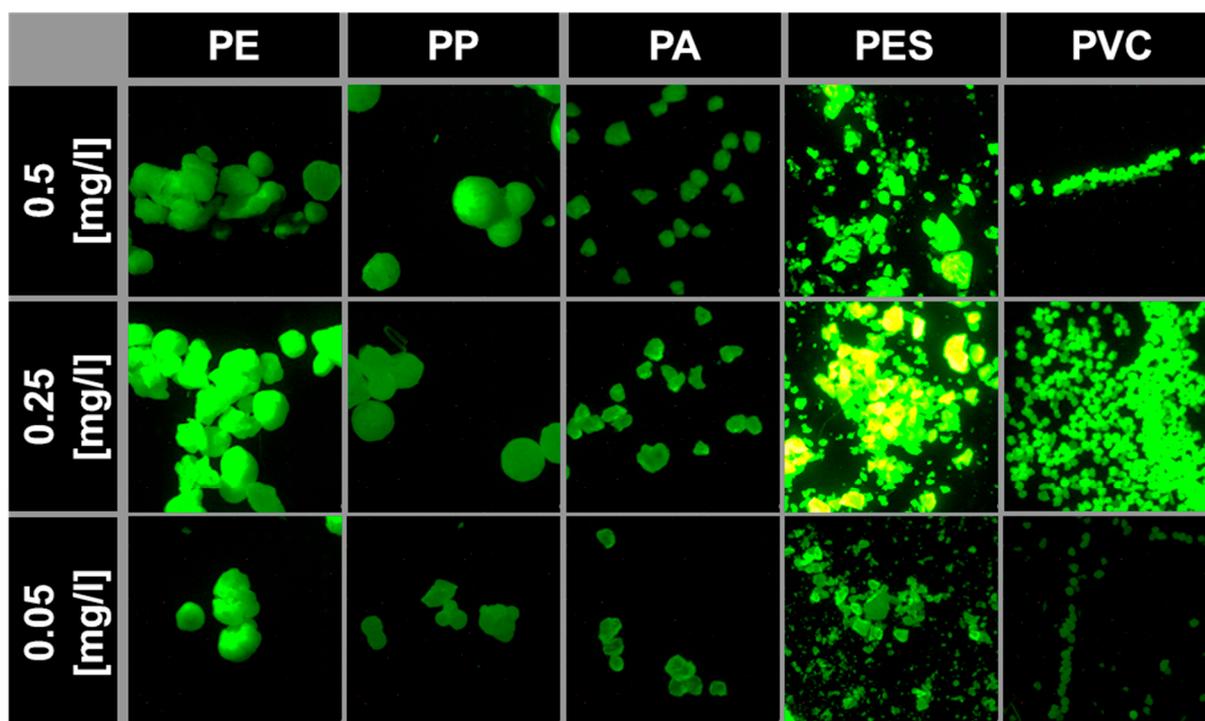


Figure 4. Influence of stain concentration—fluorescent images of microplastics from different polymers stained with abcr eco Wasser 3.0 detect mix MP-1 for 1 h at 80 °C with different stain concentrations (0.05 mg/L; 0.25 mg/L; 0.5 mg/L).

The literature on the use of fluorescent staining for MP detection indicates that various concentrations can be applied for different staining procedures and various solvents can be used for staining. Quenching at high dye concentrations is often discussed and may also be the reason for the reduced fluorescence intensity at 0.5 mg/L compared to 0.25 mg/L [16,20,32]. A previous study showed that increasing the concentrations can increase the fluorescent intensity until a certain limit, as more dye is absorbed onto the polymer surface and into the polymeric network [32]. Therefore, we observe the optimal signal at 0.25 mg/L, at which point higher concentrations induce quenching [19].

3.1.4. Surfactants

Some of the scientific literature discusses improved fluorescent staining in water through the addition of surfactants [27]. The surfactant can increase the solubility of the fluorescent dye in water and therefore improve the transport onto the MP surface and into the polymer network. The surfactants can also change the hydrophilicity of the MP surface and therefore alter the sorption properties [33]. For this study, Tween 20 (0.5 mg/L) was tested for staining with a 0.5 mg/L stain for 1 h at room temperature, as a possible alternative to heating up the samples to 80 °C.

