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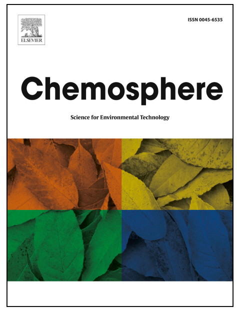

Flow cytometry as a tool for the rapid enumeration of 1-μm microplastics spiked in wastewater and activated sludge after coagulation-flocculation-sedimentation

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Flow cytometry as a tool for the rapid enumeration of 1-µm microplastics spiked in wastewater and activated sludge after coagulation-flocculation-sedimentation

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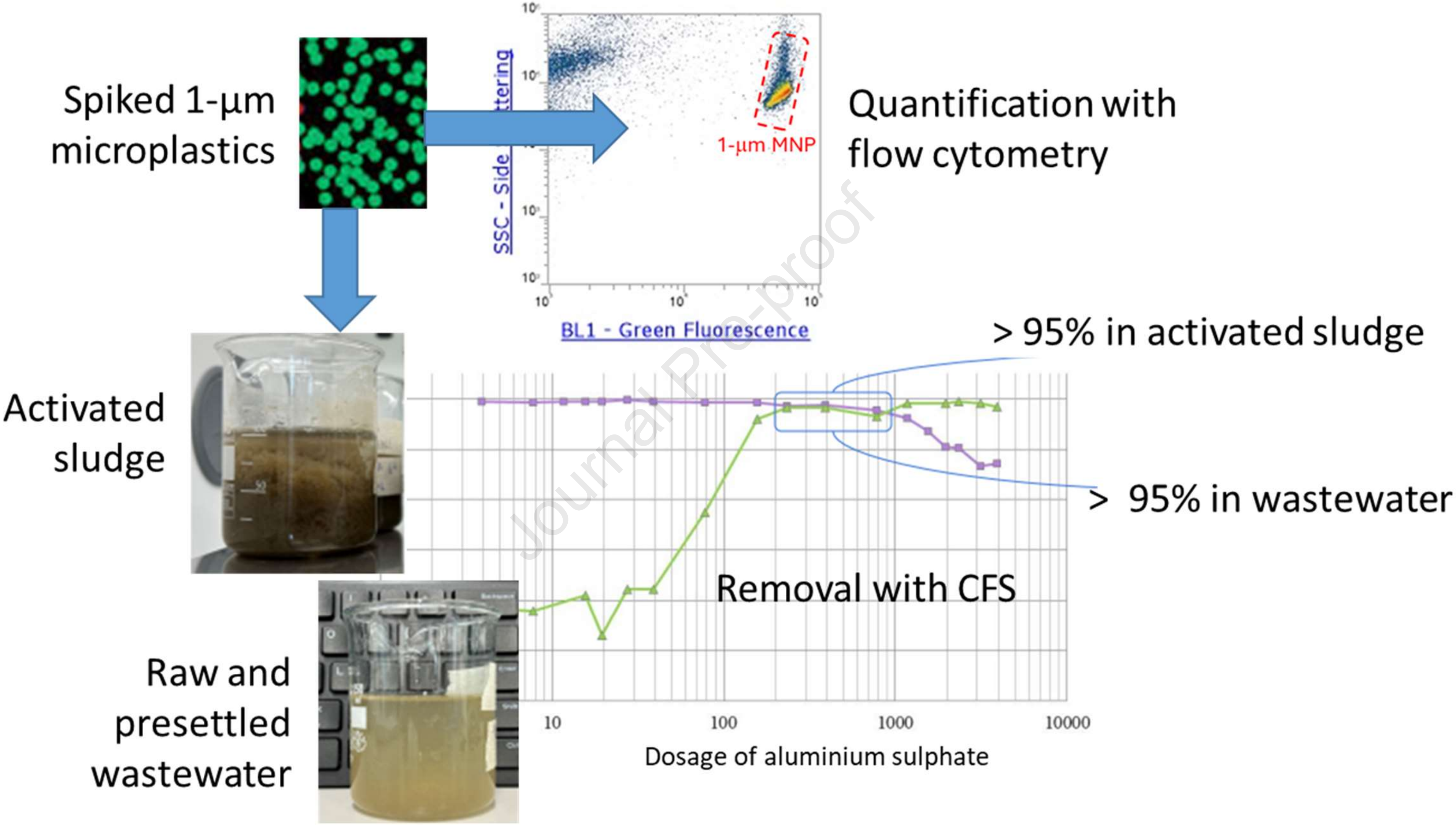
Spiked 1-µm microplastics
SSC - Side Scattering
BL1 - Green Fluorescence
1-µm MNP
Quantification with flow cytometry
> 95% in activated sludge
Activated sludge
> 95% in wastewater
Removal with CFS
Raw and presettled wastewater
10
100
1000
10000
Dosage of aluminium sulphate

Abstract

Considering the limited literature and the difficulty of quantifying 1-µm micro-nanoplastics (1-µm MNP) in complex aqueous matrices such as wastewater and sludge, the removal rate of these very small particles in wastewater treatment plants (WWTP) represents a major challenge. In this study, coagulation-flocculation-sedimentation (CFS) with aluminum salts was investigated to evaluate the removal of 1-µm MNPs spiked in tap water, raw wastewater, pre-settled wastewater, and activated sludge. Quantification of 1-µm MNP was performed using the high-throughput flow cytometry (FCM) analysis which takes only a few minutes and produces results with high accuracy and reproducibly.

The results indicated that the 1-µm MNPs were highly stable in pure water and unable to settle rapidly. In raw wastewater, sedimentation without coagulants removed less than 4% of 1-µm MNP. Conversely, CFS treatment showed a significant improvement in the removal of 1-µm MNP from wastewater. At dosages of 0.3-3 mg Al^{3+}/L, the removal of MNPs in wastewater reached 30% and no flocs were observed, while floc formation was visible with increased dosages of 3-12 mg Al^{3+}/L, obtaining MNP removal greater than 90%. CFS in activated sludge with a solids content of 6800 mg MLSS/L registered the highest removal efficiency (95-99%) even for dosages of 0.3-60 mg Al^{3+}/L and pH dropping to 5. However, activated sludge showed extremely high removal efficiency of MNPs (97.3±0.9%) even without coagulants. The large, dense flocs that constitute activated sludge appear particularly efficient in capturing 1-µm MNPs during the sedimentation process even in the absence of coagulants.

Keywords: Microplastics; coagulation-flocculation; wastewater; activated sludge; flow cytometry.

1. INTRODUCTION

Microplastics (MP) and nanoplastics (NP) are discharged into wastewater from various sources connected to the sewage network (Tian et al., 2023; Monira et al., 2023a) and reach municipal wastewater treatment plants (WWTPs), where they can be only partially removed. Particles remaining in treated effluents from WWTPs reach receiving surface water bodies where MP/NPs can interact with aquatic systems and biota (Kukkola et al., 2021; Caputo et al., 2021; Ali et al., 2021; Zhao et al., 2022; Monira et al., 2023b). In these systems, extensive research has been conducted on the detection and quantification of larger MPs from 10 µm to millimeters in size (Ziajahromi et al., 2017; Lares et al., 2018; Reddy and Nair, 2022), but experimental studies on smaller particles with dimensions of around a micrometer are currently scarce, especially in municipal WWTPs (Wang et al., 2023; Wang et al., 2022; Tse et al., 2023).