The fluorescent images (Figure S1) show no change in the fluorescence signal due to the addition of the surfactant. As the previous study showed an increased fluorescence signal through the addition of Tween 20, this difference might be due to the different chemical structure of the dye itself [27].

3.2. Fluoropolymers

Fluoropolymers are a group of polymers based on fluorocarbon monomers with multiple carbon–fluorine bonds [34]. They are known to be very resistant to solvents, acids, and bases, and have chemical- and water-repellent surface properties which make them indispensable in many areas of application. They are also very resistant to degradation processes, which cause a high persistence when released into the environment [35]. As with other plastics, sources in the environment can be improper disposal (e.g., food packaging coated with fluoropolymers, dental floss, non-stick coating) or loss during manufacturing, processing, transport, or recycling [24,36]. When degrading, they release further persistent and often toxic fluor-organic compounds. Therefore, fluoropolymers are of high environmental concern. The fluorescent staining of three different fluoropolymers, FEP (Fluorethylenpropylene), ETFE (Ethylene-Tetrafluorethylene), and PVDF (Polyvinylidene fluoride), was examined.

The fluorescent images (Figure 5) show the best signal for PVDF followed by ETFE, making them both detectable in environmental samples. FEP, which has very similar properties to PTFE (Polytetrafluorethylene or Teflon) and has a highly repellent surface, shows only very weak fluorescent signals and is not detectable. Thus, two of the three polymers can be detected using the presented staining method.

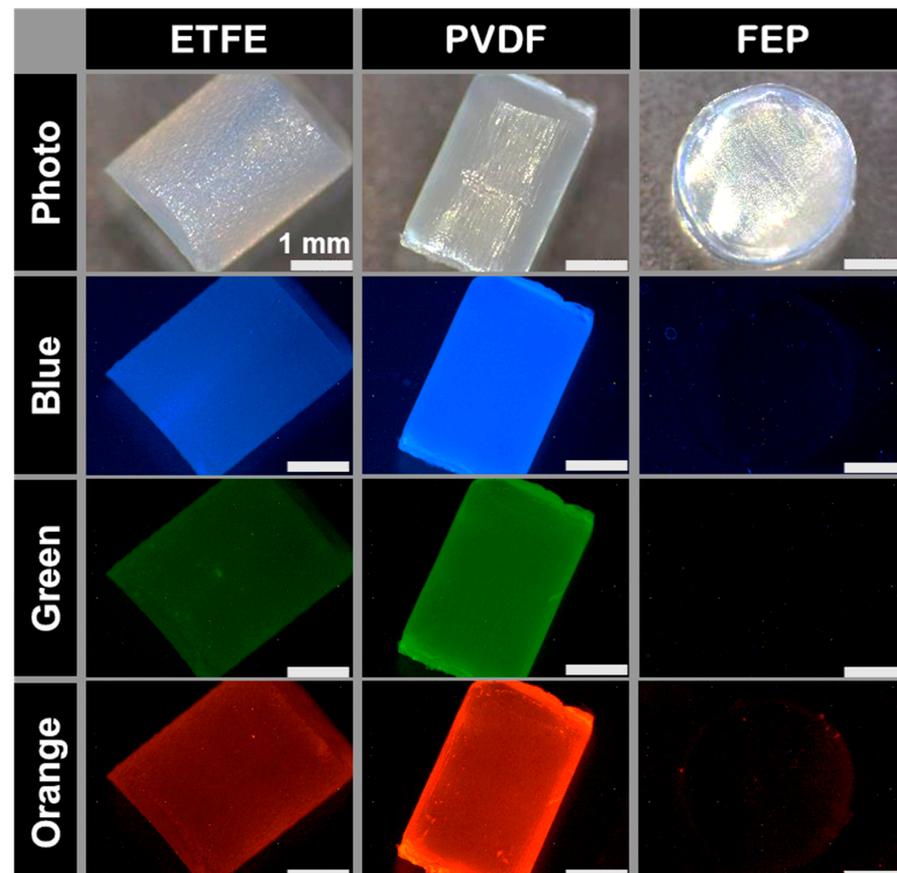


Figure 5. Fluorescent images of stained fluoropolymers—fluorescent images of microplastics from different fluoropolymers stained with 0.25 mg/L of abcr eco Wasser 3.0 detect mix MP-1 for 1 h at 80 °C. FEP = Fluorethylenpropylene; ETFE = Ethylen-Tetrafluorethylene; PVDF = Polyvinylidene fluoride.

3.3. Tire Abrasion

One of the main sources for the direct release of primary MP into the environment is tire abrasion [36,37]. Tires consist of natural rubber and/or synthetic rubber, mainly SBR (styrene-butadiene rubber) [38]. To give them their specific properties, the rubber is mixed with additional ingredients, such as fillers (carbon black or silica), plasticizers, (oils, fats), anti-aging agents, vulcanizing agents, vulcanization accelerators, pigments, or dyes. Depending on the specific tire composition, the rubber content is typically 40–60%, while the fillers make up between 15 and 30%, and the remaining components are 20–35%.

In this study, MP from a bicycle tire and inner tube with black color were compared to white rubber (from natural rubber, SBR, and a mix of both SBR and natural rubber) (Figure 6). While all white rubbers show very strong fluorescent signals and are often overexposed, the black tire particles show almost no fluorescence. This is caused by quenching due to the black color of the tire and hinders the detection of tire abrasion using fluorescent staining. Recent studies using Nile red for fluorescent staining found similar results, whereby the tire rubber did not indicate any fluorescent signals [39].