Until recently, there was no general consensus on the size threshold between MP and NPs (Elsayed et al., 2021; Gigault et al., 2018), but a recent proposal has defined NPs as sub-micron particles with dimensions of less than 1 µm (European Commission, 2023), while particles larger than 1 µm are classified as MPs. Therefore, particles with an exact size of 1 µm can be called MNPs, thus combining the two terms 'micro-' and 'nano-' (Rout et al., 2022). NPs have higher toxicity than MPs because of their ability to traverse cell walls and accumulate in cells (inter alia Ma et al., 2016). When discharged into the environment, small MPs (< 10 µm) and NPs can transfer and magnify through food chains with risks to the health of aquatic organisms (Kumar et al., 2021; Caputo et al., 2021; Tse et al., 2023). For this reason, increasing attention is now being paid to the capacity of WWTPs to remove particles in the sub-micron range (Caputo et al., 2021). However, 1-µm MNPs and NPs are extremely difficult to analyse and this restricts the acquisition of in-depth knowledge for this category of particles. For example, in the extensive review by Kukkola et al. (2021), which considers and compares 76 freshwater field studies, no investigations with MNPs of 1 µm or less are presented.

The detection of 1-µm MNPs in complex matrices such as wastewater and sludge in WWTPs is particularly difficult because their high solids content can mask the particles and requires strong chemical pretreatments for the separation of MNPs. Furthermore, when MNPs can be observed, their enumeration still takes a long time, presents a lot of interference, and only a limited number of samples can be processed per day. Difficulties in measuring MNP concentrations in influent and effluent wastewater cause high uncertainty in calculating removal efficiency in WWTPs. For these reasons, further research is needed to develop accurate methods for counting 1-µm MNPs in wastewater, so that better understanding can be gained of the fate of MNPs in WWTPs and their release into the environment in general.

Flow cytometry (FCM) is a high-resolution single-particle analysis based on the detection of fluorescence and scattering signals emitted by each particle as it passes in front of a laser beam. FCM is well known and widely applied in the medical sector, but it has few applications in the environmental field and has only recently been suggested as a promising procedure for MP/NP quantification (Long et al., 2017; Summers et al., 2018; Arkesteijn et al., 2020; Kaile et al., 2020; Elsayed et al., 2021; Tse et al., 2022; Tse et al., 2023). Fluid imaging FCM, based on high-resolution digital images, has also been proposed as a means to count and characterize MP fibers in wastewater (Hyeon et al., 2023). Flow cytometers are commonly used to detect fluorescently labeled microplastic particles having sizes in the nanometer to micrometer range and thus can successfully distinguish plastic spheres of 1-µm size from the background (Tse et al., 2022).

MP/NP removal in WWTPs can be improved by chemical precipitation based on coagulation-flocculation and sedimentation (CFS) (Kumar et al., 2021; Hidayaturrahman and Lee, 2019), but removal efficiencies reported in the literature differ significantly according to particle concentrations, size, type of coagulants, etc. Among coagulants, polyaluminum chloride (PAC), aluminum- and iron-based salts have been used for CFS (Zhang et al., 2021; Zhou et al., 2021). In particular, Zhang et al. (2021) applied CFS with PAC for the removal of MPs with sizes of 400–500 µm and found efficiency close to 100%, while the efficiency was only 40% or less in the case of particles < 400 µm, demonstrating that MPs with larger sizes can be destabilized and separated more easily than those with smaller sizes.

Therefore, the evidence that the separation of very small MNPs in municipal WWTPs is difficult and the limited knowledge about the removal of this range of particles, which are rarely investigated, highlights an important gap in this field that is an urgent challenge to address.

The research reported in this paper sought to demonstrate the great utility of FCM analysis for the rapid monitoring of 1-µm polystyrene MNPs during their removal with CFS. In particular, chemical precipitation with aluminum salts was applied to remove MNPs in various WWTP streams, such as raw wastewater, presettled wastewater and activated sludge. To the best of our knowledge, no similar research is reported in the literature due to the difficulties of monitoring such small 1-µm MNPs during the large number of experimental tests needed to evaluate the removal efficiency of CFS treatment with jar tests and various coagulant dosages and type of water.

We conducted high-throughput FCM analysis, which enabled rapid and accurate identification and quantification of 1-µm MNPs. In order to overcome the difficulty of detecting a limited, unknown and fluctuating number of MNPs naturally present in wastewater and sludge, we used traceable microsphere spikes (Long et al., 2017; Tse et al., 2022). The addition of spiked 1-µm MNPs coupled with FCM brought the following benefits: (i) well-defined dimensions and reproducible tests; (ii) accurate quantification of initial and final MNP concentrations and precise calculation of removal efficiency: (iii) accurate comparison between tests carried out under different operating conditions; (iv) simple, fast and cheap FCM analyses; (v) large number of samples that could be analysed with FCM in a reasonable period of time. Polystyrene

MNPs were chosen because polystyrene is the fourth most used synthetic polymer in consumer products such as plasticizers, antioxidants and retardants (Caputo et al., 2021) and the most common plastic material used for particles < 1000 nm in laboratory studies (Kukkola et al., 2021). Furthermore, polystyrene microspheres can be clearly detected by FCM since the scattering and fluorescence signals produced by these particles can be easily distinguished from the background/noise of the instrument. In particular, the chosen 1-µm MNPs exhibit strong fluorescence intensity which is a physical property not commonly found in MNPs naturally present in wastewater, thus allowing spiked particles to be effectively distinguished in FCM analysis, while non-fluorescent MNPs are excluded from the analysis.

Our research provides evidence that the microplastics analysis method based on FCM and spiked MNPs can validly support future studies such as deeper knowledge of the removal mechanisms of these small particles in WWTPs, and contribute to the development of new processes and technologies to improve the performance of WWTPs.

2. MATERIALS AND METHODS

2.1. Municipal WWTP and sampling of wastewater and activated sludge

Wastewater and activated sludge were collected from the full-scale municipal WWTP of Trento Nord (Trento, Italy) serving a 120,000 population equivalent. The plant treats an average daily flow rate of 21,000 m^3 d^{-1} and an average daily organic load of 11,000 kg COD d^{-1} (COD, Chemical Oxygen Demand). The layout of the plant comprises mechanical pre-treatments (fine screening, grit chamber) followed by the primary settling (2,478 m^3). Then, the biological treatment consists of an activated sludge stage (4,200 m^3) followed by secondary settlers (5,648 m^3). The treated effluents are discharged into a receiving river.

In this study, raw wastewater samples were collected at the inlet of the WWTP in pretreatments after fine screening. Pre-settled wastewater was collected after primary settling. Activated sludge samples were taken from the secondary biological treatment tanks. The physico-chemical characterization of raw wastewater, pre-settled wastewater, and activated sludge are summarized in Table 1.

Table 1. Physico-chemical characterization (Avg. ± St. Dev.) of raw wastewater, pre-settled wastewater and activated sludge collected in the WWTP. Legend: BOD_5: Biochemical Oxygen Demand, TSS: Total Suspended Solids, TKN: Total Kjeldahl Nitrogen.

Parameter	Units	Raw wastewater	Pre-settled wastewater	Activated sludge
Total COD	mg/L	711 ± 228	277 ± 75	-
Soluble COD	mg/L	285 ± 131	174 ± 58	26 ± 4
BOD_5		350 ± 111	152 ± 41	-
TSS	mg/L	338 ± 144	82 ± 44	5815 ± 1803
TKN	mg/L	65.8 ± 15.0	56.1 ± 12.3	-
NH_4^+-N	mg/L	47.6 ± 11.3	45.2 ± 10.7	2.8 ± 1.6
NO_3^--N	mg/L	1.2 ± 0.4	1.1 ± 0.4	11.5 ± 4.9
NO_2^--N	mg/L	0.2 ± 0.2	0.2 ± 0.2	0.6 ± 0.3
Total N	mg/L	66.3 ± 14.8	57.0 ± 12.8	-
Total P	mg/L	9.4 ± 3.0	8.0 ± 2.6	-
PO_4^{3-}-P	mg/L	-	-	0.8 ± 0.3
pH	-	7.6 ± 0.2	7.5 ± 0.1	7.5 ± 0.2

Raw wastewater and pre-settled wastewater differ greatly regarding total COD, BOD_5 and TSS, which are the physico-chemical parameters linked to organic matter and suspended solids.