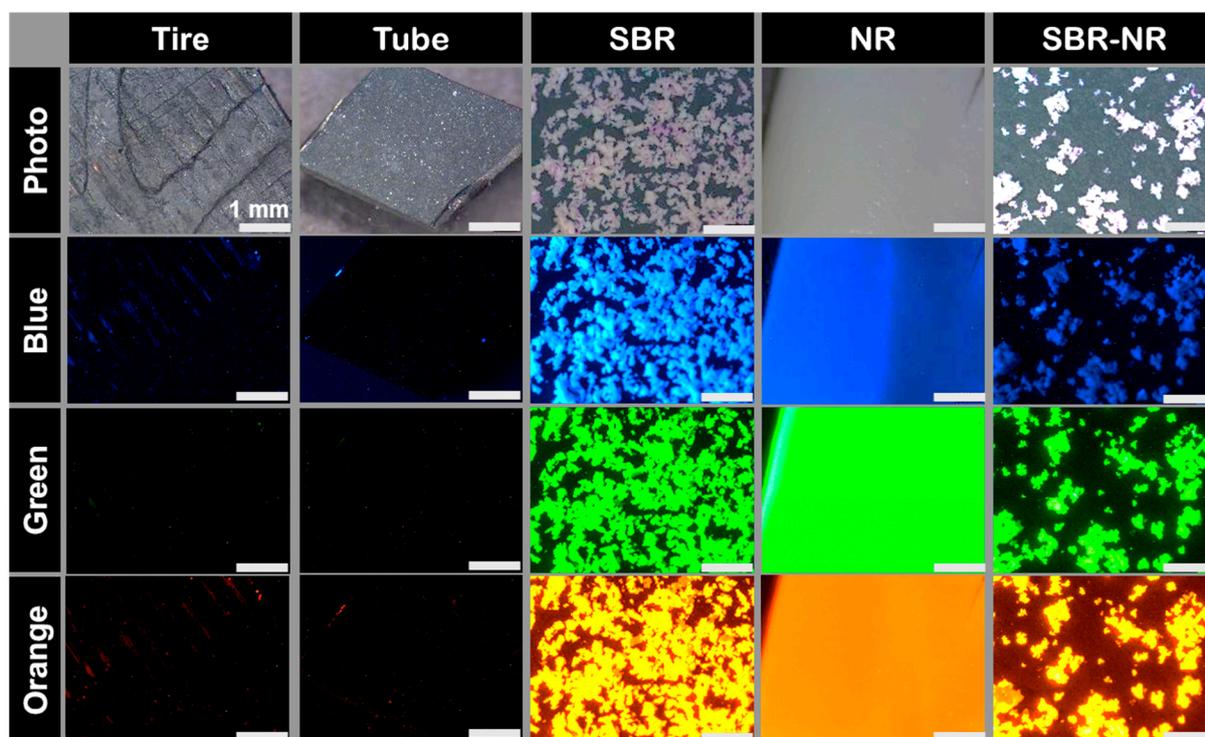


Figure 6. Fluorescent images of stained tire and rubber particles—fluorescent images of microplastics from tire and rubber particles stained with 0.25 mg/L of abcr eco Wasser 3.0 detect mix MP-1 for 1 h at 80 °C. SBR = Styrene-butadiene rubber, NR = Natural rubber, SBR-NR = blend of SBR and NR.

3.4. Accelerated Hydrogen Peroxide Digestion and Recovery Rates

To accelerate the detection process for MP in environmental samples, a fast hydrogen peroxide digestion of 1 h digestion time was compared with the original hydrogen peroxide digestion of 24 h from Sturm et al. (2023) (Table 2) [23]. Staining was performed for both digestion methods with the fast-staining procedure using 0.25 mg/L of abcr eco Wasser 3.0 detect mix MP 1 at 80 °C for one hour. The recovery rates by a particle count of MP based on five polymer types and three natural particles were investigated. The images of the samples and detailed information of the particle numbers can be found in Table S2 and Figures S2 and S4. The recovery rates for the slow staining method and slow hydrogen peroxide digestion were performed previously by Sturm et al. (2023) [23].

For all polymer types, recovery rates from 93% (PP) to 102% (PES) were reached, allowing reliable detection (Figure 7). Natural particles show low recovery rates from 3% (chitin)

to 13% (chalk) with the slow hydrogen peroxide treatment (4 h at 80 °C and 20 h at RT). For the fast hydrogen peroxide treatment (1 h at 100 °C), the natural particles had average recovery rates of 6% (chitin) to 5% (chalk, wood). An increase in temperature increases the efficiency of the hydrogen peroxide treatment and can compensate for the reduced time [31]. Both methods effectively reduce the fluorescence signal of natural particles.

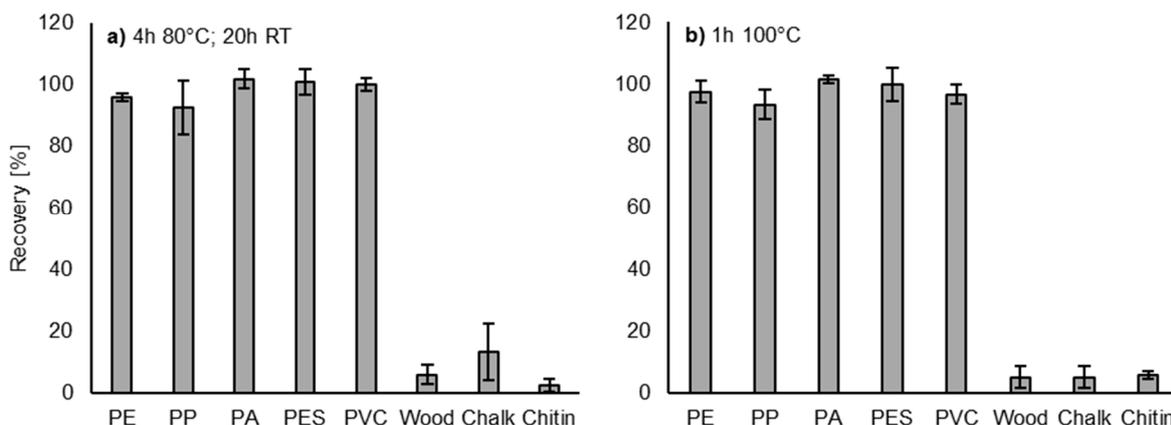


Figure 7. Optimized hydrogen peroxide treatment—recovery rates of microplastics and natural particles after a hydrogen peroxide digestion and fluorescent staining with 0.25 mg/L of abcr eco Wasser 3.0 detect mix MP-1 for 1 h at 80 °C. (a) Hydrogen peroxide treatment for 4 h at 80 °C and 20 h at RT; (b) hydrogen peroxide treatment for 1 h at 100 °C.

Further, in this study, no loss was found of the tested polymers due to the hydrogen peroxide treatment. However, only the particle number was determined; a change of the weight or particle characteristics themselves were not investigated. The heating plate used for the experiments had the option to set the temperature of the heating plate's surface, which was set to 120 °C. This prevents the beaker or steel sieve from significant heating, prevents the plastics from melting and over-boiling, and prevents possible particle loss. Other studies report that high degradation temperatures can damage microplastics, especially certain polymer types such as PA or PET (polyethylene terephthalate) [29–31].

Compared to staining at 5 °C, which was tested in a previous study, the recovery rate of PVC is strongly increased at 80 °C. Further, with the faster staining and hydrogen peroxide treatment, it is possible to measure the levels of MP contamination in samples within 2.5 to 3 h.

3.5. Comparison of Methods Using Wastewater Samples

To compare the MP levels in the wastewater effluent samples processed with the slow processing method (Sturm et al., 2023) to the fast method (current study), the same effluent samples were measured with both methods [23]. Samples processed using the rapid method resulted in an average MP count increase of $30 \pm 37\%$ compared to those processed with the original method (Figure 8). The rapid detection method leads to better fluorescent signals and detection of polymers such as PVC, as well as better signals for PE and PP, which are the two most used and produced polymers. However, the ratio between the samples is not constant and may be due to the varying polymer compositions [23].

Although the same wastewater effluent samples were used for the comparison between the fast and slow processing methods, variations in MP levels between 0 and 90% were observed in the 500 mL subsamples and should be considered when comparing the results. Deviations in the subsample measurements may also occur due to errors during the sample preparation process or uncertainties in the measurement process. For example, when taking the subsample, the improper mixing of the microplastics, which can be buoyant and float, sink, or stick to the glass walls, is a possible source of error. For 15.06. and 05.07., only one sample was measured due to errors in the sample processing of the second sample.