These parameters are largely removed in primary sedimentation with an efficiency of 61%, 57% and 76% for total COD, BOD_5 and TSS, respectively. In particular, the average TSS concentration of 338 ± 144 mg TSS/L in raw wastewater was reduced to 82 ± 44 mg TSS/L in pre-settled wastewater (Table 1).

The MLSS (Mixed liquor suspended solids) concentration in the activated sludge stages was 5.8 ± 1.8 g MLSS/L, while the sludge retention time (SRT) was approximately 12 d.

The pH values did not change significantly during the process from the influent to the activated sludge stage (Table 1).

2.2. Physico-chemical analyses

All the physico-chemical parameters indicated in Table 1 were analyzed according to Standard Methods (Lipps et al., 2023). The pH values in the various water samples were measured with a portable WTW sensor.

2.3. Polystyrene MNPs with nominal size of 1-µm

Polystyrene MNPs with a nominal diameter of 1.0 µm (commercial stock Fluoro Max G0100, ThermoFisher, USA) were used in the CFS tests. These microspheres have an individual volume of 0.52 μm^3/particle and an individual weight of 5.5E-10 mg/particle. The density of polystyrene is 1.05 g/cm^3. The refractive index of polystyrene (considered in FCM analysis) is 1.589. The solids concentration in the commercial stock was 1% w/w (corresponding to 10 mg/mL). A typical working suspension of 1-µm MNPs was prepared by dispersing a few drops of the commercial stock in deionized water (1 drop in 5 mL of water) to obtain a concentration of 100 mg/L which corresponded to approximately 1.8E+11 particles/L. Aliquots of this working suspension were then added at the beginning of the jar tests (see section 2.5). Since working suspensions prepared at different times may have slightly different MNP concentrations, it is important to measure the initial concentrations at the beginning of each jar test. The 1-µm MNPs emit green fluorescence; in particular, the excitation peak is centered at 468 nm (blue fluorescence) and the emission peak occurs at a wavelength of 508 nm (green fluorescence).

2.4. Coagulant agents used in CFS treatment

Aluminum sulphate, $Al_2(SO_4)_3$, known as “alum”, was used as the coagulation agent. Commercial stock (30% w/w) is routinely used in the full-scale WWTP to accomplish the chemical removal of phosphorus from influent wastewater. This commercial stock was diluted to obtain the working alum solution at various concentrations, and then added into jar tests to study CFS treatment.

2.5. Experimental setup and jar tests

The removal of 1-µm MNPs in raw wastewater, presettled wastewater and activated sludge during CFS treatment was investigated by jar tests. To gain better understanding of the separation of 1-µm MNPs in water, sedimentation and CFS were also applied to tap water taken from the drinking water system and deionized water produced with the Milli-Q system. Tap water and deionized water were also used as blank comparisons.

To perform the jar tests, 150-mL beakers filled with 100 mL of wastewater and activated sludge were used.

An aliquot of the 1-µm MNP working suspension (prepared as in section 2.3) was added to the beaker to obtain a final concentration of approximately 1000 MNP/µL, corresponding to 0.55 mg dry weight per liter, which is a value similar to that of microplastics naturally present in sewage. For example, Okoffo et al. (2023) reported a total plastic content in raw wastewater ranging from 0.84 to 3.12 mg/L. An aliquot of the suspension was taken and analysed with

FCM according to the procedure described in section 2.6, and the result indicated the initial concentration of MNPs (MNP_0).

Then, in the jar tests with CFS, a certain dosage of the working alum solution was added to obtain concentrations of between 3 and 300 mg Al^{3+}/L. This suspension underwent rapid mixing at 300-500 rpm for 1-2 min and then flocculation was promoted using slow mixing at 80-100 rpm for 15 min. Mixing was stopped and sedimentation occurred for 1.5-2.0 h.

Finally, a 4-6 mL aliquot of the upper layer was taken and analyzed with FCM according to the procedure described in section 2.6, and the result indicated the final concentration of MNPs (MNP_f). The variability of the FCM analysis of 1-µm MNPs was evaluated using three replicates for all matrices and measured by the coefficient of variation (CV). Results indicated small CVs with values of 1.7% for deionized water, 3.4% for tap water, 3.3% for raw wastewater and 5.2% for pre-settled wastewater.

The jar tests were performed at room temperature (19-20°C), and pH values were measured during the tests.

2.6. Flow cytometry and analytical methods for 1-µm MNP enumeration

FCM analyses of MNPs were conducted with the Attune NxT Acoustic Focusing Flow Cytometer (ThermoFisher, USA) equipped with a 488 nm laser.

In the flow cytometer, photodiodes can collect 2 scattering signals and different fluorescences for each individual MNP passing through the flow cell and in front of the detectors. In particular, the following signals were used for the identification and enumeration of MNPs: Forward Scattering (FSC), Side Scattering (SSC), and Green Fluorescence (FL1) acquired with a 525/50 nm bandpass filter.

Aliquots of the samples collected at the beginning and at the end of the jar tests (carried out according to the procedure described in section 2.5) were filtered with 30-µm filters (CellTrics, Partec) to remove coarse solids that might clog the nozzle of the flow cytometer. This 30-µm filtration did not affect the concentration of 1-µm MNP in the samples because the area of a filter pore is 4600 times larger than that of a microsphere. Each filtered sample was placed in 1.5 mL Eppendorf for FCM analysis.

The procedure for the FCM analysis was as follows: (i) blank analysis to define the threshold of the FL1 signal and remove background and noise; (ii) setting the flow rate of the pressurized fluid at 25 µL/min; (iii) acquisition of at least 50,000 MNPs to obtain statistically representative results; (iv) acquisition of FSC, SSC and FL1 signals and histograms/cytograms to distinguish MNPs from other solids naturally present in wastewater and sludge.

The Limit of Quantification (LOQ) of FCM was established by analyzing 8 different concentrations of 1-µm MNPs (serially diluted) in triplicate, and the CV was calculated for each concentration. A CV < 20% was considered to confirm the lowest MNP concentration that can be quantitatively detected with the acceptable precision. In this way LOQ was estimated at 0.1 counts/µL.

2.7. Removal efficiency of 1-µm MNP after CFS

The removal efficiency of MNPs (%) obtained during the CFS treatment with jar tests was calculated by means of the following expression:

$$Removal\ efficiency\ (\%) = \frac{MNP_0 - MNP_f}{MNP_0} x\ 100$$

where MNP_0 and MNP_f are the initial and final concentrations of MNPs in the jar test, respectively, measured by FCM and expressed as the number of MNPs per µL. In particular, polystyrene MNPs are conservative and therefore the difference between MNP_0 and MNP_f indicates the concentration of particles separated by the matrix.

3. RESULTS

3.1. FCM analysis for the identification of 1-μm MPs in water samples

The 1-μm MNPs spiked in wastewater and activated sludge are extremely small microplastics, but they were clearly identified by the FCM.

Figure 1 shows the cytograms obtained for 1-μm MNPs spiked in raw wastewater, in comparison with the cytograms of raw wastewater and 1-μm MNPs spiked in deionized water. In these cytograms the three signals FSC, SSC and FL1 are combined to optimize the identification of the microplastics. The scale used on the axes represents the intensity of the respective signals expressed in arbitrary units, as is usual in FCM analyses. Each dot in the cytogram depicts a single event (a single particle) observed by the FCM. In particular, the dashed lines indicated in the cytograms delimit the area that includes the 1-μm MNPs.