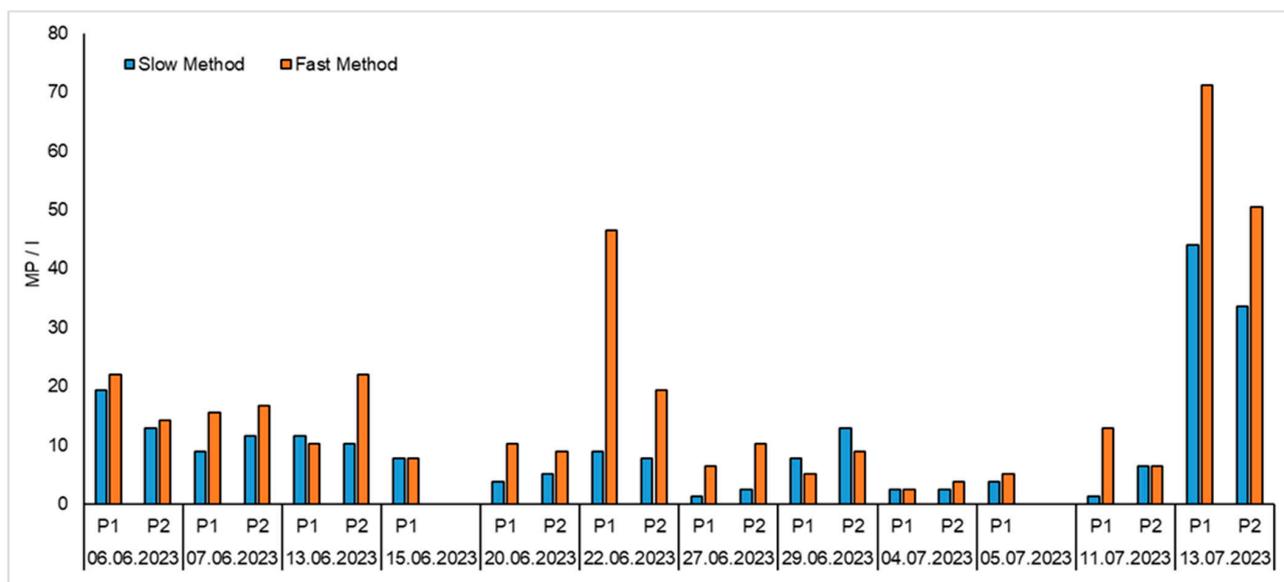


Figure 8. Comparison of wastewater effluent samples processed with the original method (slow method) compared to the accelerated method (fast method) with faster hydrogen peroxide treatment and staining.

This case study shows that the detection method is applicable for environmental samples. With contamination levels ranging from 2.6 to 46.6 MP/L, the results are in the typical range for a wastewater treatment plant [40]. The photos and fluorescent images of the samples (Figure 9) show that fluorescent staining is very helpful to separate microplastics from non-fluorescent particles and natural particles.

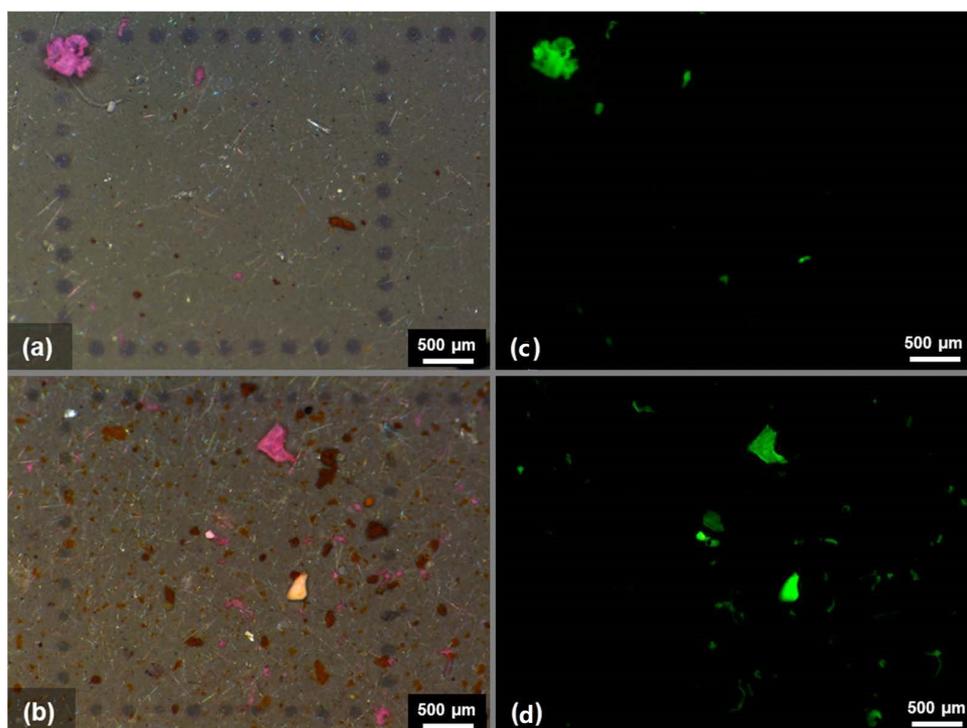


Figure 9. Photos and fluorescent images of the processed and stained wastewater samples using the fast method. (a) Photo of the sample from 13 June 2023. (b) Fluorescent image of the sample from 13 June 2023. (c) Photo of the sample from 15 June 2023 (d) Fluorescent image of the sample from 15 June 2023.