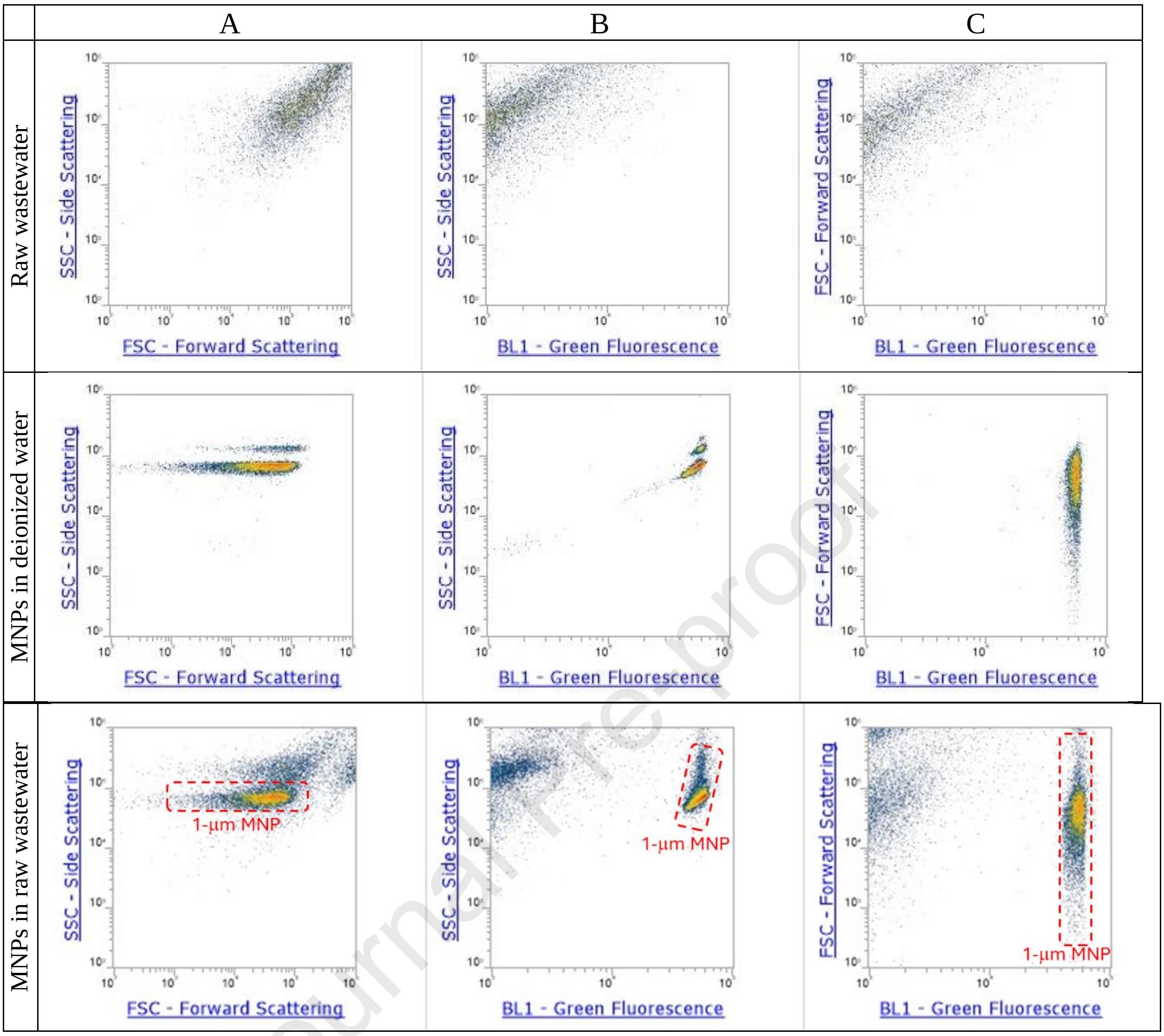

Figure 1. Cytograms of raw wastewater and cytograms of 1-µm MNPs spiked in deionized water and in raw wastewater: (A) SSC vs. FSC provides an indication of particle size and roughness; (B, C) SSC vs. BL1 and FSC vs. BL1 enable the clear distinction of fluorescent 1-µm MNP from other non-fluorescent particles in wastewater. All signals on the axes are expressed in arbitrary units.

Considering the cytograms SSC vs. FSC (Column A in Figure 1), the FSC signal provides an indication of the size and refractive index of the particles (Foladori et al., 2008), while SSC depends on the inclusions and roughness of the particles. These graphs show that 1-µm MNPs exhibit an intensity of both signals similar to that of other particles found in raw wastewater.

Therefore, because MNP signals overlap suspended solids, MNPs in raw wastewater cannot be distinguished by using FSC and SSC signals alone (Column A in Figure 1).

Instead, in the cytograms FL1 vs. SSC (Column B in Figure 1) and FL1 vs. FSC (Column C in Figure 1), 1-µm MNPs can be identified with high resolution because the fluorescence signal produced by MNPs falls in a completely different region than that of suspended solids in raw wastewater.

Similar observations were deduced from the cytograms obtained for 1-µm MNPs spiked in pre-settled wastewater (data not shown), except for the lower quantity of solids present.

The cytograms of 1-µm MNPs spiked into activated sludge are presented in Figure 2. The cytograms plotting BL1 fluorescence on the horizontal axis (Columns B, C in Figure 2) allow clear distinction of fluorescent 1-µm MNPs from other particles in activated sludge. Most solids in activated sludge are non-fluorescent and therefore they may fall below the threshold of positive BL1 signals and thus be excluded from FCM analysis and visualization in cytograms, while 1-µm MNPs remain clearly identifiable.

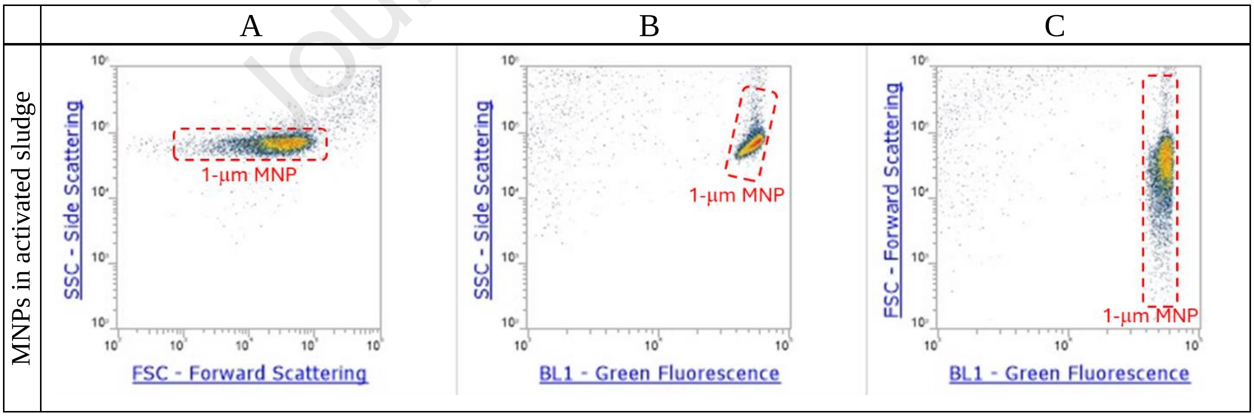

Figure 2. Cytograms of 1-µm MNPs spiked in activated sludge: (A) SSC vs. FSC; (B) FSC vs. BL1 and (C) SSC vs. BL1. All signals on the axes are expressed in arbitrary units.

These cytograms in Figure 1 and Figure 2 clearly confirm that FCM analysis makes it possible to identify and enumerate 1-µm MNPs in aqueous suspensions quickly, accurately, and precisely. In particular, signals from approximately 50,000 MNPs can be acquired in just a few minutes. In this way, MNP removal during CFS treatment can be evaluated with statistically representative results. At present, there is no other instrumentation able to provide such precise and accurate quantification of large numbers of 1-µm MNPs in complex environmental matrices like wastewater and sludge.

3.2. Coagulation-flocculation of 1-µm MNP in raw and pre-settled wastewater

First investigated was the ability of sedimentation without coagulants to remove 1-µm MNP spiked in raw wastewater. The removal efficiency was negligible and did not reach 4%, indicating that sedimentation alone cannot remove 1-µm MNP from raw wastewater, and consequently that coagulation-flocculation is required to improve the separation of microplastics with dimensions close to the nanometer-micrometer interface (1 µm).

When aluminum sulphate coagulation was used, the removal efficiency of 1-µm MNPs in wastewater was significantly improved. The graphs in Figure 3 show MNP concentrations (expressed as No./µL) in raw and pre-settled wastewater, before and after CFS, as a function of Al^{3+} dosage. In particular, in Figure 3, the Al^{3+} dosage has been distinguished into low and high values, where “low dosages” fall within the range commonly applied in full-scale WWTPs for the removal of orthophosphates; in detail, the stoichiometric ratio is approximately 1 mg Al^{3+}: 1.15 mg P plus the excess necessary for the optimization of chemical precipitation. Studies investigating MP removal in WWTP using PAC or aluminum sulphate have indicated Al^{3+} dosages ranging from 7 to 9 mg Al^{3+}/L (Hidayaturrahman and Lee, 2019; Kwon et al., 2022; Talvitie et al., 2017; Reddy and Nair, 2022). It is therefore apparent that chemical precipitation with alum for the removal of orthophosphates in WWTPs can help separate MNPs. For completeness, the “high dosages” indicated in Figure 3 were applied to explore a wider range of alum concentration and the resulting removal of 1-µm MNPs by coagulation-flocculation.

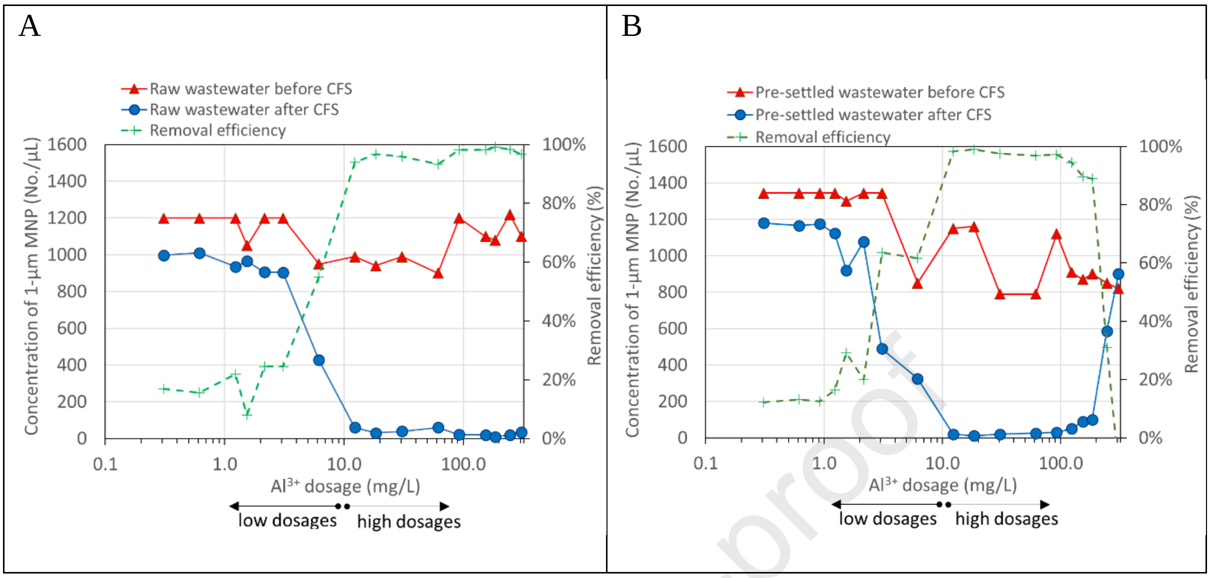

Figure 3. Concentrations of 1-µm MNPs before and after CFS treatment with various Al^{3+} dosages and removal efficiency in two different matrices: (A) raw wastewater; (B) pre-settled wastewater.

Low Al^{3+} dosages. In raw wastewater (Figure 3A), the removal efficiency of 1-µm MNPs at coagulant dosages < 3 mg Al^{3+}/L was less than 25% (average removal of 18%). Similar results were found for pre-settled wastewater (Figure 3B): the average removal efficiency was 17% for dosages < 2 mg Al^{3+}/L. More specifically, at dosages < 3 mg Al^{3+}/L, the concentration of MNPs in raw wastewater ranged from an average of 1174 MNP/µL before CFS to 953 MPs/µL after CFS, with a corresponding removal of 220 MNP/µL. Similarly, the pre-settled wastewater ranged from an average of 1337 MPs/µL before CFS to a final value of 1106 MPs/µL after CFS, resulting in a removal of 230 MPs/µL. With dosages increasing from 3 to 12 mg Al^{3+}/L (Figure 3), the removal efficiency of 1-µm MNPs increased to a maximum plateau (97% average removal) in both raw and pre-settled wastewater. These results demonstrate statistically equal MNP removal capacity in raw and pre-settled wastewater at coagulant dosages < 10 mg Al^{3+}/L. In this range, pH did not decrease below neutrality (data shown in

Figure 7B).

High Al^{3+} dosages. Dosages above 12.3 mg Al^{3+}/L in raw wastewater (Figure 3A) and pre-settled wastewater (Figure 3B) provided the highest removal efficiency of 1-µm MNPs (97% average). In both cases, this high dosage of coagulant favored the formation of flocs incorporating MNPs. The pH values were between 3.7 and 6.8, indicating that pH did not significantly influence the removal of MNPs (pH values are shown in Figure 7B).

For extremely high Al^{3+} dosages, ranging from 200 to 300 mg Al^{3+}/L, the removal efficiency of MNPs in pre-settled wastewater dropped to zero (Figure 3B), while removal in raw wastewater remained high. The decline in coagulation performance when very high dosages of coagulant are applied is expected because the flocs tend to loosen and break easily (Wu et al., 2011, Zhou et al., 2021). In this way, the effect of incorporation of surrounding MNPs by precipitates produced by coagulants, which is also an important step in coagulation, may be less efficient (Tang et al., 2015). Here, the decrease in MNP removal efficiency was clearly observed only in pre-settled wastewater. The difference between pre-settled and raw wastewater (Figure 3B) may be due to the different solids content (see Table 1) in raw wastewater (average 338 ± 144 mg TSS/L) and pre-settled wastewater (average 82 ± 44 mg TSS/L) which could have influenced the coagulation process, making the removal of MNPs more efficient when the quantity of suspended solids was higher.

3.3. Coagulation-flocculation of 1-µm MNP in activated sludge

The coagulation-flocculation process applied to the activated sludge allowed greater efficiency in the removal of 1-µm MNPs, which reached 99% even at low Al^{3+} dosages (up to 12 mg Al^{3+}/L), as shown in Figure 4. The improved separation of MNPs in activated sludge compared to wastewater can be attributed to the presence of biological flocs with a concentration of 6800 mg MLSS/L, an order of magnitude higher than 100 and 500 mg TSS/L in pre-settled and raw wastewater, respectively. However, when the Al^{3+} dosage increased above 100 mg Al^{3+}/L, the removal efficiency of 1-µm MNPs gradually decreased to below 90%, but always remained above 70% for dosages up to 300 mg Al^{3+}/L.