One of the main limitations of this method is that the particles are not chemically identified, what leads to the remaining risk of false positives (see Figure 7) and missing information on polymer types [17,19]. Further, numerous different polymers with different additives and colors are present, and only the most common polymer types were tested [41,42]. The same applies to natural particles with a very high chemical diversity. In further research, an evaluation of a higher variety of natural particles and plastic types, also covering the variations within the plastic types due to additives, different monomers, or different molecular structures, should be completed [16,18]. As shown in the results from the tire abrasion (Figure 6), colorants, especially dark colors, can also negatively influence the detection. Moreover, a cross-evaluation with FTIR or Raman microscopy can be helpful to ensure the accuracy of the method [20].

4. Conclusions

In summary, the presented study could optimize and accelerate the fluorescent staining method for MP detection, enabling results within 2.5–3 h. A staining time of one hour is sufficient; staining times over one hour do not lead to improved fluorescent signals. Increasing the staining temperature to 80 °C could strongly increase the fluorescent signals for PE and PP, making them easier to detect. Even PVC, which was hardly detectable when staining at 5 °C, could be detected after staining at 80 °C. The optimum concentration of the stain applied in an aqueous solution is 0.25 mg/L. The surfactants showed no positive effect on the staining process. Thus, the final parameters determined for efficient staining were one hour staining at 80 °C with 0.25 mg/L of abcr eco Wasser 3.0 detect mix MP-1.

When investigating the staining of the fluoropolymers, ETFE and PVDF could be detected due to their good fluorescent signals, while FEP showed a very weak fluorescence due to its repellent surface properties. Tire abrasion, bicycle tire abrasion, and tubing abrasion showed no fluorescence signals due to quenching by the black color. White natural rubber and SBR rubber showed strong fluorescent signals, which shows that the basic polymer would be detectable.

For the final detection method, increasing the temperature to 100 °C could reduce the hydrogen peroxide treatment to one hour. Using hydrogen peroxide treatment, fluorescent staining, fluorescent imaging, and automated particle detection, recovery rates from 93.3% for PP to 101.7% for PES were obtained. For natural particles, the average recovery rates were 5% for chalk and wood, and 6% for chitin. This allows reliable MP detection, applicable for routine measurements in the process control or rapid screening of MP contamination. The long-term study on the effluent of the WWTP in Landau-Mörlheim confirmed this finding. The method is well suited for contamination control and routine measurements as it is fast and easy to apply.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/microplastics2040026/s1>, Figure S1: Surfactant (Tween 20) as staining aid – fluorescent images of microplastics from different polymers stained with 0.5 mg/L abcr eco Wasser 3.0 detect mix MP-1 for 1 h with and without Tween 20 (0.5 mg/L). Figure S2: Fotos of the microplastics (PE, PP, PA, PES, PVC) and natural particles (wood, chalk, chitin) used for the recovery rates. Table S1: Measurement data for the recovery rates for microplastics and natural particles using the slow hydrogen peroxide digestion (4 h 80 °C, 20 h R.T.). Table S2 Measurement data for the recovery rates for microplastics and natural particles using the fast hydrogen peroxide digestion (1 h 100 °C). Figure S3.1: Caption—see Figure S3.3. Figure S3.2: Caption—see Figure S3.3. Figure S3.3: Recovery rate experiment images using the slow hydrogen peroxide digestion (4 h 80 °C, 20 h R.T.)—Photos, green fluorescent images and images from automated particle detection of subsamples for the recovery rates using the slow hydrogen peroxide digestion (4 h 80 °C, 20 h R.T.) and fluorescent staining with 0.25 mg/L MP-1 for 1h at 80 °C. Figure S4.1: Caption—see Figure S4.3. Figure S4.2: Caption—see Figure S4.3. Figure S4.3: Recovery rate experiment images using the fast hydrogen peroxide digestion (1h at 100 °C) - Photos, green fluorescent images and images from automated particle detection of subsamples for the recovery rates using the fast hydrogen peroxide digestion (1h at 100 °C) and fluorescent staining with 0.25 mg/L MP-1 for 1h at 80 °C.

Table S3: Measured microplastics contamination and deviations of the wastewater samples from the effluent of the WWTP Landau. The same samples were measured with the fast method based on the current paper and the slow method based on Sturm et al. (2022) and the results compared.

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