To understand the role of MLSS in activated sludge, jar tests were performed by adding 1-µm MNPs to activated sludge alone without any coagulant. In this test the MNP removal rate was 97.3%, which means that the CFS treatment does not add a particular benefit to the mixed liquor. This evidences that the biological process and secondary sedimentation in WWTPs can contribute on their own to a high separation of small microplastics.

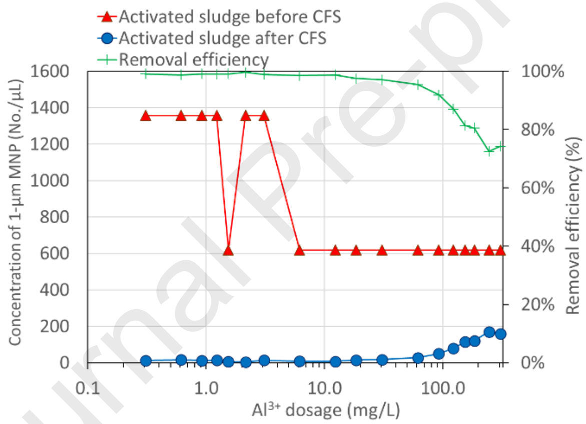

Figure 4. Concentrations of 1-µm MNPs in activated sludge before and after CFS treatment with various Al^{3+} dosages and removal efficiency.

3.4. Coagulation-flocculation of 1-µm MNP in water

To gain better understanding of the results of the removal of 1-µm MNPs due to CFS treatment in WWTPs and the role played by the presence of salts and solids, water with a negligible presence of solids but different salt content was also investigated: (1) deionized water and (2) drinking water supplied in the urban area served by the WWTP.

CFS treatment in deionized water. Figure 5 shows 1-µm MNP concentrations in deionized water before and after CFS treatment with various Al^{3+} dosages and MNP concentrations in the low range of 40-70 MNP/µL and high range of 850-890 MNP/µL. On using Al^{3+} dosages of up to the high value of 78 mg Al^{3+}/L, the removal efficiency of 1-µm MNPs did not exceed 34%: with an initial MNP concentration of approximately 900 MNP/µL, the maximum removal in deionized water was 300 MP/µL.

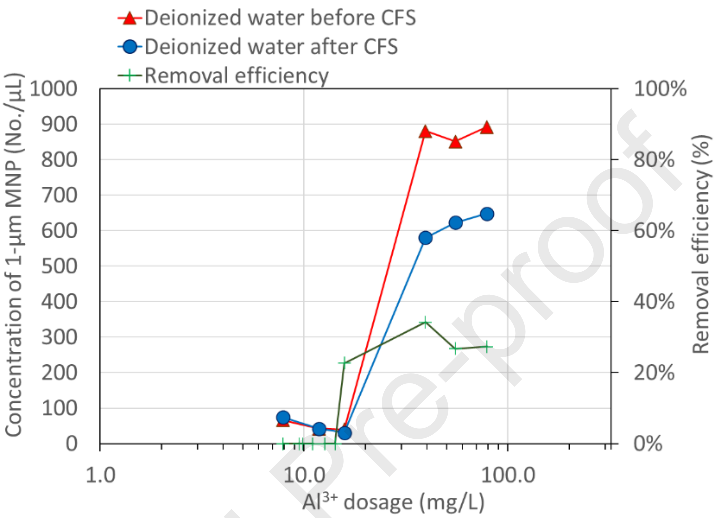

Figure 5. Concentrations of 1-µm MNPs in deionized water before and after CFS treatment with various Al^{3+} dosages and removal efficiency.

CFS treatment in drinking water. The greater presence of salts in tap water compared to deionized water is sufficient to encourage the formation of flocs by the coagulant, which is able to incorporate a greater number of 1-µm MNPs. Figure 6 shows MNP concentrations in tap water before and after CFS treatment. With Al^{3+} dosages from 3 to 30 mg Al^{3+}/L, removal increased by up to 91%. These results are in agreement with those of Skaf et al. (2020), who applied CFS and aluminum salts to MPs of size 1-5 µm in drinking water and found removals of 84-91% at dosages of 4.8-43.8 mg Al^{3+}/L. In Figure 6, the maximum removal efficiency of 97% was achieved at dosages of 46-92 mg Al^{3+}/L. The number of MNPs separated by CFS reached the maximum value of 728 MNP/µL. When Al^{3+} dosages become extremely high, above 120 mg Al^{3+}/L, the removal efficiency of 1-µm MNPs rapidly drops to 70% and below, leaving a large amount of free microplastics in the suspension that are no longer able to flocculate and settle.

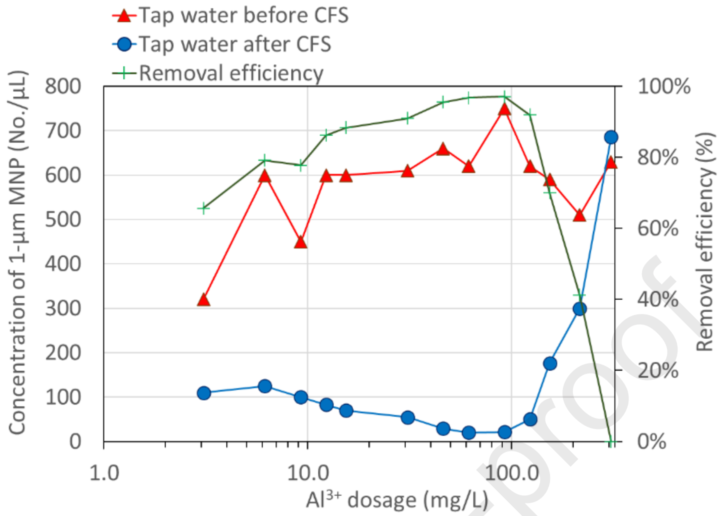

Figure 6. Concentrations of 1-µm MNPs in tap water before and after CFS treatment with various Al^{3+} dosages and removal efficiency.

4. DISCUSSION

4.1. Negligible removal of 1-µm MNPs in wastewater by sedimentation alone

In our research, we investigated the spontaneous sedimentation of 1-µm MNPs spiked in raw wastewater without coagulants, and the removal efficiency was negligible, less than 3.6%. In fact, polystyrene 1-µm MNPs with a density of 1.05 g/cm^3 are stable in water and settle very slowly when they are not adsorbed on solids or flocs. MNPs are negatively charged due to the negative sulphate group chemically bonded on their surface (Lu et al., 2018). This results in high stability in aqueous solutions due to surface electrostatic repulsion or steric repulsion between particles. These observations are in agreement with the findings of Bayarkhuu and Byun (2022), who observed that small polystyrene particles of size < 10 µm had high stability in deionized water, while only particles larger than 50 µm showed a greater sedimentation.

Only an addition of cations makes it possible to destabilize the MNPs, obtain a significant decrease in the interparticle repulsive force, and promote attraction (Lu et al., 2018).

4.2. Comparison of 1-μm MNP removal with CFS in water, wastewater and sludge

The addition of alum generates positive ions with Al^{3+} as the core hydrolyzed from the aluminum sulphate. These cations cause a decrease in the negative surface charge of the MPs (Zhang et al., 2021) and a weakening of the electrostatic repulsive force between particles which results in more probable collision, flocs formation and settlement, thus improving the removal of MPs. In this way, MPs lose their stability in water, are adsorbed on flocs, or vice versa, and precipitate by gravity. To be noted is that the efficiency of this process can be improved by the presence of a certain amount of solids in the water.

In our tests with deionized water, due to the lack of particles other than the 1-μm MNPs, the addition of Al^{3+}, even at high dosages of up to 78 mg Al^{3+}/L, was moderately effective and the removal did not exceed 34%. In fact, MNPs in deionized water can only agglomerate with each other and sedimentation is poor precisely due to the negligible formation of flocs (Bayarkhuu and Byun, 2022).

Conversely, tap water contains various types of ions (Ca^{2+}, Mg^{2+}, SO_4^{2-}, HCO_3^- and other ions typical of alpine water sources according to Pedron et al., 2022), which can influence the coagulation efficiency (Wang et al., 2013) and the removal of polystyrene MPs (Zhou et al., 2021). In particular, the presence of CO_3^{2-} is associated with greater removal efficiency, as the solution becomes alkaline and promotes the hydrolysis of coagulants (Zhou et al., 2021). Comparison of 1-μm MNP removal after CFS treatment in tap water, raw and pre-settled wastewater and activated sludge is shown in Figure 7A.

Addition of alum to raw and presettled wastewater produced nearly coincident curves (Figure 7A). At dosages of 0.3 to 3 mg Al^{3+}/L, the removal of MNPs in wastewater was less than 30%.

No flocs were observed in the wastewater treated with 3 mg Al^{3+}/L, indicating that no flocculation occurred. This means that, for low concentrations of Al^{3+}, the main process is the reduction of the negative charge, while flocculation is not visually appreciable. These results demonstrate that small microplastics can be removed to a limited extent from wastewater for dosages < 3 mg Al^{3+}/L. This matches the findings of Xue et al. (2021), who report less than 50% removal using 1.6 mg Al^{3+}/L aluminum sulphate in river water with 6-µm MPs. However, Bayarkhuu and Byun (2022) found a higher efficiency, approximately 80%, in removing 1.1-µm MPs from pond water with PAC at a dosage of 1.92 mg Al^{3+}/L. This result may be due to the properties of PAC that increase the positive charge density, which results in the better destabilization of negative charges on solids in water compared to alum. At Al^{3+} dosages increased from 3 to 12 mg Al^{3+}/L (Figure 7A), floc formation was visible, and the adsorption of MNPs onto the flocs became significant. The removal of 1-µm MNPs increased to over 90%. Organic matter in wastewater can be captured during coagulation, resulting in higher floc quality and easier sedimentation from the water (Zhang et al., 2021).

Activated sludge showed the highest removal efficiency (>95%) even for very low Al^{3+} dosages (Figure 7A). These high removal values remained stable for dosages up to 60 mg Al^{3+}/L and pH decreasing to 5.

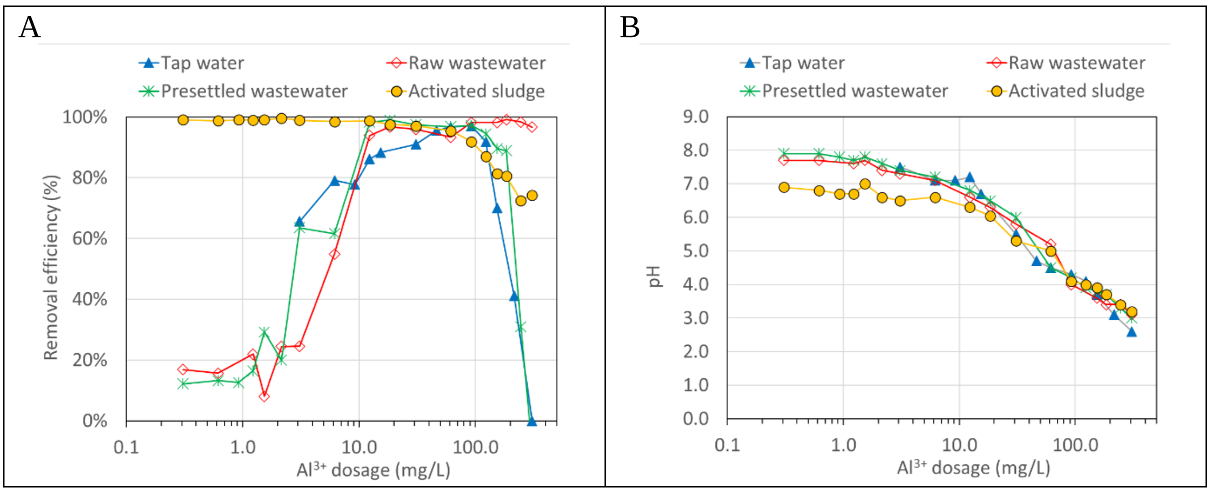

Figure 7. Comparison among tap water, raw and presettled wastewater, and activated

sludge: (A) 1-µm MNP removal after CFS treatment as a function of Al^{3+} dosage; (B) pH values in the tests.

The difference between wastewater and activated sludge in the removal of MNPs can be attributed to a different coagulation process. The flocs formed by coagulation in wastewater are small in size and are therefore not efficient enough to capture and separate MPs during sedimentation. This is particularly marked in the presence of low Al^{3+} dosages (< 12 $mgAl^{3+}/L$), since coagulation in wastewater is less effective than in activated sludge (Figure 7A). Moreover, flocs formed in wastewater are looser than those in activated sludge, which may be the reason why wastewater is less efficient than activated sludge. In fact, when the aggregates are too loose, their capacity to adsorb and trap microplastics is reduced. In contrast, a higher amount of dense flocs present in activated sludge or induced with coagulants is undoubtedly beneficial for the removal of MPs (Zhang et al., 2021).

As dosages increased above 100 mg/L (Figure 7A), the removal efficiency of MNPs decreased in pre-settled wastewater, tap water and activated sludge, while the removal rate in raw wastewater did not decrease significantly. In particular, the drop in removal efficiency observed in pre-settled wastewater was similar to tap water (Figure 7A), probably due to the reversal of the surface charge of MNPs. In detail, the surface charge inversion stabilizes the particles again and hinders their aggregation, resulting in a decrease in the number and size of the flocs and worsening the MNP separation (Skaf et al., 2020). In coagulant hydrolysis, Al^{3+} cations combine with OH^-, and extremely low pH values can cause acidic conditions that inhibit further coagulant hydrolysis. However, the cause cannot be attributed to the pH effect alone, since the pH profiles in wastewater and sludge (Figure 7B) are quite similar. This in in agreement with the observations of Zhang et al. (2021) in PAC coagulation, who indicated that pH = 3 had only a small impact on MP removal performance.

Compared to the rapid decrease in MNP removal efficiency in tap water and pre-settled wastewater at dosages above 100 mg/L, the decline in raw wastewater was negligible up to 300 mg Al^{3+}/L (Figure 7A). The overdosing of coagulants can lead to charge reversal (from neutral to positive) and restabilization of particles but the threshold that originates this effect depends on the water composition (Malik, 2018). High alum dosages inhibit interparticle interactions and form fragile floc that break down irreversibly (Jarvis et al., 2005; Marques and Ferreira Filho, 2017), reducing their ability to trap MPs, and therefore raw wastewater that has more suspended solids and flocs (compared to tap water and pre-settled wastewater) may have a greater capacity to retain MNPs.

4.3. Comparison of 1-µm MNP removals with and without CFS

To gain better understanding of MNP removal in raw wastewater and activated sludge, tests without coagulant and with CFS were compared. The 1-µm MNP removals are summarized in Figure 8. In particular, the average and standard deviations in Figure 8 were calculated by considering only the removal rates obtained for low Al^{3+} dosages (< 12 mg Al^{3+}/L), in order to reflect the conditions routinely applied in full-scale WWTPs for the chemical precipitation of phosphorus.

In raw wastewater, the absence of coagulants limited the removal efficiency of MNPs to 2.1±1.4%, while the removal increased to 17.0±7.0% after the CFS treatment with dosages < 12 mg Al^{3+}/L (Figure 8). Surprisingly, activated sludge with and without coagulants showed a similar and extremely high removal efficiency of MNPs. Even without coagulant, MP removal by activated sludge was 97.3±0.9%. In activated sludge the flocs are larger in volume and denser than those in raw wastewater and are therefore particularly efficient in capturing 1-µm MNPs during the sedimentation process even in the absence of coagulants.

Comparison between wastewater and activated sludge in Figure 8 shows that the weakening of the electrostatic repulsive force generated by coagulation with Al^{3+} (which occurs in both matrices) cannot be the only explanation for these very different results. To be noted is that the amount of solids differs by an order of magnitude between the two aqueous matrices: 6800 mg MLSS/L in activated sludge; 500 mg TSS/L in raw wastewater.

The remarkable difference in behavior between wastewater and activated sludge reveals that the solids concentration plays a significant role in the removal of MPs. In particular, TSS contain microbial extracellular polymeric substances (EPS) that influence the adsorption and flocculation of MPs in activated sludge (Geyik et al., 2016; Zhang et al., 2020, Wang et al., 2023). Wang et al. (2023) indicated that the degree of flocculation between NPs and EPS of activated sludge varies with carbon sources, suggesting that behaviors may be different between sludges. Furthermore, the floc size and shape affect the force distribution surrounding the particles, which ultimately influences the coagulation and flocculation process in activated sludge (Shahi et al., 2020; Jiang and Guan, 2006).

Especially in the activated sludge sample, a coagulant might not be needed since the removal increase would only be 1.7% (from 97.3% to 99.0%; statistically different).

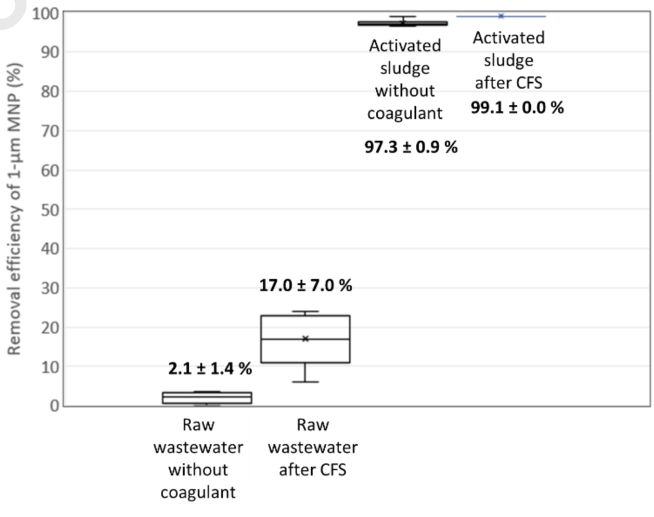

Figure 8. Removal efficiency of 1-µm MNPs without coagulant and with CFS applied to raw wastewater and activated sludge. Average ± standard deviation are indicated.

In summary, activated sludge alone can improve the adsorption of polystyrene 1-µm MNPs (97.3% removal) without it being necessary to implement an additional dosage of coagulant, which is therefore irrelevant for this liquid matrix. This observation confirms the results of previous studies that found >80% removal of plastic particles in WWTPs, which thus accumulate in the excess sludge produced in the plant (inter alia Gies et al., 2018), but pose potential environmental risks in subsequent sludge disposal.

4.4. Study limitations

This study was designed to demonstrate the great utility of FCM analysis for the rapid monitoring of 1-µm MNPs during their removal with CFS in various WWTP streams, rather than to further understand the mechanisms involved in the CFS process with alum and with different types of water.

There are many studies investigating the mechanisms of CFS in different operational conditions and in various aqueous matrices with various coagulants, and therefore the results obtained in this study may appear partial and not exhaustive. To investigate the mechanisms responsible for the removal of 1-µm MNPs when CFS is applied to complex and very different matrices such as tap water, raw and pre-settled wastewater and activated sludge, further research is needed. Consequently, measurement of additional parameters such as zeta potential, microscopic observation of flocs size and shape, could be beneficial to better explain the results of this study.

Another study limitation that we recognize is the use of spike of 1-µm polystyrene MNPs that is practical but does not represent the MPs actually present in water. Ideally, we should have used MNPs naturally present in wastewater but the main reason for using artificial MNPs was: (i) difficult identification and quantification of MNPs in each WWTP stream (wastewater, sludge), (ii) inability to analyze hundreds of samples in a reasonable time frame; (iii) absence of a standardized methodology, (iv) low MNP concentrations that do not allow the maximum removal rate to be estimated.

Studies on the removal and fate of MNPs naturally present in wastewater require methods that can provide information on both quantification and chemical identification. Therefore both mass-based methods (e.g. Py-GC/MS, TED-GC/MS) and particle-based methods (e.g. IR spectroscopy combined with μ-FTIR) have been proposed for MNP analysis. Morphology and surface properties can be observed with SEM/EDX even for small MNPs in the order of 100 nm. In this context, FCM is very efficient for rapid quantification, but cannot provide information on individual polymer types and particle shape. It could give indications on the size distribution but this only at a potential level as further research is needed to convert the scattering signal into real dimensions. However, FCM allows the analysis of very small MNPs, around 0.5 μm and smaller. MNPs with these dimensions can be analysed with μ-Raman spectroscopy allowing chemical characterisation and quantification, but this analysis is time-consuming and requires suitable spectral libraries and model-based classification methods. At present, standardization of these methods is still under development, especially for MNPs around 1 μm and smaller.

4. CONCLUSIONS

High-throughput flow cytometry proved to be a fast and accurate procedure with which to identify and quantify in a few minutes individual 1-μm MNPs spiked in raw and pre-settled wastewater and activated sludge. By means of quantification with FCM, the removal efficiency of 1-μm MNPs during CFS treatment and using varying dosages of aluminum salts was rapidly calculated. In particular, the FCM enabled the analysis of over 130 samples containing 1-μm

MNPs, which could not be determined otherwise, considering that such small microplastics are currently considered very difficult to enumerate in wastewater and sludge.

In wastewater, sedimentation alone, but without coagulants, removed less than 4% of 1-µm MNPs. Instead, CFS with aluminum sulphate resulted in destabilization and aggregation of 1-µm MNPs. On adding 0.3-3 mg Al^{3+}/L to wastewater, the average removal of MNPs was 17%, while it increased to over 90% at dosages of 3-12 mg Al^{3+}/L.

Activated sludge, with solids content of 6800 mg MLSS/L, displayed extremely high MNP removal efficiency (>95%) with negligible benefit from the addition of the coagulant.

In summary, the spike of very small microplastics coupled with FCM analysis highlighted a useful and alternative approach with which to investigate the fate of MPs in WWTPs, improve knowledge in this field, and support future studies on new processes and technologies for the removal of MPs.

Authors' contributions

PF: conceptualization, methodology, investigation, supervision, writing the original draft, funding acquisition; **GL**: conceptualization, definition of the experimental protocol, investigation, methodology, writing and editing; **AT**: methodology, validation, writing-review and editing; **LB**: methodology, formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests.

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- High-throughput flow cytometry to enumerate 1-µm microplastics in few minutes
- Coagulation-flocculation to investigate microplastics removal from wastewater and sludge
- 1-µm microplastics in wastewater are removed by 30% with 0.3-3 mgAl^{3+}/L
- 1-µm microplastics in activated sludge are removed by 99% with 0.3-3 mgAl^{3+}/L
- Activated sludge showed removal of 1-µm plastics of 97% even without coagulants

Declaration of interests

☒ 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.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: