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Microplastics in the urban water cycle: focus on analytical determination methods and presence in drinking water treatment plants effluents

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Abstract (English)

The European Union has assessed in the new Drinking Water Directive the need for the definition of an analytical protocol for microplastics (MPs) monitoring in water for human consumption by 2024. This specification stems from the awareness of the public and the authorities of MPs as a pervasive and persistent pollutant, that needs to be understood in its presence and fate to assess the associated health risk. MPs in drinking water however cannot be thought and studied without consideration of the whole urban water cycle, due to the strong interconnections between its elements.

The work presented in this thesis addresses therefore the topic of MPs presence in the urban water cycle focusing on their determination (analytical methods and data reporting), having as specific object the MPs in the effluents of drinking water treatment plants (DWTPs).

The research is also framed in the context of the integrated water management and the topic is observed considering the point of view of the water utilities too. In this context, the aim is to offer an overview according to the state of the art on the presence of MPs in the urban water cycle and the main influencing parameters, as well as to develop a method for the routine analysis of MPs in DWTPs effluents, balancing the time-cost-reliability triad. These goals were achieved by extensive literature research on MPs presence and analytical methods. Moreover, laboratory activities were run at the facilities of Acea Elabori, society of engineering and services of Acea S.p.A, the main water utility of central Italy. Aim of the laboratory activities was the full understanding of potential and limitation of the μ -Raman technique for the routine analysis of MPs in the effluent of DWTPs, and the definition of the analytical method.

The application of a spectroscopic technique coupled to a microscope has however inner limitations, such as the lack of MPs volume and mass among the analysis outputs. These metrics are of utmost relevance for proper management of the urban water cycle by water utilities (e.g. for mass balances, source apportionment, risk assessment). To overcome these limitations, the experience abroad, carried out at the Aquatic Ecology and Water Quality Management Group of the Wageningen University and Research (WUR) (Netherlands), was devoted to the study and evaluation of methods for the estimation of MPs volume from the available outputs of spectroscopic techniques.

The findings of the research allow the consideration that MPs are continuously fed and recycled within the water cycle. The influencing factors on MPs abundance need however to be better framed at various time-space scales. Analytical methods are improving not only from a technological point of view, but also on the quality of provided data: procedures for quality assessment and control are being developed by the scientific

community to frame the reliability and uncertainties of the methods, and propose data that can be critically read and evaluated on solid bases.

The μ -Raman is a strong method for MPs analysis, especially for routine analysis, due to the high level of automatization that can be reached. However, the potential and analytical limits of the μ -Raman should be considered also as a function of such an automatization: for example, the precision of the microscope stage might increase the minimum size analysable and the chemical identification of the particles should be critically evaluated.

The cost for MPs analysis with the method developed appears from preliminary evaluations to be comparable to that of other Contaminants of Emerging Concern (CECs).

The time required for the analysis depends however on the efficiency of the pre-treatments applied and the volume analysed. These two aspects need to be balanced to accommodate the need of the laboratory organization in the context of routine analysis.

The results from the application of the studied method to the effluent of a DWTP confirm the data on the presence of MPs with size $>20\mu\text{m}$ reported in the scientific literature. However, their quantification was possible only in one sample of one litre over five samples total. This testifies on one hand the presence of MPs $>20\mu\text{m}$ in the effluents from DWTPs, but also their low concentration, especially when evaluated per polymer group (<1 MPs/L). The method applied is considered suitable for the evaluation of the analytical procedure and the screening of DWTPs effluent. However, it needs to be modified if the goal is the complete characterization of the MPs pollution in the DWTPs effluent: samples of higher volume are in this case to be taken, and the whole procedure slightly modified. The core aspects of the analysis are however the same and the main observations and points of attention still valid. The innovation of the research lies in the definition of a screening method for MPs $>20\mu\text{m}$ in effluent from drinking water plants, considering its cost and taking into account the perspective of water utilities. Further innovation lies in the approach used for the research conducted at WUR to estimate volume from data obtainable from micro-spectroscopic techniques.

Abstract (Italiano)

L'Unione Europea ha stabilito nella nuova Direttiva sulle acque potabili la necessità di definire un protocollo analitico per il monitoraggio delle microplastiche (MPs) nelle acque destinate al consumo umano entro il 2024. Questa specifica nasce dalla consapevolezza del pubblico e delle autorità che le MPs sono un inquinante ubiquitario e persistente, di cui è necessario approfondire le conoscenze per valutarne il rischio associato. La presenza di MP nelle acque potabili, tuttavia, non può essere pensata e studiata senza considerare l'intero ciclo urbano dell'acqua, a causa delle forti interconnessioni tra gli elementi del ciclo stesso. Il lavoro presentato in questa tesi affronta quindi il tema della presenza di MPs nel ciclo urbano delle acque, concentrandosi sulla loro determinazione (metodi analitici e reporting dei dati), avendo come oggetto specifico la loro presenza negli effluenti degli impianti di trattamento delle acque potabili (Drinking Water Treatment Plants, DWTPs).

La ricerca è inquadrata nel contesto della gestione integrata delle acque e l'argomento viene osservato considerando anche il punto di vista delle società del servizio idrico integrato. In questo contesto, l'obiettivo è quello di offrire una panoramica sullo stato dell'arte della presenza di MP nel ciclo urbano delle acque e sui principali parametri che la influenzano, e di sviluppare un metodo valido per l'analisi di routine delle MPs negli effluenti dei DWTPs, bilanciando tempo-costo-affidabilità.

Questi obiettivi sono stati raggiunti attraverso un'ampia ricerca in letteratura sulla presenza delle MPs nel ciclo idrico urbano e sui metodi analitici applicati. Le attività di laboratorio sono state condotte presso le strutture di Acea Elabari, società di ingegneria e servizi di Acea S.p.A., il principale gestore del servizio idrico integrato del centro Italia. L'obiettivo delle attività di laboratorio è stato quello di comprendere appieno le potenzialità e i limiti della tecnica μ -Raman per l'analisi di routine dei MP negli effluenti dei DWTPs e di definirne il protocollo analitico.

L'applicazione di una tecnica spettroscopica accoppiata a un microscopio come il μ -Raman ha delle limitazioni intrinseche, tra le quali spicca l'impossibilità di determinare il volume e della massa delle MPs, come risultati direttamente ottenibili dall'analisi. Il lavoro effettuato presso l'Aquatic Ecology and Water Quality Management Group della Wageningen University and Research (WUR) (Paesi Bassi), durante un periodo di 6 mesi svolto nell'ambito del Dottorato, è stato dunque dedicato allo studio delle strategie per produrre anche questi dati. Nello specifico, si sono valutati i metodi per la stima del volume di MPs partendo dai risultati direttamente ottenibili da tecniche micro-spettroscopiche.

I risultati delle attività di ricerca permettono di concludere la continua alimentazione di MPs e il riciclo delle stesse nelle varie fasi del ciclo idrico urbano. I fattori che influenzano l'abbondanza di MPs devono tuttavia essere meglio inquadrati a diverse scale spazio-temporali. I metodi analitici stanno migliorando non solo dal punto di vista tecnologico, ma anche per quel che riguarda la qualità dei dati forniti: la comunità scientifica sta sviluppando procedure di valutazione e controllo del metodo, per definire l'affidabilità e le

incertezze dei metodi e proporre dati che possono essere criticamente letti e valutati su basi solide.

Il μ -Raman è un metodo affidabile per l'analisi delle MPs, soprattutto per quelle di routine, grazie all'elevata automatizzazione raggiungibile. Tuttavia, le potenzialità e i limiti analitici del μ -Raman devono essere considerati anche in funzione di tale automatizzazione: ad esempio, la precisione dello stage motorizzato del microscopio potrebbe aumentare la dimensione minima analizzabile. Inoltre deve essere valutata criticamente l'identificazione chimica delle particelle.

Il costo dell'analisi delle MPs con il metodo sviluppato sembra essere paragonabile a quello di altri contaminanti di interesse emergente (Contaminants of Emerging Concern, CECs).

Il tempo richiesto per l'analisi dipende tuttavia dall'efficienza dei pretrattamenti applicati e dal volume analizzato. Questi due aspetti devono essere bilanciati per soddisfare le esigenze dell'organizzazione del laboratorio nel contesto delle analisi di routine.

I risultati ottenuti dall'applicazione del metodo studiato all'effluente di un impianto di potabilizzazione confermano la ridotta presenza di MPs con dimensioni $>20\mu\text{m}$, come riportato anche nella letteratura scientifica. Tuttavia, la loro quantificazione è stata possibile solo in un campione di un litro su un totale di cinque campioni. Ciò testimonia da un lato la presenza delle MPs $>20\mu\text{m}$ negli effluenti dei DWTP, ma anche la loro bassa concentrazione, soprattutto se valutata per gruppo polimerico (<1 MPs/L). Il metodo applicato è dunque considerato idoneo e per lo screening degli effluenti dei DWTPs. Tuttavia, esso deve essere modificato se l'obiettivo è la caratterizzazione completa dell'inquinamento da MPs negli effluenti dei DWTPs: in questo caso è necessario prelevare campioni di volume maggiore e modificare leggermente l'intera procedura. Gli aspetti fondamentali dell'analisi rimangono comunque gli stessi e le principali osservazioni e punti di attenzione ancora validi. L'innovatività della ricerca risiede nella definizione di un metodo di screening per le MPs $>20\mu\text{m}$ negli effluenti degli impianti di potabilizzazione, considerandone i costi e tenendo conto del punto di vista delle water utilities. Ulteriore elemento innovativo è il l'approccio utilizzato per il lavoro prodotto presso la WUR per la stima del volume a partire dai dati ottenibili da tecniche micro-spettroscopiche.

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Foreword

The research work was developed at the laboratories of Acea Elabiori, society of engineering and services of Acea S.p.A, one of the biggest utilities in Italy. Acea Elabiori indeed made available the use of a μ -Raman, with the aim of developing methods for the analysis of Microplastics (MPs) in the urban water-cycle: due to the review of water policies by the European regulators (see paragraph 1.3), water utilities are expected to become able to monitor MPs in the urban water cycle in the years to come.

The research questions addressed by the present research are therefore all linked by this real and impending need for monitoring.

The method applied approached the topic first from a general point of view, to set the context of the research. Therefore, the state of the art on analytical methods for wastewater, sludge, and drinking water were addressed, as the presence and fate of MPs in the urban water cycle were reviewed. Once the context and the related issues and turning points were set and cleared, the focus was shifted to microplastics in the effluent of a drinking water treatment plant (DWTP), which represents the matrix for which the need for monitoring is most imminent. A method was developed for the analysis of MPs with μ -Raman in the DWTP effluent. The DWTP effluent was monitored for two weeks and the results were carefully evaluated.

Spectroscopic techniques, among which there is the μ -Raman used during this research work, do not offer information on MPs volume or mass. However, such metrics are of utmost relevance for the assessment of MPs fate and related risks in the urban water cycle. Therefore, the strategies available for the volume estimate from MPs 2D information, which are available from the application of μ -spectroscopic techniques, were evaluated during the period abroad. This is a first step towards the completion of the information offered by the spectroscopic techniques. It is an added value that might be used in the future by water utilities for a better management of MPs pollution.

The specific research questions addressed by the present study are listed below:

- RQ1: MPs definition, describing parameters, legislation
Defining the MPs characteristics, categories used to describe and classify them, recent trends followed for a complete description and comparison of the MPs continuum
- RQ2: State of the art on MPs presence and fate in the urban water cycle

Knowledge and fate of MPs in the urban water cycle, the major influencing factor of their diffusions, needs of additional data

- RQ3: State of the art on MPs analysis in the urban water cycle
The steps of a protocol for the analysis of MPs in waters, the critical points, weak spots and possible solutions, reliability and significance or misguidance of the data retrievable by MPs analytical protocols
- RQ4: Definition of an analytical protocol for MPs routine analysis in the effluent of a drinking water treatment plant (DWTP)
Laboratory activities to set an analytical protocol for routine analysis
- RQ5: Results of the monitoring activity of MPs in a DWTP (case study)
Application of the protocol defined in RQ4 to one full-scale DWTP, results evaluation and critical analysis.
- RQ6: Strategies for MPs volume estimation
Developed at the Aquatic Ecology and Water Quality Management Department (AEW) of the Wageningen University and Research, under the supervision of Professor Albert A. Koelmans, focused on MPs dimension 1-5mm, to evaluate a new approach to assess the reliability of current strategies for volume estimation from the whole MPs mix

Table 1 GANTT of the Research activity addressed during the Ph.D. period (November 2019-January2023) (: laboratory work)

year	2020					2021					2022			
trimester	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV
RQ1														
RQ2														
RQ3														
RQ4														
RQ5														
RQ6														

The thesis is organized in eight Chapters. The general aspects related to MPs are described and critically evaluated in Chapter 1, including MPs definition, public outreach and legislation. Chapter 2 presents an overview on the influencing factors for MPs abundance in the urban water cycle. Chapter 3 comments on the analytical protocols in use for the

analysis of MPs in the urban water cycle. Chapter 4 describes in more detail the application of the μ -Raman to the MPs analysis. Chapter 5 illustrates the development of a method for the analysis of MPs in the effluent of a DWTP, using specifically the μ -Raman DXR3 Thermo Fisher. Chapter 6 reports the results of the application of such method to a case study. The work developed during the period abroad is reported in Chapter 7. Conclusions and future perspectives are addressed in Chapter 8.

Chapter 1. Microplastics (MPs)

1.1. The theme and public outreach

Microplastics studies (MPs) represent a recent research field, taking its first steps just two decades ago. Microplastic research stems from the analysis of marine water quality status, and its causes. Indeed, MPs were first mentioned in relation to marine environments (Thompson et al., 2004); then, wondering about the sources of such new pollutants, the research expanded to surface waters and wastewater treatment plants (WWTPs). At this point, observing the ubiquity of MPs in every matrix and location analyzed, the research was extended to all environmental compartments, including therefore air, water and soil. There is no doubt nowadays about MPs ubiquity and persistence (Barchiesi et al., 2021). The steep learning curve observed at the beginning of the MPs research, however, has now tilted a bit, due to the need of comparable and reliable data to guarantee an effective advancement in knowledge. There is indeed no internationally recognized and applied method for MPs analysis yet. If the presence of MPs can now be given for granted, their quantitative abundance, spatial and temporal patterns, dynamics transport, and risk are still unclear. More scattered evidence of presence at this point of the research do not add insights on the MPs pollution phenomenon. The research question the scientific community is now trying to answer is not IF MPs are present somewhere, but how many, their characteristics and dynamics. Necessarily, the development of methods that guarantee the uncertainties to be circumscribed, quantified and declared, is a field progressing in parallel. The definition of common, consistent and reliable analytical methods is indeed the fundamental step to ensure studies comparison for a true advancements in MPs understanding. This tendency and shift in interest by the scientific community has been clear in these three years of PhD. research, with an increased number of papers published, particularly on the quality assessment and control (QA/QC) of the analytical methods and also on the quality criteria to present MPs data (De Ruijter et al., 2020; Koelmans et al., 2019; Schymanski et al., 2021).

The public outreach of the issue “MPs pollution and related risk” has been anyhow wide and the public answer strong and clear. The particular sensibility to the topic might be linked to the multiple aspects of the MPs pollution that can stir response in the public: these aspects are mainly ethical, aesthetic and last but not least, linked to the environmental risk (Koelmans et al., 2017). The ethical debate is related to the introduction and force of an exogenic material into the environment. The esthetic issue is linked mostly to macroplastics, however also MPs play their role in reduction of the visual appeal of locations and settings. Furthermore, there is the potential environmental risk. However, considering the public concern, this aspect is either the worst communicated, or the worst

understood (Catarino et al., 2021; Kramm et al., 2022). The concentration discovered in the environment and those of effect on biota recorded in the laboratories are still often afar. Moreover, there is quite a debate on the methods of assessing the MPs risk and effects (De Ruijter et al., 2020; Koelmans et al., 2022). The certainties on the topic are therefore scarce. This uncertainty seems to be not well transferred by the bold headers that present the issue (e.g the reporting of the findings of Wit and Bigaud (2019), estimating the ingestion of a “credit card” worth of MPs (Wit and Bigaud, 2019)). However, due to their persistence and continuous discard of macroplastics into the environment, it is a foreseeable outcome the increase of MPs concentration until the limit threshold of acceptable risk (Koelmans et al., 2022). This awareness calls therefore for the engagement of the scientific community for a full understanding of the MPs pollution and its related risks.

1.2. Definition and describing parameters

The wide research carried out so far on MPs still have not solved definitively the question on how to define MPs. Indeed, the lack of a common official definition has not impeded the progress of knowledge on MPs. However, the idea of a definition to help the comparison arises, now that the issue is mostly related to the availability of comparable results. Furthermore, there is the need of a definition for regulators to circumscribe rules and norms.

To define MPs is not an easy task. First of all there is the need to define what is “plastic” and what is “micro”.

By the ISO 472:2013, plastic is defined as:

“plastic”, noun

material which contains as an essential ingredient a high polymer and which, at some stage in its processing into finished products, can be shaped by flow

Note 1: Elastomeric materials, which are also shaped by flow, are not considered to be plastics.

Note 2: In some countries, particularly the United Kingdom, the term “plastics” is used as the singular form as well as the plural form.”

According to the IUPAC (Vert et al., 2012), the definition is:

“plastic”

Generic term used in the case of polymeric material that may contain other substances to improve performance and/or reduce costs.

Note 1: The use of this term instead of polymer is a source of confusion and thus not recommended.

Note 2: This term is used in polymer engineering for materials often compounded that can be processed by flow.

Both these definitions are quite generic.

The other term to be defined to have an overall definition of “Microplastic”, is “micro”. For this, we will refer to the definitions in use by the scientific community in the field of MPs studies. *Micro* is:

- 1µm-5mm (Vianello et al., 2013)
- 100nm-5mm (Alimi et al., 2018)
- 1µm-1mm (Van Cauwenberghe et al., 2013)

MPs are therefore a broad concept. There is indeed not enough information on their behavior in the environment or effect on ecosystems to reduce or widen the field of meaning for the term MPs based on their environmental or health risk.

The definition of MPs used by few authorities and regulators are hereby reported (list not exhaustive):

WHO: *“Microplastic – a material consisting of solid polymer-containing particles, to which additives or other substances may have been added, and in which $\geq 1\%$ w/w of particles have (i) all dimensions $1\text{ nm} \leq x \leq 5\text{ mm}$, or (ii), for fibres, a length of $3\text{ nm} \leq x \leq 15\text{ mm}$ and a length to diameter ratio of > 3 . Polymers that occur naturally and have not been chemically modified (other than by hydrolysis) are excluded, as are polymers that are (bio)degradable; plastic particle $< 5\text{ mm}$ in diameter; $1 - < 1000\text{ }\mu\text{m}$ ”* (World Health Organization, 2022)

NOAA: *“Microplastics are plastic particles smaller than 5.0 mm in size (Arthur et al. 2009). The lower bound (size) of the microplastics is not defined; however, it is common practice to use the mesh size ($333\mu\text{m}$ or 0.33mm) of the neuston nets used to collect the samples (Arthur et al. 2009)”* (Masura et al., 2015)

California Water Board: *“Microplastics in Drinking Water are defined as solid polymeric materials to which chemical additives or other substances may have been added, which are particles which have at least three dimensions that are greater than 1nm and less than 5,000 micrometers (μm). Polymers that are derived in nature that have not been chemically modified (other than by hydrolysis) are excluded”.*

Evidence concerning the toxicity and exposure of humans to microplastics is nascent and rapidly evolving, and the proposed definition of ‘Microplastics in Drinking Water’ is subject to change in response to new information. The definition may also change in response to

advances in analytical techniques and/or the standardization of analytical methods.”
(California State Water Resources Control Boards, 2020)

EU (ECHA): *“‘microplastic’ means a material consisting of solid polymer-containing particles, to which additives or other substances may have been added, and where $\geq 1\%$ w/w of particles have (i) all dimensions $1\text{nm} \leq x \leq 5\text{mm}$, or (ii), for fibres, a length of $3\text{nm} \leq x \leq 15\text{mm}$ and length to diameter ratio of >3 . Polymers that occur in nature that have not been chemically modified (other than by hydrolysis) are excluded, as are polymers that are (bio)degradable.”*(ECHA, 2019)

Another definition, restricted to the primary MPs (those produced as such), is used in the Commission Regulation (EU) amending Annex XVII to Regulation (EC) No 1907/2006 of the European Parliament and of the Council concerning the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH): it defines the chemical composition of MPs as “synthetic polymers” and does not set a lower size boundary.

Considering now the field of scientific research, if a strict definition for MPs is not required for an advancement in knowledge, what is of utmost relevance is the chance of studies comparison. On this account, it might be said that the two crucial points to be always kept in mind are:

1. Comparison of results is a matter of method
2. Comparison of results is a matter of results representation

Point 1 refers to the fact that, depending e.g. on the reagents used for sample preparation, different particles of different materials might be selected, independently from the size. However, also these results might turn useful if care is given to point 2, and if method and linked uncertainties are clearly stated and defined, if not quantified.

Point 2 refers indeed to how results are shown. The clearest example is the impossibility of comparing MPs size categorized (see following paragraph) in different size bins, whereas it is possible to compare results reported by the use of continuous probability distributions (Kooi and Koelmans, 2019)).

Moreover, the object of study (MPs) should be better described as completely as possible, to help the scientific community interpret the results obtained by a study.

Microplastics, for what seen so far, are 3D solid pollutants that might be composed by many different polymers that span many orders of magnitude in size.

Four parameters are mostly used in the scientific literature to describe MPs (Cole et al., 2011; Magni et al., 2019; Pivokonsky et al., 2018):

1. Size
2. Chemical composition
3. Shape
4. Origin: MPs are divided in primary MPs (those produced to be as such) and secondary MPs (those formed as results of fragmentation and embrittlement)

Indeed, the first three variables are the outcome of the analysis of MPs by spectroscopic techniques, which are among the most used for MPs analysis (see Chapter 3). Therefore, the first three categories are chosen by habit and used as the minimum information to make available when discussing results of a MPs study.

These four categories correspond to aspects linked to the single MP, that therefore can be considered as variables. Size is a continuous variable and it is usually reported through histograms, plotting the longest dimension. The choice of the bins size however is often random or linked to the practical aspects of the analysis, due to the lack of a standardized analytical method. This causes the impossibility of comparison between studies with different bins' width. Moreover, the use of histograms is not true to the continuous nature of the variable and its proper representation. As remarkably clarified and explained by Kooi et al. (2019), MPs size is better described by the use of continuous probability distribution (Kooi and Koelmans, 2019). The use of continuous probability distribution instead of histograms allows not only the comparison among different studies on the same environmental compartment, but also a more straightforward comparison of studies related to different environmental compartments. This in turn allows for a more complete overview of the phenomenon "MPs pollution". Another work in this direction, studying MPs size as a continuous variable, was carried out by Alkema et al. (2022) (Alkema et al., 2022). The power of the approach and the possibilities it holds for a true advancement in MPs pollution understanding through comparison, makes it advisable to try and follow it as much as possible.

The same reasoning was tried also for the categories of shape and chemical composition, in terms of density (Kooi and Koelmans, 2019). However, the chemical composition is representative even if considered as a qualitative nominal variable, due to the difficulties in determining the density of the particle itself, compared to the grouping of its composition to a polymer category. Regarding shape, the use of continuous probability distribution is also advisable, using shape factors (e.g Corey shape factor, Circularity, Aspect Ratio) instead of a qualitative variable. Indeed, considering taxonomy, Cowger et al. (2019) reported the use of 19 different terms in literature, some partially overlapping or with an untidy system of sets (e.g. fibers and fragments vs fibers and a subdivision of fragments in different taxa, or beads vs spheres vs pellets) (Cowger et al., 2019).

The possible relationship between these variables are also of utmost interest: e.g. do MPs become rounder with decreasing size? Does this happen in every matrix analyzed?. Describing hence MPs with a multi-dimensional probability distribution might open new paths for pollution understanding and interpretation.

These categories however give no information on other possibly environmentally relevant metrics such as mass, volume and surface area. The topic is addressed more in detail in Chapter 7.

1.3. Present policies tackling MPs pollution

Policies on MPs can be divided until now in two groups:

1. Policies that target MPs directly
2. Policies that have an indirect effect on MPs pollution

The first group can be further divided in policies that target primary MPs (those produced as such) and those that target MPs overall (primary and secondary).

A clear overview of the EU policies on addressing plastic pollution and MPs reduction in force or foreseen for the near future by the EU regulatory organisms, is found in the SAPEA report of 2019 (SAPEA, 2019).

MPs are explicitly mentioned in:

- REACH (EC 1907/2006) Oxo-degradable plastics and intentionally added microplastics
- Single Use Plastics (SUPs) and Fishing Gear (COM (2018)340)
- The Marine Strategy Framework Directive (2008/56/ EC) and the amending Directive 2017/845/EC and Commission Decision 2017/848/EC
- The EU Plastics Strategy (COM/2018/028)

(SAPEA, 2019).

The European Union has published the final draft of the restriction proposal on intentionally added MPs on August 30 2022. It is now in discussion between the member state authorities.

Moreover, they have been explicitly mentioned also in the new drinking water directive (The European Parliament and the Council of the European Union, 2020) and the revision to the wastewater directive (European Commission, 2019). The new Drinking Water Directive states that by 2024 a method must be defined for the monitoring of water to be destined to human consumption and that by 2029 a report on the potential threat to

sources of water intended for human consumption is to be submitted (The European Parliament and the Council of the European Union, 2020).

Regarding water management, outside of Europe, it is of interest the policies that are being developed in California, where thanks to the Senate Bill 1422 a method for the analysis of Microplastics in Drinking Water was developed. It is now in force the monitoring of MPs in drinking water for four years, in order to evaluate possible restrictions (California State Water Resources Control Boards, 2021).

Conclusively, policies on MPs in water are enforced with ban and restrictions only on primary MPs and at the level of production and commercialization. Monitoring strategies are instead beginning to be enforced on environmental matrices to better understand the presence and effect of MPs on the ecosystems.

Chapter 2. Presence and fate of MPs in the urban water cycle



Presence and fate of microplastics in the water sources: focus on the role of wastewater and drinking water treatment plants

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The presence and fate of MPs in the urban water cycle has been addressed by an extensive literature research, which results have been published in the Journal of Water Process Engineering. The main outcome of the research are hereby reported and commented. The work is an overview of what is known about MPs in the water cycle, which are the influencing factors for their abundance and dynamics and where the lack of data are more evident. This information is indeed relevant in view of MPs pollution management in the urban water cycle. Regarding data abundance, the lowest information are related to MPs in groundwater sources, followed by drinking water, as shown in Figure 1. More data are instead available for wastewaters and surface waters. A possible reason for such discrepancy is the lower abundance of MPs in cleaner matrices (such as groundwater and drinking water) which makes their detection harder due to the need of an almost absent contamination during the analytical procedures. Another possible reason is the historical development of MPs studies, which started with the marine environment, then looked for the sources of MPs in such environmental compartments hence the surface waters and WWTPs. The effort exert by the scientific community to address and characterize the MPs pollution phenomenon is however continuously enriching the information available (see Chapter 5 and 6).

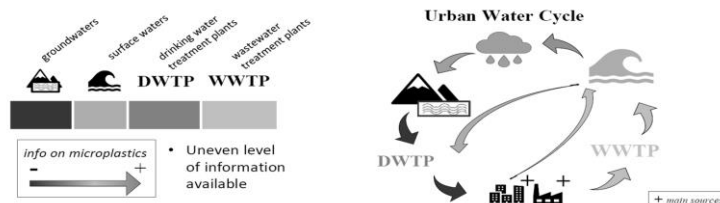


Figure 1 Abundance of MPs information in the water cycle (Barchiesi et al., 2021, modified).

Regarding MPs sources, with consideration to the urban water management, it is put under the spotlight the role of the WWTPs as sources of MPs to surface waters, the influencing factors for their abundance in WWTPs and the influencing factors in surface water. The results obtained are summed up in the following tables. First, the abundance of MPs in inner waters was observed and the information on the main influencing parameters retrievable from the literature were categorized (analysis methodology, socio-economical features, weather and climate, hydrological features) (Table 2)

Table 2 Factors influencing MPs abundance in surface water (Barchiesi et al., 2021)

Factor	Ref.	Parameter	Main conclusions
Analysis methodology	1	Sampling device	Statistically significant difference in MPs concentration upstream and downstream WWTPs effluent with a manta trawl net, no statistically significant difference with a pumping device sampler
	2	Sampling device	MPs abundance and the fibre content detected with a pump sampling device was significantly higher compared to that recovered by plankton nets
	3	Sampling point	Differences in MPs concentration between the left, middle and right section of the stream
	4	Sampling point	MPs mainly distributed on the surface compared to the other depth levels
Influence of socio-economical features	5	GDP, population density, (land use)	Significant correlation of parameters with MPs concentration, (positive correlation though not significant with build-up land use)
	6	GDP, population density	Significant correlation of parameters with MPs concentration
	7	Per capita GDP, primary industry	Significant correlation of parameters with MPs concentration, (correlation though not significant with population density, basin area, runoff and tertiary industry)
	8	industrialization	MPs concentration significantly higher in industrial areas
	9	industrialization	Packing industry as probable source of the MPs
	10	urbanization	The predictors indicative of urbanized areas (as population density and urban percent) were among the highest in explaining MPs abundance variation
	11	urbanization	The predictors related to urban filters explained the 22% of the variation, while 15% was linked to MPs possible source
	12	urbanization	No significant correlation among MPs abundance and distance from the city centre
	13	urbanization	No significant difference in MPs abundance between urban and rural area
	14	urbanization	Urban areas were not always responsible of a significant increase in MPs abundance
Weather and climate influence	15	seasonality	MPs concentration were always higher in dry season compared to wet season
	16	seasonality	MPs concentration were always higher in dry season compared to wet season (limited)
	17	rainfall	Positive correlation between MPs abundance and rainfall events
	18	Seasonality, flow	No seasonality in UK river MPs abundance and the relation with flow was indicated as "inconsistent"
	19	Rainfall, flow	Could not determine clear patterns between MPs abundance and river flow rate or rainfall (sampling difficulties in flood conditions)

Factor	Ref.	Parameter	Main conclusions
Spatial vs temporal variations	20		Spatial variations (as in geographical, demographical or WWTPs inputs) had higher influence on MPs abundances than temporal variation (as in flow or wind conditions)
Influence of hydrological features	21		Higher MPs concentration was found at rivers mouth
	22		Higher concentration in the inner and outer bends of the river compared to the straight tracts

Ref. 1 (Liu et al., 2020), 2 (Zhang et al., 2020), 3 (Wong et al., 2020), 4 (Lestari et al., 2020), 5 (Huang et al., 2020), 6 (Zuo et al., 2020), 7 (Zhang et al., 2020), 8 (Liu et al., 2020), 9 (Wu et al., 2020), 10 (Grbić et al., 2020), 11 (Crew et al., 2020), 12 (G. Wang et al., 2020), 13 (Corcoran et al., 2020), 14 (Stanton et al., 2020), 15 (Han et al., 2020), 16 (Wu et al., 2020), 17 (Wong et al., 2020), 18 (Stanton et al., 2020), 19 (Constant et al., 2020), 20 (Mintenig et al., 2020), 21 (Lestari et al., 2020; Migwi et al., 2020; Xu et al., 2020), 22 (Corcoran et al., 2020)

The influence of the analytical protocol is not in doubt, and it is once more underlined its relevance. Moreover, also the sampling point within a river section appears to held some relevance on the detected MPs concentration. Regarding socio-economical features, it appears that GDP (Gross domestic product) correlates well with MPs concentration, whereas the role of urbanization is not yet clear with relation to MPs abundance. MPs appears also to be in higher concentration during dry season compared to wet season, whereas mixed results are obtained when correlating MPs and rainfalls (possibly related to runoff concentration and dilution and sediments resuspension dynamics). Finally, MPs in water seems to be present in higher concentration where the current speed is lower, such as in inner and outer bends or at rivers mouth.

Following, it was studied which influencing factors were reported to held relevance with regards to MPs abundance at the inlet of WWTPs (Table 3).

Table 3 Factors influencing influent characteristics from Barchiesi et al. 2021

Factors	Ref.	N° of plants/ research period	Notes
Socio-economic	1	50 WWTPs/fall months	-MPs load strongly correlated with population served and influent volumetric flow rate -The relative contribution of indirect source remains unclear
Socio-economic / weather- related	2	3 WWTPs/more than 1 year	-Location (urban or rural) and treatment of industrial WW relevant for the explanation of the dynamics and relative MPs concentration, characteristics and per-capita load. -Significant correlation with rainfall found only for a WWTPs located in a rural area hence runoff relevant for less densely populated areas
Socio-economic	3	7 WWTPs/3 days	- Satisfactory linear relationship for MPs abundance and the number of plastic-related manufacturers in the area studied. -No indication of correlation between MPs concentration and population density
	4	3 WWTPs/1 year	-Average influent concentration was not statistically different between the WWTPs. - Fibers per-capita load of one of them was significantly higher compared to the others: this was explained by the more diverse sewages received by the plant, including residential, commercial, and industrial (the other two treated mostly residential and commercial sewages)
	5	2 WWTPs/ 1 week	-Influence of industries on concentration and characteristics
Time related patterns	6	1 WWTP/more than 1 year	- Statistically significant differences in influent MP concentration: summer MP concentration in influent was indeed higher - (Noticeably they didn't find time related pattern for the effluent)

Factors	Ref.	N° of plants/ research period	Notes
Time related patterns	7	1 WWTP/6 month	- No significant seasonal patterns
	8	3 WWTPs/more than 1 year	- No apparent trend for influent MP concentration
	9	3 WWTPs/1 year	- Inflow in-season variability comparable to the all-year degree of variability
	10	1 WWTP/1.5 year	-Daily variability observed: not statistically significant increase in mean MPs concentration was found between morning and afternoon
	11	1 WWTP/1year c.a	- Reported variation between morning and afternoon but remarked how the absence of replicates could demote the power of the observation.

Ref. 1 (Park et al., 2020), 2 (Akarsu et al., 2020), 3 (Long et al., 2019), 4 (Conley et al., 2019), 5 (Gündoğdu et al., 2018), 6 (Bayo et al., 2020b), 7 (Bayo et al., 2020a), 8 (Akarsu et al., 2020), 9 (Conley et al., 2019), 10 (Bayo et al., 2020b), 11 (Blair et al., 2019)

The information researched were related to correlation between MPs and the following parameters: population served, flow rate, seasonality, time related patterns and rainfall events. The information available though are too scattered to drive conclusive deductions. There are however evidences of MPs load proportional to the population served, whereas the correlation is not clear with the population density. However, in some cases, the location of the WWTP (rural-urban-industrial) seemed to held some relevance in determining the MPs inflow concentration.

Finally, WWTPs were addressed as possible source of MPs to waterbodies. The evidences of such hypothesis, along with the influence on the MPs concentration in waterbodies upstream and downstream are reported in

Table 4. The table is divided in two sections: first, the papers analyzing only upstream and downstream concentrations to WWTPs, then papers that analyzed the WWTPs effluent too. It was also tried to evaluate how the role of the WWTPs was assessed: only based on number/mass concentration, or if morphology and/or size and/or chemical composition were also considered in deriving the MPs source.

MPs are always found in WWTPs effluents; however, it is not always clear how this affects the concentration of MPs in the receiving waterbody. Indeed, it is often recorded an increase in MPs concentration downstream a WWTPs, but it is anyway not always the case. Moreover, the relevance of the difference sources was evaluated mostly based on the concentration, then morphology and chemical composition. Interesting is the analysis of bacterial communities on MPs, as a tool to understand their origin (McCormick et al. 2018).

Table 4 Studies reporting up-stream and down-stream concentration to WWTPs (ref. 1-9), up-stream, down-stream concentration to WWTPs and effluent characterization (ref. 10-17) (Barchiesi et al., 2021)

Ref.	Country	Water body	Matrix analysed	WWTP capacity	Streamflow	Results and WWTP as source attribution methods
1	CA	river	water, sediment	n.a	n.a	Significant increase of MPs abundance downstream only in one over ten WWTPs evaluated <i>No evaluation of apportionment if not based on concentration, (but environmental predictors based on MPs concentration and morphology)</i>

Ref.	Country	Water body	Matrix analysed	WWTP capacity	Streamflow	Results and WWTP as source attribution methods
2	CHINA	river	water	n.a	26.4*10 ⁹ m ³ /y	Different results with different sampling devices, but in conclusion WWTPs deemed as one of the main discharge sources of MPs in the river <i>Evaluation of apportionment based on concentration, (sources on MPs morphology and chemical characterization)</i>
3	UK	river	water	10 - 900*10 ³ P.E	n.a	MPs generally increased downstream WWTPs, no variation of MPs types hence <i>Evaluation of apportionment based on concentration and morphology</i>
4	GER	river	water	(Teltow Canal discharge) / (WWTP effluent) = [0.65-2.6] during the sampling days		Significant increase of MPs concentration for one over three WWTPs considered. No spatial or temporal variation for MPs shape and size, hence <i>Evaluation of apportionment based on concentration and morphology</i>
5	CA	river	water, biota	200*10 ³ P.E (24.1 – 77.4*10 ³ mc/d during sampling days)	n.a	Higher concentration upstream the WWTP <i>Evaluation of apportionment based on concentration and morphology</i>
6	USA	river	water	n.a	n.a	WWTP had no effect on MPs concentration or type <i>Evaluation of apportionment based on concentration and morphology</i>
7	USA	river	WWTP, water	WWTPs effluent was 0-98% of streamflow	(5-185mc/s)	No correlation between MPs types and WWTPs, role of WWTPs as source remains unclear <i>Evaluation of apportionment based on concentration and morphology</i>
8	USA	river	water	Contrib. of effluent to flow (%) [13.17-110.82]		MPs concentration higher downstream of 7 out of nine WWTPs (two significantly higher) <i>Evaluation of apportionment based on concentration and morphology and bacterial communities</i>
9	USA	river	water	8.69 – 18.9*10 ³ mc/d	n.a	Significant increase of MPs downstream of some WWTPs as function of size class and morphology (I and II MPs) <i>Evaluation of apportionment based on concentration and morphology (size class, I and II MPs)</i>
10	NL	River	WWTP, river surface water	M 0.041-3.4 mc/s D 0.53-9.7 mc/s	Meuse 350 mc/s av Dommel 3.1 mc/s av	Confirmation of WWTPs as source No general increase between up and downstream WWTPs Higher concentration attributed to diffuse sources or other riverine dynamics and processes <i>No evaluation of apportionment, if not based on concentration</i>
11	CA	Creeks, lake	WWTP, water	n.a	n.a	Confirmation of WWTPs as “dominant” source <i>Evaluation of apportionment based on concentration and MP characterization</i>
12	ES	Lagoons	WWTP, sediments	207685-929962 discharge	m ³ /y authorized	Confirmation of WWTPs as source Higher MPs concentration in artificially recharged lagoons <i>Evaluation of apportionment based on concentration and MP morphology and spectra</i>

Ref.	Country	Water body	Matrix analysed	WWTP capacity	Streamflow	Results and WWTP as source attribution methods
13	USA	River	WWTP, water, sediment, air	n.a	n.a	Confirmation of WWTPs as source No significant increase up and downstream WWTP Study focused on microfibers Other sources or reservoirs of synthetic microfibers suggested <i>No evaluation of apportionment, if not based on concentration</i>
14	CHINA	River	WWTP, water sediment	0.55-1.2*10 ⁶ mc/d	n.a	Confirmation of WWTPs as source No significant relation between MPs pollution in urban river and the geographical location of the WWTPs <i>No evaluation of apportionment, if not based on concentration</i>
15	CA	River	Water, sediments	n.a	n.a	Confirmation of WWTPs as source MPs concentration significantly higher downstream WWTPs than upstream <i>No evaluation of apportionment, if not based on concentration</i>
16	FR	River	WWTP, water, atm fallout	n.a	n.a	Confirmation of WWTPs as source MPs. A subsequent study deepened these aspects but focused only on the fibres class. <i>No evaluation of apportionment, if not based on concentration</i>
17	NL	River, sea	WWTP, Water, sediments, biota	385-30000 m ³ /h Dry weather flow	n.a	Confirmation of WWTPs as source No evidence of dilution for effluent MPs, hence the presence of many diffuse sources of MPs is suggested <i>No evaluation of apportionment, if not based on concentration</i>

Ref. 1 (Crew et al., 2020), 2 (Liu et al., 2020), 3 (Kay et al., 2018), 4 (Schmidt et al., 2018), 5 (Campbell et al., 2017), 6 (Hylton and Ghezzi, 2017), 7 (Baldwin et al., 2016), 8 (McCormick et al., 2016), 9 (Estahbanati and Fahrenfeld, 2016), 10 (Mintenig et al., 2020), 11 (Grbić et al., 2020), 12 (Edo et al., 2020b), 13 (Peller et al., 2019), 14 (Lin et al., 2018), 15 (Vermaire et al., 2017), 16 (Dris et al., 2017), 17 (Leslie et al., 2017) n.a not available

Conclusively, the MPs presence in the urban water cycle is pervasive as in any other environmental compound. MPs are continuously fed into the cycle by WWTPs, runoff and atmospheric deposition. If the urban water cycle can be considered as a closed one, based on similarities with the natural water cycle, the MPs cycle in the urban water cycle is of both natures. Indeed, some of the MPs present in water sources (surface and/or groundwater) get recycled in the urban water cycle, whereas other MPs end up to the sea and from there follow one of many possible destinies (resuspension in air and back to the cycle, sinking within sediments etc.). The relative contribution of urban areas and industrialization to MPs pollution in an already polluted environment is not easily accounted for. The information on the influence of socio-economic factors and hydraulic features and time related patterns on MPs pollution of waterbodies are present, but the information retrieved are still strongly linked to the local level and can be hardly generalized.

These trends and observation have been confirmed also by subsequent review works. The relevance of the anthropogenic factors on the special variability and abundance of MPs

have been indeed confirmed by the work of Talbot et al. (2022) and Gao et al.(2023)(Gao et al., 2023; Talbot and Chang, 2022). The same authors confirmed also the relevance of meteoric events in determining the temporal variability of MPs in WWTPs influents and surface waters (Gao et al., 2023; Talbot and Chang, 2022). Talbot et al. (2022) confirmed also the impossibility to derive conclusive deductions on spatial and temporal variability for MPs. This lack of certainty is however linked not only to the use of inhomogeneous analytical protocol, but also on the different scale at which the studies available have been developed. This is indeed another aspect that hinders the chance of fruitful comparison Talbot et al. (2022).

Chapter 3. Protocols for MPs analyses in water treatment plants

The “RQ3: State of the art on MPs analysis in samples from water treatment plants” was addressed by a broad literature research.

It was a step forward in understanding the critical turning points regarding the MPs pollution topic, adding the comprehension of the reliability, pitfalls and strength of the analytical power for MPs analysis, to the understanding of the characteristics of the analyte itself, described in Chapter 1. The main goal was to understand and characterize the different options available for each step of the analysis, their pros and cons and applicability to the matrix of interest based on their characteristics.

Specifically, the review work limited to MPs analysis in wastewater and sludge and its results was presented at the SIDISA (International Symposium on Environmental Engineering) 2021.

First, the main features and issues of MPs analytical method are generally presented, next the results presented at the SIDISA 2021 are reported, and finally, each step of the analytical protocol is described and critically commented in detail for MPs analysis in wastewater, sludge and drinking water.

3.1. Main feature and issues in MPs analyses

Analytical methods for MPs have been officially defined only in few cases, e.g the method proposed by the National Oceanic and Atmospheric Administration (NOAA) for microplastics in the Marine Environment, and newly for the drinking water by the California State Water Board. However, there is still no agreement on methods by the international community. An ISO is still under development (ISO/FDIS 24187), the EU plans to define a method for monitoring in drinking water by 2024 (see paragraph 1.4).

The task of “method definition” is complex as MPs analysis involves a succession of activities aimed at isolating MPs from the sample and their characterization. The main steps to be followed are reported below.

1. Sampling
2. Sample pretreatment (if needed)
3. MPs identification – quantification – characterization (IQC)

The procedure to be followed for each step changes according to the matrix to analyze and the analytical technique used for the step 3. Among the most used techniques are the thermo-analytical methods, that make use of the thermal degradation of the MPs, and the spectroscopic techniques that exploit the behavior of MPs when solicited by an

electromagnetic radiation. The possible technical and analytical combination and compatibilities for the analysis of MPs for each step of the protocol are reported in detail in the following paragraphs (specifically, paragraph 3.3).

The main issues linked to the MPs analysis are, for every matrix and analytical protocol:

- Definition of a representative volume
- Contamination control
- Lack of reference material to define protocol quality parameters

The discussion on how to define proper quality parameters for MPs analysis has begun only in the most recent years. The lack of representative reference material makes impossible the definition of precision, accuracy and recovery rates in the same way as for other analytical techniques (e.g. liquid chromatography).

Quality parameters need then to be defined in such a way to be meaningful and representative of the procedure, albeit not corresponding to the aforementioned parameters as traditionally understood.

Limit of detection (LOD) and limit of quantification (LOQ) for the analysis of MPs are defined according to instrumental limits and the effects of environmental or protocol contamination. The former determines the minimum characteristics of the individual particle that can be analyzed, while the latter provides the minimum number of particles that can be attributed to the sample with sufficient certainty. In the former case, typical limits for spectroscopic techniques are $1\mu\text{m}$ for μ -Raman and $10\mu\text{m}$ for μ -FTIR. However, it will be shown in Chapter 6 that in the case of automatic particle analysis with μ -Raman (specifically DXR3 Thermo – Fisher), the limit for automatic analysis is unlikely to fall below $2\text{-}5\mu\text{m}$. On the other hand, the instrument's ability to recognize the chemical composition of MPs is to be considered, but the reference material to be able to define the reliability range is currently lacking.

Environmental contamination, which can confound the data obtained and make them misleading if not properly assessed, is the other fundamental parameter to define the limits of detection and quantification. Several methodologies are used to define these limits, either borrowed from analytical methods referable to other types of contaminants (e.g., dissolved and analyzed by techniques such as HPLC) or newly defined. The following table presents the main methods for defining LOD and LOQ found in literature (Table 5, list not exhaustive). The California Water Board only defines the Minimum Reporting Level equal to the Minimum Detectable Amount.

Table 5 LOD and LOQ for MPs analysis

	(Rødland et al., 2020)	(Bäuerlein et al., 2022)	(Johnson et al., 2020)	(Wong and Coffin, 2022)*
LOD	M+3*SD	Blanks 95% CI	3.3*SD	$M + 3 + 3.29 * SD * \sqrt{1 + \frac{1}{n}}$
LOQ	M+10*SD	Blanks 2*95% upper CI	10*SD	

M average; SD standard deviation; CI confidence interval; MRL minimum reporting level *MRL

These LODs and LOQs are thus defined on the basis of the results of blanks. In the context of MPs research, however, the "blanks" that can be considered are different, depending on the type of assessment to be made. Indeed, blanks can be evaluated as:

- Environmental blanks
- Procedural blanks
- Laboratory blanks

Environmental blanks are useful for understanding what role the analytical laboratory may play in sample contamination during laboratory procedures, but they do not take into account, for example, the role of reagents or cleaning methodologies. They are often evaluated by leaving a filter next to the work surface for as long as needed for analysis.

Laboratory blanks, on the other hand, consider both environmental contamination and contamination related to all laboratory activities: a sample of MilliQ or otherwise purified water of similar quantity to the real sample is processed equally to the real sample. It is missing in this case the consideration of contamination that may occur at the time of sampling and transport.

Finally, procedural blanks take into account all possible sources of contamination: an amount of MilliQ or otherwise purified water that is equal to or similar in quantity to the actual sample is processed in all respects like the actual sample, from sampling to pretreatment to the MPs detection and characterization stage.

As mentioned in the previous paragraphs, a critical assessment must also be made for recoveries: the lack of a representative reference material of the contaminant present in the matrix of interest greatly limits the informational weight of recoveries. Or rather, recoveries too, in the context of the analysis for MPs, cannot be defined as they are for other types of analysis. In fact, recoveries, necessarily made with unrepresentative, or only partially representative, spike material, are useful for comparing the method in use, what is happening internationally and for understanding the dynamics of fluxes and thus the possible causes of contaminant loss. Their representativeness for the recovery of the MPs

mixture is however valid only under heavy hypotheses (e.g same behavior for various shapes and size ranges).

3.2. MPs analysis in the wastewaters and sludge

The trends in MPs analysis in wastewater and sludge for the years 2018-2020 are presented in Figure 2. Regarding wastewater, most studies relied on grab samples, with few composite samples or samples acquired with pump or pump and filtration devices. Sample separation was obtained almost equally by filtration or filtration and density separation; hence a clear tendency cannot be derived. Regarding sample purification, only few studies avoided its use, whereas hydrogen peroxide and Fenton solutions were the most implemented, with preference for simpler protocols. Regarding Identification quantification and characterization (ICQ), spectroscopic techniques were the most applied; increasing interest is building on thermo-analytical methods due to the easier sample treatment procedures (see paragraph 3.4). Regarding sludge, grab samples were the only choice taken (therefore no pie chart is shown); density separation, conducted by different solutions, was also almost always applied. Fenton and H₂O₂ solution were again preferred as the spectroscopic techniques.

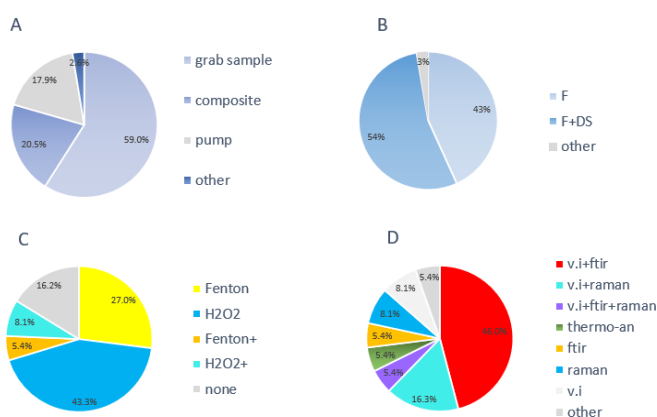


Figure 2 Strategies applied for wastewater MPs analysis, year 2018-2020. A : sample acquisition; B: particles separation; C: sample purification; D: particles identification, quantification and characterization. H₂O₂+ and Fenton+ represent a strategy that includes multiple steps

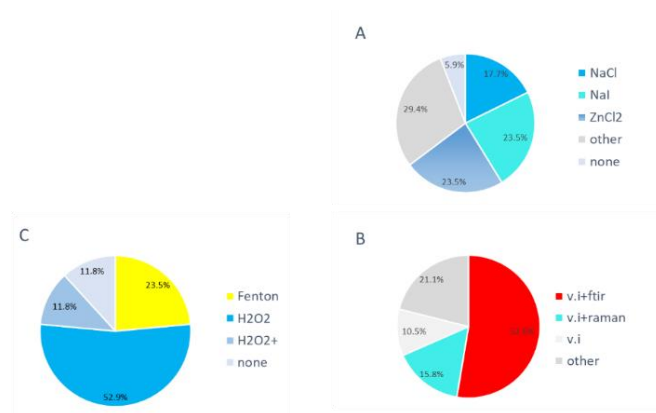


Figure 3 Strategies applied for sludge MPs analysis, year 2018-2020. A: particles separation; B: sample purification; C: particles identification, quantification and characterization

These trends noticed for the years 2018-2020 have been confirmed for their most part also in the subsequent years. Indeed, the only significant discrepancy regards the sampling methods. Pump and filtration system have indeed become more common for wastewater, due to the confirmed acquisition of more representative samples (Gao et al., 2023). Regarding MPs separation, oil extraction methods are also becoming of interest, although the most common techniques remain oxidation and alkali-acid digestion followed by density separation (when needed) (Gao et al., 2023). The most applied techniques for ICQ are still spectroscopic techniques (El et al., 2022; Gao et al., 2023).

3.3. MPs analysis in the urban water cycle

3.3.1. Sample acquisition and volume

Traditionally, there are two ways for water sampling: grab sampling or composite sampling. A grab sample is representative only of the time and space at which the sample is taken, and it is used for the monitoring of unstable parameters such as pH, dissolved oxygen, temperature, nitrites. Composite samples could either be collected as a sequence of grab samples, of same volume at a defined interval over a defined period of time -usually 24h- (typically defined as “sequential sampling”), or as a flow proportional sample, which requires a purposefully designed sampler in association with a flowmeter (typically defined as “composite sampling”).

The daily fluctuations in parameters are characteristics of wastewater treatment plants (WWTPs) influents; drinking water treatment plants (DWTPs) influents change instead mainly as a function of the meteorological conditions. Therefore, the representativeness of a grab sample compared to composites or sequential samples is of utmost relevance for the understanding of the different processes especially for WWTPs.

At the beginning of the studies on MPs in wastewater, many studies relied on grab samples focusing on ascertaining the presence of MPs in wastewater or on the methodologies of characterization and quantification (Fortin et al., 2019; Lares et al., 2019, 2018; Wolff et al., 2019) rather than on retrieving information on the daily average behavior of the WWTPs in relation to MPs. In some cases, grab sampling was repeated, e.g. to explore daily fluctuations (Blair et al., 2019) or to reduce the intrinsic variability linked to weather and/or changes in the urban release (Magni et al., 2019). Methods for WWTP routine monitoring were also evaluated (Gies et al., 2018). Moreover, there are also few experiences with 24h composite sampling (Conley et al., 2019; Grbić et al., 2020; Gündoğdu et al., 2018; Simon et al., 2018). On this regard, it is worthy of note the work realized by Talvitie et al. (2017b), where grab, composite and sequential samples were all taken. Even if results are mainly reported as “microlitter” and not as “microplastics”, conclusions are anyway informative: it is underlined the daily variation in microlitter concentration, detected through the sequential sampling, hence influencing the concentration evaluated by grab sampling. It is also pinpointed how the concentration assessed by grab samples is in the same range of the 24h composite samples (Talvitie et al., 2017b; Wolff et al., 2019). Dyachenko et al.(2017) found however a significant difference in microplastics concentration between 2h sampling at peak flow and 24h composite sampling (Dyachenko et al., 2017): therefore, due to the expectable and confirmed daily variation, composite sampling or long-term large-scale sampling is deemed to be the suitable approach to evaluate the role of WWTPs as source of MP (Talvitie et al., 2017b).

Sampling devices held paramount influence over the volume eligible for analysis. Sample may be collected by containers, autosampler or separate pumping and filtration (Sun et al., 2019). Separate pumping and filtration allows the collection of microplastics from a higher volume than containers or autosamplers (Sun et al., 2019). However, as a method it is more prone to airborne contamination (Okoffo et al., 2019) and eventual loss of MPs (Blair et al., 2019). Another aspect that has to be taken into account is the possible fragmentation of MPs (Bannick et al., 2019).

Moreover, the pumping and filtration devices are developed by each research group, leading to another source of differentiation between studies (Mintenig et al., 2017; Talvitie et al., 2017a; Ziajahromi et al., 2017)

Regardless of the mode of acquisition, the representative volume to be sampled is to be evaluated based on the MPs cut of interest. MPs concentration is indeed function of the size: the lower the size the higher the concentration (Bayo et al., 2020b; Blair et al., 2019; Conley et al., 2019; Edo et al., 2020a; Koelmans et al., 2019; Lusher et al., 2017a; Magni et al., 2019; Talvitie et al., 2017a; Wolff et al., 2019; Ziajahromi et al., 2017). The volume that can be practically sampled, is instead a function of the amount of solids and organic

material present in the water at the sampling point. Another aspect that should not be forgotten is the limit of detection of the chosen analytical methodology, strictly linked to the MPs size range under study.

The relevance of these aspects may differ according to the sample collection point. For example, the concentration of solids and organic material at the inlet of a WWTP devices (or DWTP, even with the due differences) is usually high and could lead to the fast clogging of filtration. The effluent instead is usually characterized by lower microplastics concentration and higher volume needs to be collected to have a representative sample. Indeed, a lower volume hinders the chance of finding microplastics, downgrade the power of a study and raise the margin of error (Gies et al., 2018; Koelmans et al., 2019; Magnusson and Norén, 2014; Talvitie et al., 2017b; Wolff et al., 2019; Ziajahromi et al., 2017). It is also to be born in mind that composite samples could give false zero if MP concentration is very low (Talvitie et al., 2017b). It was indeed noticed that the wastewater volume sampled increased with the proceeding on the plant process line, following the hypothetical decrease of MPs concentration in the flow (Bayo et al., 2020b; Bretas Alvim et al., 2020; Michielssen et al., 2016). Even if the approach is common, the magnitude of the sample taken by various studies is highly different, as well shown in the review by Bretas Alvim et al. (2020). Often there are variations even at the same point of sampling due to fast clogging of the finest mesh, as shown in Table 6.

Table 6 Volume [L] sampled related to sampling point and sieve mesh size. Example studies.

Sampling point	Mesh [μm]	500	250	125	62.5	25
1	Influent	18	18	9	7	2
	Grease and grit removal	32	32	10	2	2
	Activated sludge	26	8	3	3	3
	Secondary sludge	11	5	1	1	1
	UV in	200	200	200	200	59
	Mesh [μm]		300	100		20
2	Influent		0.1	0.1		0.1
	Pre-treatment		50-333	70-10		0.5
	Activated sludge		200-333	20-30		1
	OUT		1000	100		2
	Mesh [μm]	500	190	100		25
3	Primary clarifier a	16.5	16.5	3		3
	Primary clarifier b	100	100	12		12
	Primary clarifier c	41	41	8		8
	Secondary clarifier b	150	150	27		27
	Tertiary c	200	200	200		200
	Reverse osmosis c	200	200	200		200
	Mesh [μm]		355	125	63	43
4	Influent a		97.06	97.06	25.09	25.09
	Effluent a		285.38	285.38	285.38	160.22
	Influent b		53.55	11.57	2.98	2.98
	Effluent b		256.98	256.98	256.98	256.98

Reference: 1 (Lv et al., 2019), 2 (Talvitie et al., 2017a) 3 (Ziajahromi et al., 2017) three plant study and 4 (Long et al., 2019)

No common strategy has been defined to identify the volume needed to obtain a representative sample. However, Koelmans et al. (2019) set a lower limit of 500L or a reported clogged filter for WWTPs effluent volume to assign the highest score in their evaluation of microplastics studies (Koelmans et al., 2019). Another strategy, based on a statistical approach is suggested by Bannick et al. (2019) (Bannick et al., 2019).

The evaluation of the proper volume to sample is also impeded being MPs concentration hardly foreseeable.

3.3.2. Particle separation

Once the sample is acquired (water or sludge), to perform the analysis it is needed to reduce the volume of the sample and isolate the particles to be sent to the analysis. Considering that the target of the analysis is MPs, these techniques have been developed to concentrate specifically MPs, discarding water and solids of no interest.

Microplastic concentration is performed either by filtration and/or density separation. The first one exploits the size difference between the filtering surface and the particles to be retained, whereas the latter is based on the difference in density between microplastics and the sample medium.

Filtration separation

Filtration and sieving exploit the difference in dimension between the solid to be retained and the filter mesh. This allows the acquisition of MPs from samples of conspicuous volume, e.g. with pumping-and-filtration systems, and the concentration of MP on the filter (Mintenig et al., 2017; Talvitie et al., 2017b; Wolff et al., 2019; Ziajahromi et al., 2017). It is also possible to carry out a first dimensional classification using a series of sieves with decreasing mesh size. Using a stack of sieves may also help to avoid the fast clogging of filters with finer meshes. In many articles it is reported the dimensional categorization through a series of filters, but the size intervals (or the meshes chosen) are hardly comparable (Sun et al., 2019). Mesh-based size categorization though is affected by some possible inaccuracies e.g. fibers may pass longitudinally through meshes due to their morphology, or irregular shapes might affect the passing of particles through the sieve (Sun et al., 2019), or the formation of aggregates could hinder the pass through of particles (Fortin et al., 2019). Filtration is often coupled with chemical purification, but even alone it appears to be a decent system for microplastics retrieval due to the high recovery rate established by Lares et al. (2019)(test medium: influent wastewater)(Lares et al., 2019). However, this result is limited to the MPs size range used, which comprises MPs of significant dimension (between 300-6700 μ m). In the same study it is anyway underlined

the lower recovery rates for fibers compared to particles, passing lengthwise through filters openings, increasing slightly when using tighter meshes (from 250 μm to 20 μm) (Lares et al., 2019).

Density separation

MPs buoyancy is a function of the polymers that compose the particles, additives and possibly by the effects of biofilms (Lusher et al., 2017a). Density separation is mainly used for inorganic solids separation such as sand and rust (Mintenig et al., 2017). Density separation is widely applied to soil and sediments (Corradini et al., 2019; Li et al., 2020; Masura et al., 2015). Mismatched opinion are recorded for wastewaters and sludge: indeed it is reported the possible loss of microplastics (Lares et al., 2018) even if as a function of the solution density, and how it might be not appropriate for wastewater from secondary treatment due to the presence of inorganic salts, coagulants and flocculants with density close to that of microplastics, as many naturally occurring particles (Dyachenko et al., 2019, 2017). Moreover, the complex nature of WWTPs solids may reduce the success of density separation (Lusher et al., 2017a). However the need to reduce the volume of sample to manage, to reduce the chance of filters clogging, to reduce the number of particles to analyze, makes density separation often a valid ally (Bretas Alvim et al., 2020; Magni et al., 2019). This is also true when analyzing MPs from surface waters (Mintenig et al., 2020), whereas there are hardly references of density separation applied to raw waters from DWTPs studies (Pivokonský et al., 2020) (Table 7).

Many hypersaline solutions have been used: the most commonly encountered are NaCl, ZnCl_2 and NaI (Li et al., 2019). NaCl is officially recommended by the NOAA (Masura et al., 2015) for the extraction of MPs from marine water, beach and sediment samples, and by the Marine Strategy Framework Directive (MSFD) technical subgroup (2013) (MSFD, 2013). It is also applied by many to samples from WWTPs (Bayo et al., 2020b; Leslie et al., 2017; Magni et al., 2019; Mintenig et al., 2017; Ziajahromi et al., 2017). It is a cheap solution, commonly available and environmentally friendly (Picó and Barceló, 2021), even though the density reachable allows an acceptable separation only of low-density polymers such as PP, PE, PS, PA, PMMA and PC and low recovery of higher density polymers such as PVC, PET and POM, hence their underestimation (Okoffo et al., 2019). With ZnCl_2 and NaI it is possible to reach higher densities ($\rho = 1.6 \text{ kg/L}$ and $\rho = 1.8 \text{ kg/L}$ respectively (Mintenig et al., 2017; Ziajahromi et al., 2017)), hence the opportunity to isolate polymers of higher density and with higher recovery rates, but with significant drawbacks: they are indeed more expensive than NaCl and hazardous to the environment (Joana Correia Prata et al., 2019; Zarfl, 2019). Moreover, even if it is possible the reuse of NaI for multiple cycles, it seems to be particularly sensible to temperature (Raju et al., 2020). Density separation alone was not considered as a suitable method by Lares et al. (2019) in their study on the

comparison of commonly used methods to determine microplastics, due to the low recovery rate and the inability of cellulose fibers separation from microplastics (Lares et al., 2019).

3.3.3. Sample purification

A crucial step in the sample preparation is the removal of solids, in order to reduce the chance of errors when proceeding with visual identification and to obtain clearer spectra when using spectroscopic methods of identification. Improvements due sample purification for the application of thermo-analytical techniques are also reported (La Nasa et al., 2020). Organic solids are removed by digestion. Inorganic solids in the form of soluble salts are also removed by a step of sample purification. In some cases indeed, the downsides of a density separation (high use of reagents, multiple steps, high chance of contamination and particles loss) make its use not advantageous, whereas a sample purification step can lead to satisfactory results. A typical example is the application of a purification step applied to the effluent of a DWTPs, where the solids expected are mainly of inorganic nature and in the form of salts (Minténig et al., 2019).

Organic matter digestion could be obtained by chemical or enzymatic digestion. Chemical digestion is attained by the use of alkali or acid solutions, oxidizing agents. The use of alkali or acid solution has to be very careful and provide for a polymers resistance test (also called “stress test”), because it might damage or destroy MPs resulting in flawed results (Bretas Alvim et al., 2020; Strungaru et al., 2019). Moreover, it is to be considered that environmentally weathered microplastics might have lower resistance than the virgin microplastics used in the stress tests (Lusher et al., 2017b). Indeed, it is establishing as digestion method for WWTP samples, both liquid and solid, the use of H₂O₂ based methods, due to literature evidences of poor to no degradation of polymers (Lusher et al., 2017a; Joana Correia Prata et al., 2019; Sun et al., 2019). No standard set up has been yet identified and the H₂O₂ solution concentration and the process temperature varies among studies, as therefore the time needed for the digestion. Indeed, temperature of 50°C is often encountered as concentration around 30% H₂O₂ (Edo et al., 2020a; Fortin et al., 2019), but also lower concentration (Magni et al., 2019) and higher temperature (Blair et al., 2019). These three factors, time – temperature - H₂O₂ solution concentration, must be carefully balanced to obtain a proper digestion and not to damage the MPs. To accelerate the process without increasing too much the temperature (temperature higher than 60°C have been reported to be dangerous for polymers integrity (Bretas Alvim et al., 2020), and deformation has been detected even at 50°C (Lares et al., 2019)), Fenton-type solutions have been explored. These solutions are indeed suggested by the NOAA protocol (Masura et al., 2015) but also indicated as faster procedure that do not require high temperature,

having showed nearly no harm to MPs and hence with no impact on the subsequent identification techniques (Li et al., 2019; Lusher et al., 2017a; Okoffo et al., 2019; Sun et al., 2019). Hu et al. (2019) reports also possible economic return due to the lower amount of hydrogen peroxide needed (Hu et al., 2019). Key factors to mind in the use of Fenton-type solutions are pH and temperature, as underlined by Simon et al. (2018) who kept the temperature between 15-30°C and pH around 3, to avoid the precipitation of ferric compounds and the formation of complex species of ferric or ferrous ions that slow down the reaction at pH below 2 and above 4, reducing moreover the amount of free iron(II). Another possible solution is the application of enzymes to obtain the digestion of organic matter. It is a milder treatment that is proven to cause little to no harm to polymers, reduce the aggregation between particles and not to hinder the fluorescence signal of labelled plastic particles (e.g. in toxicological studies) (Schwaferts et al., 2019). It is moreover less hazardous (e.g. could be used without a fume hood), even though the efficiency depends on the type of organic material to be digested (Joana Correia Prata et al., 2019). It appears though that to achieve a suitable level of digestion the time required is longer than the previously mentioned protocols. Regarding wastewater samples, enzymes were often coupled with another digestion step, for faster procedures: in the study of Lares et al. (2018) it is coupled with wet peroxide oxidation (WPO) to gain a proper removal of organic fibers and it is applied only for 24h; Simon et al. (2018) employed it with a Fenton-type protocol to remove the cellulose fibers and the enzymatic treatment took 48h. An aspect that mustn't be forgotten though is the economic burden of each technique, and apparently the enzymatic treatment is still too costly to be widely applied (Joana Correia Prata et al., 2019). It is also to be considered that if the goal of the study is the evaluation of adsorbed pollutants, a digestion step could lead to the deterioration or elimination of such compounds.

Regarding the removal of salts, the goal is the increase of the salts solubility, without damaging the MPs. Two are the strategies that can be pursued:

1. Increasing the solubility of salts by directly or indirectly changing the parameters that determine it: acting on temperature, pH, reaction time, which are also the defining parameters of the method. Care must be taken not to stress the MPs.
2. Exploiting the ability of certain reagents to increase the solubility of salts through specific properties, such as chelating agents

The first strategy implies the use of acids for pH modification and possible temperature raising. Regarding temperature, from literature it is not recommended to exceed 50-60 °C, due to obvious effects on MPs, as previously mentioned. The pH modification is to be related to the dissociation equilibria to be modified. Specific target in the case of DWTPs effluents, is the bicarbonate equilibrium. The goal is the shift toward carbonic acid

formation, to favor the solution of calcium and magnesium carbonates by the release of bicarbonates as a reaction to the modification of the dissociation equilibrium of the ion in common with the salts. Almost all of the bicarbonates will be found in the form of carbonic acid if the pH is shifted to 3-4. This therefore allows the target pH to be defined at c.a pH=3. Acid often used in literature is HCl (Tong et al., 2020; Weber et al., 2021), whereas there are also trials with Acetic Acid, which use a higher pH (weak acid) and exploits the complexing properties (Strand et al. 2018).

The implementation of the second strategy is through the use of ethylenediaminetetraacetic acid, commonly referred to as EDTA. EDTA is a weak polyprotic acid whose pKa are 2.0, 2.7 for carboxyl groups, 6.16, 10.26 for amine groups. This implies that at typical pHs in drinking water (range allowed by regulations pH [6-9]), EDTA occurs with a significant fraction of molecules suitable for exerting chelating action. In addition, the chelating action of EDTA occurs in a molar ratio of 1:1. This strategy is also the one that probably generates the least alterations in MPs.

Examples of analytical protocols for the analysis of samples from DWTPs or drinking water are reported in Table 7

Table 7 Analytical protocols for the analysis of samples from DWTPs or drinking water

ref	sampling point	V	reagents	dose	pH final	T°C	t (h)	hardness	turbidity	technique
[1]	IN-OUT DWTP	1-200L	H2O2 30%	80mL/L	n.a	50°C	6	n.a	36 (av IN) NTU	μ-Raman
[2]	DWTP various	1L	H2O2 30%	20mL/L	n.a	Rt	24	n.a	2.9 av IN, 0.12 OUT NTU	μ-FTIR
[3]	IN-OUT DWTP tap	10L	WPO, HCl ZnCl2 (IN)	0.02M (Hcl)	n.a	Rt rt	n.a n.a	n.a	n.a	μ-Raman
[4]	tap	0.25-1.3 mc	HCl 37%	n.a	0-1.5	Rt-50	24-48	2.5 IN 4.3OUT mmol/L	n.a	μ-Raman
[5]	Mineral water	1000-1453L	CA 0.55M	30mL	n.a	rt	24	n.a	n.a	μ-FTIR
[6]	tap	1L	HCl 37%	10mL/L	n.a	rt	24	n.a	n.a	μ-Raman
[7]	IN-OUT DWTP	2L	H2SO4	n.a	3.5	n.a	n.a	1.1 IN 1.4 OUT mmol/L	3.8 IN <30 OUT FNU	μ-Raman
[8]	IN-OUT DWTP	300-2500L	HCl+ H2O2 35% (+ZnCl2 IN)	30mL H2O2 35%	2 (HCl)	Rt 40	24 24	n.a	n.a	μ-FTIR
[9]	mineral	250mL	EDTA	Equimolar to Ca, Mg	n.a	rt	0.2 5	n.a	n.a	μ-Raman
[10]	tap		AA 32%	n.a	n.a	rt	1h	n.a	n.a	μ-FTIR
[11]	IN-OUT DWTP	1L	WPO	n.a	n.a	n.a	n.a	n.a	n.a	μ-Raman μ-FTIR

[1] (Wu et al., 2022) [2] (Chu et al., 2022) [3] (Shen et al., 2021) [4](Weber et al., 2021) [5](Weisser et al., 2021) [6](Tong et al., 2020)[7](Pivokonský et al., 2020)[8](Mintengin et al., 2019) [9](Strand et al., 2018) [10](Strand et al., 2018) [11](Pivokonsky et al., 2018)

3.3.4. Identification- Quantification -Characterization

Final step of a MPs analysis procedure is the identification – quantification – characterization step. Identification means the definition of the MPs chemical composition, quantification is the delivering of a quantitative metric (based on number or mass) for the samples acquired, characterization refers to the categories of paragraph 1.2.

Microplastic characterization includes therefore the grouping in shapes, size and color classes, referred to as physical characterization, and the definition of the polymers that make up the particles and fibers, also known as chemical characterization. Here below are described the mostly used and the most promising techniques, in a WWTPs and DWTPs monitoring perspective, for physical and chemical characterization of microplastics. Techniques linked specifically to research purposes (such as SEM-EDS, suited for the evaluation of ageing and superficial characteristic, as for the elemental analysis (Kershaw et al., 2019)), not suited/practical for such an approach are therefore left behind.

Visual identification

Physical characterization in its simplest form could be performed by visual identification using at most a microscope or a stereomicroscope. Visual identification though is time consuming as it cannot be automated. For this same reason, it is also biased by the personal judgment of the operator. Hence it is prone to over or underestimation of microplastics, no matter how precise the indications for microplastics identification. It allows though the definition of size, color and shape and remains a cheap method for an overview of MPs presence in the sample, especially for microplastics of higher dimension (Li et al., 2018; Joana Correia Prata et al., 2019; Strungaru et al., 2019). The lower boundary for identification is linked to the magnifying properties of the instrument used, but it gets tougher with decreasing size, especially under 1 mm (Peñalver et al., 2020). To improve the visual identification of microplastics an option is the use of dyeing solutions. These exploit the staining properties of defined substances, that gives to microplastics, or the surrounding, color or fluorescence that ease the identification of microplastics themselves. Nile Red is the most used and also the dyeing solution that gives best results in staining plastic materials (Joana C. Prata et al., 2019), while Rose Bengal is the reference to stain natural and non-plastics particles such as natural fibers. Nile Red links to neutral lipids and synthetic polymers (Zarfl, 2019) hence it is possible the co-staining of organic material (Shim et al., 2016) and an overestimate of the amount of MP. Staining with Rose Bengal is though prone to error too: even including a previous organic matter digestion step in the sample processing, 22-90% of the suspected microplastics visually identified with the help of Rose Bengal staining resulted as non-plastic particles when verified with FTIR (Ziajahromi et al., 2017). Also Lares et al.(2019) found the staining with Rose Bengal not particularly

useful, because some cotton fibres remained un-stained and the recovery rates of stained and not stained samples did not differ significantly (Lares et al., 2019). It is to be underlined how the use of Rose Bengal allows anyway the recognition of MP colors, which is not possible with e.g. Nile Red, because it stains specifically MPs. Still, the chance of finding a protocol that may avoid the use of more expensive techniques such as spectroscopic or thermogravimetric ones, especially when thinking at monitoring purposes, or whenever the chemical characterization of MPs is not required, is appealing. Another try is attempted by Prata et al. (2019), aiming at dodging the individual inspection of each particle on the filter surface and testing the method on environmental samples: the group tried out eight staining dyes, different wavelengths for identification and they optimized a software for the automatic identification of MPs by fluorescence-based microscopic techniques. They determined the Nile Red to be the best suited for the aim of the study, as a wavelength of 254 nm. The fluorescence of LDPE, PET, PP, EPS was intense, while weak for HDPE, PS and PVC (might be linked to their crystalline surface), absent for nylon and weathered PE (both fragments and fibers). Among fibers, strong fluorescence was recorded for linen, polyamide and rayon, weak for viscose, nylon, and polyester, absent for cotton and wool. Among the organic matter tested, only fish muscles showed fluorescence (test should still be performed on wastewater solids). Glass fibers were the only with no fluorescence under Nile Red staining. Apparently, there was also no change in MPs FTIR spectra after staining, which is convenient if a confirmation step with the spectroscopic technique is introduced. Conclusions reported satisfactory results, though caution in application of the method was recommended due to the possible underestimation of PS, PVC, nylon and weathered PE (Joana C. Prata et al., 2019). Further optimization indeed showed recovery rates of 95.9 ± 10.3 on environmental samples, confirming the promising results towards a cheap and reliable method for a fast microplastics overview (Prata et al., 2020).

Spectroscopic techniques

The most used spectroscopic techniques are FTIR and Raman spectroscopy. The Fourier Transformed Infra-Red (FTIR) spectroscopy operates hitting the sample with a ray sum of different wavelengths in the infrared spectrum, and recording with a decoder the resulting adsorption spectra. The spectra are the result of the selective adsorption of energy by the different molecular bonds present in the molecule, changing their dipole momentum. Raman spectroscopy instead hits the sample with a laser beam, hence a monochromatic ray, and then the light scattering, due to the change in polarizability (the looseness, or propensity to distortion, of the electron cloud) of the molecular bonds, is recorded and analyzed (Ribeiro-Claro et al., 2017). Hence, these two techniques are considered “complementary”: what gives weak response with FTIR might give strong or clear signals with Raman and vice-versa. Indeed, FTIR is very sensitive to molecules with polar functional

groups (Li et al., 2018) like carbonyl groups (C=O bonds), while Raman responds better to substances with aromatic, C-H and C=C bonds (Zarfl, 2019).

FTIR

FTIR is mostly used in three different modes: transmission, reflection and attenuated total reflectance (ATR). In transmission mode the radiation passes through the sample and is detected afterwards; in reflectance mode the incident light is reflected by a substrate and passes back through the sample and it is collected; in ATR the sample is put in contact with a high reflective material, called “ATR crystal” and the surface is irradiated with an evanescent wave (Xu et al., 2019). Each mode has its strength and weakness: in the first case IR-transparency of the sample support is required and dark or thick (>100µm (Strungaru et al., 2019)) sample cannot be analyzed due to the too high adsorption; in the second case MP morphology has to be taken into account and the signal detected could be disturbed by scattering; in the third case (i.e. ATR) attention has to be paid to the crystal not to get scratched or anyhow degraded, moreover particles might not produce a useful signal if smaller than the crystal itself (Xu et al., 2019). ATR mode is indeed best suited for particles with size wider than 500µm (Zarfl, 2019).

For small particles and to obtain also information over morphology, distribution and size, FTIR can be coupled with a microscope, in the so called µ-FTIR set up.

To reduce the time required for the analysis of the sample, Focal Plane Array (FPA) FTIR has been developed: FPA asset allows indeed the recording of multiple spectra within one measurement, hence e.g. of filter area, eluding the otherwise unavoidable, time demanding and error prone previous visual selection of the particles (Minténig et al., 2017). Time required is still noticeable though, hence further development in technique automation is expected. The accuracy of microplastics identification with FTIR solutions is or might be affected by the presence of water, biofilm or other organic matter and moreover irregularly shaped particles might cause reflection errors (Okoffo et al., 2019; Strungaru et al., 2019; Xu et al., 2019). Limit of detection is often set at 20 µm. It is also required a trained operator for the operation of the delicate and expensive instrumentation and the interpretation of the spectra (Li et al., 2018).

Raman

Raman spectroscopy does not need the laser beam to be in the infrared, hence shorter wavelength lasers might be used increasing the spatial resolution, therefore it could be employed to particles down to 1 µm (Schwaferts et al., 2019). It is reported to be more reliable also for bigger MPs particles (Zarfl, 2019): the resulting spectra are not disturbed by water; non-transparent and dark particles can be analyzed; the results is not hampered

by the particle thickness or shape. (Li et al., 2018). It could be coupled with a microscope for μ -Raman set up too. What can hamper instead the readability of the Raman spectra though is the fluorescence due to the presence of organic matter, dyes and additives (Okoffo et al., 2019). Moreover, attention has to be paid also to the laser beam, not to overheat the microplastic particles leading to their degradation. Also, the filter supporting surface might hinder the success of the analysis being another possible source of fluorescence (Araujo et al., 2018). Fiber glass filters have no spectral interference but their uneven surface makes tricky to focus all small particles on the same plane (Fortin et al., 2019). Satisfactory results seemed to be obtained with silicon filters, characterized by a smooth surface and uniform filter pores having, moreover, a Raman signal that do not cover the MPs spectra and do not cause fluorescence (Wolff et al., 2019). Silicon filters are recommended for FTIR too, inasmuch they provide adequate transparency in the broad mid-infrared region of $4000\text{-}600\text{ cm}^{-1}$ (Xu et al., 2019).

For both spectroscopic methods, to confirm the identification of a particles as microplastics, the comparison of the obtained spectrum with known ones is necessary. If a match within plastic spectra is found, then the polymer is confirmed, otherwise it isn't. Though weathered plastics might be the origin of spectra that may partially differ from those of virgin plastics (Xu et al., 2019). Hence, how to define when there is a satisfactory "match"? Which spectra library should be used? The MSFD Technical Subgroup on Marine Litter set a lower percentage of similarities to 70% to define the match "good", and define as non-plastic material spectra with less than 60% match (Zarfl, 2019). The issue is though that the use of different libraries might lead to different results. Moreover, often, it is not specified in the studies how the "match" was evaluated. The data processing is indeed often neglected and not deepened enough or described, but it is as important as any other step in MPs identification (Renner et al., 2019). Another aspect often discarded is the report of the instrumental set up, that bear also significant influence on the results obtained, and hence of paramount importance for method validation and the replicability of research (Andrade et al., 2020). Nowadays, two research work present guidelines for the analysis and reporting minimum requirements for MPs analysis, in order to improve comparability and assessment of MPs studies (Cowger et al., 2020a; Schymanski et al., 2021).

Thermo-analytical methods

Thermo-analytical methods are based on the analysis of the compounds released by the thermal degradation of the polymers that constitutes the microplastic particles. Those are all destructive methods, hence if not acquired previously, no information on size, shape and color are retrieved. However, it is possible to identify the different compounds and get reliable information regarding mass quantification, operation not possible with

spectroscopic techniques. It is possible to retrieve indirect information on size using a stack of sieves and classifying the MP based on the different mesh size (Bannick et al., 2019).

py-GC-MS

Pyrolysis – Gas Chromatography – Mass Spectrometry (py-GC-MS) entails: the pyrolyzation of the sample, normally under inert atmosphere; the separation of the various compounds produced by gas chromatography; the analysis and identification of the produced compounds by mass spectrometry. By the intensity of the signal it is possible to obtain an indication on the quantity of the compounds too. It is also possible to recover information on additives and pollutants present in the sample (Zarfl, 2019). Limitation to its applicability are linked to the few comparison libraries available and the handling of each particle (due to the need to manually introduce the single particle into the pyrolysis tube, with tweezers) that set a necessary lower boundary around 50-100 μm , instead the upper dimension is around 1.5 mm in order to avoid the clogging the thermal desorption tubes. The amount of sample required is also low, and the limit of detection (LOD) for various polymers is set at the order of magnitude of μg (Peñalver et al., 2020). The different values of LOD for the polymers might lead to ambiguous results: e.g. Funck et al. (2020) did not detect PE in samples from low mesh filters, due to its higher LOD compared to PS characteristic pyrolysis products (Funck et al., 2020).

Thermogravimetric methods

Thermogravimetric methods exploit, instead, the change in mass of the heated sample related to time or temperature, according to the set-up (constant temperature or ramp) under a specific atmosphere (inert or air) (Peñalver et al., 2020). It could also be coupled with mass spectrometry and gas chromatography to improve the amount of information obtained: relevant studies on this account were conducted to lead the development of the thermal extraction-desorption – gas chromatography – mass spectrometry (TED-GS-MS) (Dümichen et al., 2017; Elert et al., 2017). Main advantage compared to py-GC-MS is in the amount of sample that could be analyzed (up to 100mg) and the possibility to analyze sample of complex matrices without or with simpler pre-treatments (Strungaru et al., 2019). According to Elert et al. (2017) thermogravimetric methods are faster than spectroscopic methods, this is also confirmed by Schwaferts et al. (Schwaferts et al., 2019) which affirms that the time required for a single particle analysis is higher with spectroscopic techniques than with thermo-analytical methods. Though, the ongoing development in both technologies recalls for a continuous comparison to evaluate the newest updates.

3.3.5. QA/QC

Quality assessment and Quality control main concepts have already been mentioned in paragraph 3.1. The main aspects that have to be taken into consideration, regard mainly the possible sample contamination, loss or features changes in the microplastics content of the sample, ability of the protocol to recover the microplastics from the sample. Contamination is a risk that has to be minimized through all MPs analysis procedure stages, from sampling, to transportation, to laboratory handling. At all steps it is important to avoid as much as possible the use of plastic devices and to reduce the sample transfers from containers that expose the sample to air, causing possible MPs deposition. Contamination may indeed arise from sampling and laboratory equipment, clothes and rinsing water included (Wolff et al., 2019), and from air deposition, again possible at all operational stages. Changes in the sample content is referred to possible loss of microplastics mainly during sampling procedures, filtration and sample purification. It might also be related to the fragmentation of those MPs present in the sample mainly due to the sampling devices used (Bannick et al., 2019) (to asses this potential source of error, fragmentation tests might be useful (Blair et al., 2019)). When using a digestion method, it is proper to run also a stress test, if it hasn't been done before.

To assess the effect of all these factors, two are the strategy to apply: use of procedural and background blanks, evaluation of spiked samples (recovery rates).

Thanks to the systematic review work run on MPs in WWTPs samples, it was possible to notice few interesting aspects with regards to recovery rates evaluation habits. Indeed, during the year 2018-2020 the recovery rates in studies on samples from WWTPs were evaluated in less than 50% of the studies, and less than 70% of those, evaluated the matrix of interest. Moreover, less than 50% tested MPs of high density (PET, PVC) and again, less than 50% tested fibers recovery.

Another interesting aspect to be noticed, is that rarely the recovery rates are run with MPs in the same size as the inferior size limit declared in the study, as shown in Figure 4

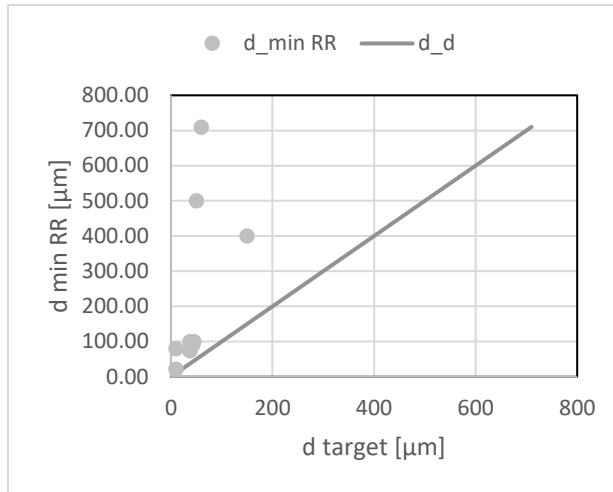


Figure 4 comparison between minimum diameter studied and spiking particles diameter for studies on WWTPs samples. D_{minRR} is the minimum diameter of the spiking particles d_{target} is the minimum size studied

This observation is to be coupled with the results obtained by Simon et al (2018), that noticed how to lower size ranges of spiking particles corresponds lower recoveries. Moreover, it isn't standardized the spiking parts characteristics nor the number nor any other factors included in the process of quality assessment. The scientific community is however beginning to provide a framework for the quality assessment and control of MPs analysis protocols, which, as mentioned in paragraph 3.1, allow for methods assessment by comparison. Even if recovery rates are not fully representative of the MPs mix, they can indeed be used to evaluate the replicability of the measures and to compare methods between one another.

Regarding environmental contamination and its evaluation, few works are at disposal evaluating the efficacy of the correction methods of the analysis results based on the blanks outcome. Of these, the work of Dawson et al. (2023) offers an overview and comparison of various correction methods, concluding that the use of LOD and LOQ is the most reliable in evading the influence of environmental contamination (Dawson et al., 2023). The work by Tsering et al. (2022) also underlines the relevance of proper procedures for results correction by blanks, underlining however the care to pay to those correction, especially when dealing with low MPs concentration samples (Tsering et al., 2022).

3.4. Considerations for monitoring and routine analysis

Routine monitoring activities must be designed compatibly with the work schedules of both sampling and laboratory activities of the water utility. Sample retrieval must be as easy and/or quick as possible, easy transport must be possible, the analysis must not take an unbearable amount of time, and above all, the necessary time of the operator's presence

must be reduced as much as possible. All these aspects must be balanced with obtaining representative and reliable results.

It is therefore imperative for the scientific community to define what parameters are needed for monitoring MPs in wastewater. Could only the number or mass concentration be sufficient? What will be the size interval of interest? Is it also needed the chemical composition? Once this is defined, it will also be possible to define the routine protocol. Some observations, however, can also be made regardless of these aspects.

First of all, therefore, we emphasize the need to establish quality protocols, with proper quality assessment.

On the other hand, with regard to the possible choices for each step of the analysis, Table 8 represents a summary of the potentials and limitations.

Regarding sampling, the acquisition of a representative volume for the complete characterization of the MPs pollution is function of the expected MPs concentration (and therefore also of the size range of interest). For screening purposes however, lower volumes might be justifiable. With regards to WWTPs, the daily fluctuations must be considered when planning the screening campaign. To avoid underestimation, grab samples might be collected during peak flow. The validation of the data obtainable by the automatic sampler available at the WWTPs facilities is still under study.

With regard to sludge, whose composition is not variable within a single day, the grab sample is accepted, as long as it is of a representative volume.

Regarding the separation of MPs from the study matrix, separation by density and digestion of organic matter are difficult to avoid for samples from WWTPs, except in the case of the study of MPs of considerable size ($>300\mu\text{m}$) and even in this case only for those sampling points with reduced presence of organic matter and inorganic solids (e.g. WWTPs effluent). Separation by density should be performed with solutions able to recover also higher density MPs. Possibly, protocols for reuse of solutions to reduce costs might be studied (without complicating too much the procedure).

Regarding the digestion of organic matter, it would be appropriate to define protocols according to the characteristics of the water to be treated (solids concentration). The choice should be in preference for milder, lower-cost reagents, even possibly at the expense of digestion time (the most manageable parameter within the routine, as long as there is no need for continuous operator control). Here, then, hydrogen peroxide is preferred wherever possible, if necessary in combination with more aggressive agents. The

application of Fenton is not recommended, because of the constant control needed by the operator for the success of the step (pH and temperature control).

Finally, regarding step IQC, the use of visual identification is not recommended in any case, because of the extreme relevance of the operator in determining the result of the analysis and the possibility of bias, as already mentioned in the afferent paragraph.

Spectroscopic techniques at the moment are the most popular because they allow the acquisition of many of the parameters of interest for the characterization of MPs. Moreover, their potential for automatization is another upside of the technique. Higher automatization indeed results in more comparable results and in lower engagement of the operator, which in turn results in lower cost operational cost for the water utility. Thermogravimetric techniques are however made attractive by the need for less refined pretreatments and the opportunity to obtain mass concentration, especially for complex matrices (sludge, influent).

Should the determination of the number of MPs prove sufficient, the potential of dyes is certainly of extreme interest.

Table 8 Evaluation of protocols options for WWTPs and DWTPs samples MPs analysis. DW DWTPs samples, WW wastewater, *for DWTPs influent

	options	dw	ww	sludge	time	cost	reliability	info	perspectives
sampling	grab samples (containers)	✓	✓/✓	x	x	x	x	Time related	validation (monitoring for sludge)
	pump and filtration	✓/✓	✓/✓	xx	xxx	xx	xx	Time related	monitoring/validation
	autosampler	✓/✓	✓/✓	x	x	x	x	Daily average	monitoring if validated
extraction	filtration	✓/✓	✓/✓	x	x	xx	xx	Linked to mesh size	
	density separation NaCl	✓	✓	x	x	x	x	Underestimation of heavier MPs, cheapest	Monitoring, with appropriate recovery rates and/or when heavier MPs are present in lower percentages
	density separation ZnCl ₂	✓/✓*	✓/✓	x	xx	xx	xx	Higher costs and environmental burden	Monitoring with optimization/recovery of solutions use, validation
	density separation NaI	✓/✓*	✓/✓	x	xxx	xxx	xxx	Higher costs and environmental burden	Monitoring with optimization /recovery of solutions use, validation
purification	Acid/alkali	✓/✓*	✓	xx	xx	x	x	Possible loss of MPs or MPs characteristics	Possibly to be avoided. Acid to be used for salts dissolution in DWTPs effluent samples
	H ₂ O ₂	✓/✓	✓/✓	xx	xx	xx	xx		among the most suited, standards required
	Fenton	✓	✓/✓	x	xx	xx	xx		among the most suited, standards required
	enzymes	✓	✓/✓	xxx	xxx	xxx	xxx		optimization required, too costly and time consuming
	visual identification (v.i)	✓	✓	xx	x	x	x	n°, size, shape, color	for qualitative overview purposes
I.Q.C	v.i + dyes	✓	✓	xx	x	x	x	n°, size, shape, (color)*	For qualitative overview purposes
	automated+dyes	✓/✓	✓/✓	x	x	xx	xx	n°, size, shape, (color)*	fast monitoring (optimization required)
	m-FTIR	✓/✓	✓/✓	xxx	xxx	xxx	xxx	n°, polymers, size, shape, color	for complete characterization (required relation to mass + pre treatments+automation increases reliability and cost)
	m-RAIMAN	✓/✓	✓/✓	xxx	xxx	xxx	xxx	n°, polymers, size, shape, color	for complete characterization (required relation to mass + pre treatments +automation increases reliability and cost)
	py-GC-MS	✓	✓/✓	xx	xx	xxx	xxx	Polymers, additives (mass)	Overview/monitoring
TGA-GC-MS	✓	✓	xx	xx	xxx	xxx	mass, polymers	Overview/monitoring	

Chapter 4. Theory and practice of Raman Spectroscopy for MPs analysis

The Raman scattering is just one of the many possible results of the interactions between matter and an incident electromagnetic radiation. Specifically, the Raman Scattering can be observed when a laser, a monochromatic wave, hits molecules with polarizable bonds. However, even in this case, it is not the only interaction possible, and not even the most common. The description of the interactions between matter and radiations should exploit both the quantum description of molecular energy states, group theory and the wave theories for electromagnetic radiations. An in depth description of the interaction between light and molecules however is beside the scope of this work. In the following paragraphs, the basic theory behind the Raman effect is first explained, then its most relevant practical aspects for MPs analysis are critically described.

4.1. Raman effect: theory and models

Molecules are characterized by energy states, which are described by electronic, vibrational and rotational energy levels. The molecule can only be at a discrete energy level. The molecule can change the energy level only if the energy provided by a photon is exactly equal to the energy difference between levels. Within each electronic level there are multiple vibrational level, and within each vibrational level there are multiple rotational levels.

When a light hits matter, the possible interactions are the following (Oßmann, 2020):

- The material is “transparent”: the light passes through without changes
- The light is absorbed by the material
- The light is scattered elastically or inelastically, interacting with the vibrational states

The different interactions depend, among others, by the energy of the incident light (its wavelength) in relation to the structure of the molecules and its energy levels.

The third point is the one of interest for what regards the Raman effect. Once the photon hits the molecules under study, assuming that those molecules are Raman active, which will be subsequently discussed, the molecule behavior is modelled hypothesizing a “virtual” state reached by the molecule, which is between two electronic states. This virtual state is labile and unstable and therefore the photons are almost immediately re-emitted. The photons reemitted might have:

1. Same energy of incident photons (Raleigh scattering)
2. Lower energy of incident photon (Stokes scattering)
3. Higher energy than the incident photon (anti-Stokes scattering)

The energy of the photons emitted depends on the interaction between the incoming photons and the molecule: if the photons is remitted at the same wavelength (hence energy), no interaction has occurred and the energy level of the molecule hasn't changed (Raleigh scattering); if the energy level of the photons emitted is higher (wavelength lower), the molecule has lost part of its energy and is therefore at a lower vibrational level than the original one (anti-Stokes scattering); if the energy of the photon emitted is lower (higher wavelength), the incoming photon has given some energy at the molecule that has therefore changed its vibrational state (Stokes scattering)(Ribeiro-Claro et al., 2017)(Figure 5).

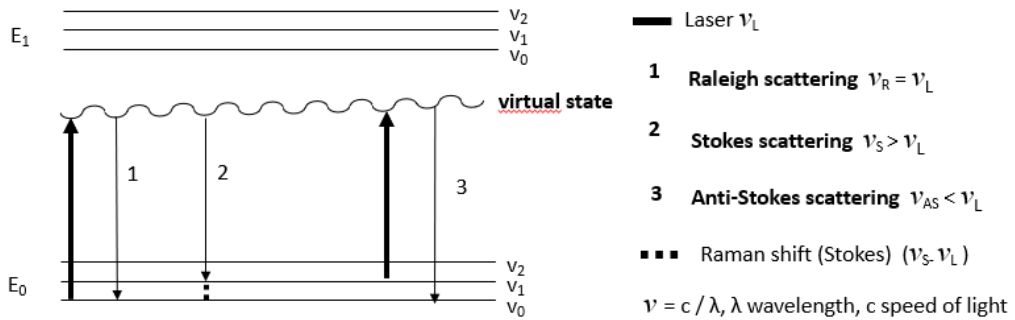


Figure 5 Raman effect: possible interactions between a Raman active molecule and incident photons. E electronic energy levels, v vibrational energy levels, v photon frequency.

The most common interaction is the Raleigh scattering, or elastic scattering: most of the photons hitting the molecule will be scattered back with no changes in wavelength, and will therefore carry no information on the molecule characteristics. One in a million photons might instead give some of its energy to the molecule, and the difference in wavelength between the incident and the scattered photons depends on the chemical bonds present in the molecule that changed its vibrational state (Hotokka, 1987). This type of inelastic scattering is named “Stokes” scattering, and it is the one commonly used in the Raman Technology. Also the anti-Stokes scattering carries information, but it is even more rare than the Stokes scattering, and therefore less exploited (Oßmann, 2020).

The intensity of the scattered Raman light (I_R) depends on:

- the intensity of the radiation source (I_0)
- the wavelength (λ) of the radiation source

- N: number of scattering molecules
- $\delta\alpha/\delta Q$: change in polarizability

$$I_R \propto v^4 x I_0 x N x \left(\frac{\delta\alpha}{\delta Q} \right)^2 \text{ with } v = \frac{c}{\lambda}$$

Eq. 1 Raman scattering intensity. C: speed of light, v: frequency of the radiation source

(Oßmann, 2020)

The intensity of the Raman scattered light depends therefore on the polarizability of the bonds present in the molecules. Polarizability is the looseness or propensity to distortion of the electron cloud (Ribeiro-Claro et al., 2017).

Which gives the first hint on why it is not always possible to retrieve a meaningful Raman spectrum for all molecules. Indeed, only bonds that have a non-zero derivative of the electronic polarizability at the equilibrium geometry along the q-th normal coordinate scatter light inelastically (i.e. $\left(\frac{\delta\alpha}{\delta Q} \right)_{Q_0} \neq 0$) (Toporski et al., 2018).

This understanding can be applied to simple molecules to intuitively understand the selection rules for Raman scattering, however, the vibration of complex molecules can only be evaluated using quantum mechanics and group theory (Vandenabeele, 2013).

For example, the vibration might not be Raman active in the case of tri-atomic or more complex molecules with strong dipole present. If this can be considered a disadvantage in some cases, it is also one of the main advantages offered by the Raman technology compared to the FTIR: Raman technology is not affected by the presence of water. FTIR instead, which exploits the change in dipole of polar bonds, is highly affected by water, which might even obscure the FTIR spectrum of the sample.

The characteristic Raman spectrum of a molecule represents therefore the intensity of the Raman shift at each wavenumber (the wavenumber is reciprocal of the wavelength, usually reported in cm^{-1}). The Raman shift is the difference in wavelength, or as wavenumber, between the incident light and the scattered light (Figure 5). As the Raman shift depends on the bonds present in the molecule, hence the Raman spectrum is characteristic of each molecule.

Considering the multiple possible interactions between matter and radiations, there are also possible interferences: if the incident light (laser) carries enough energy to excite the molecule to an excited electronic state, the energy is absorbed. The absorbed energy is released by radiationless transition to heat, that is transferred to the environment.

(Vandenabeele, 2013). It is also possible that the molecule, to get back to the ground electronic state, emits radiation: this is the process that gives fluorescence (Figure 6). Moreover, the efficiency of fluorescence might be even 6 order of magnitude higher than the Raman interaction, cancelling the visibility of the Raman scattering (Toporski et al., 2018).

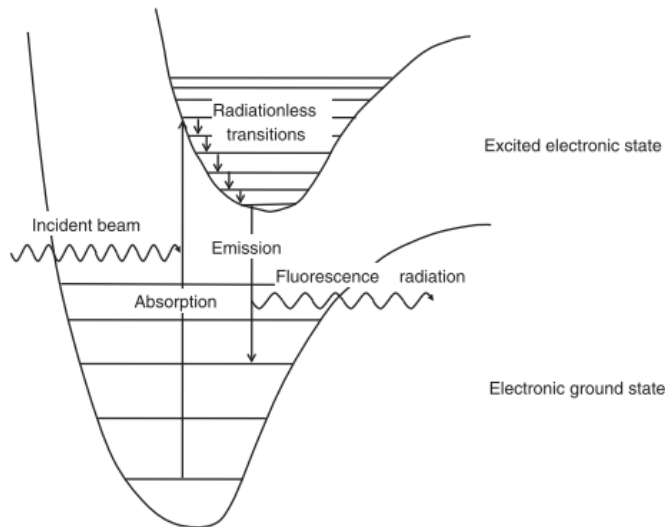


Figure 6 Energy diagram of the fluorescence effect, from Vandenabeele et al. (2013)

4.2. Structure of a μ -Raman confocal spectrometer

A confocal μ -Raman spectrometer combines the power of the Raman analysis technology with the potential of the optical microscopy. The confocality allows the sampling from a defined volume of the sample, at a defined focus plane.

The main components of a μ -Raman confocal spectrometer are hereby listed and briefly commented:

- *Microscopy optics (magnification lens)*: the magnification of the microscopy optics, along with the laser wavelength, determine the lateral resolution δ of the analysis, according to the Raleigh criterion (Vandenabeele, 2013):

$$\delta = 1.22\lambda \frac{\lambda}{N.A}$$

with λ as the laser wavelength and N.A the optics numerical aperture.

- *Laser*: laser are characterized by their wavelength. Lasers with wavelength from the UV until the near infrared are often used in Raman spectroscopy. Relevant

carachteristics required for lasers for Raman spectroscopy are: frequency stability, narrow bandwidth, few sidebands, low divergence, good definition of the profile of the laser (Vandenabeele, 2013).

- *Filter*: the filter is used to filter out the Raleigh scattering
- *Pinhole*: the pinhole is used to select only the scattering coming from the volume in focus
- *Slit*: it determines the spectral resolution along with the grating and the detector resolution. To narrower slits correspond higher resolution. Limiting parameter to such gain is however the pixel width on the detector.
- *Grating*: the grating is used to split the incident light in clearly defined rays with determined wavelengths. It indeed disperses the light on the detector by deflecting each wavelength at a slightly different angle (Toporski et al., 2018). The higher the number of lines/mm in the grating the higher the resolution (Oßmann, 2020)
- *Detector*: detectors transform the incident photons in electrons and then in voltage that can be sampled and processed. The signal is proportional to the light intensity hitting the detector. CCD detectors (Charged Coupled Devices) are often used for Raman Spectroscopy (Vandenabeele, 2013).

The schematic representation of a confocal μ -Raman spectrometer is reported in Figure 7.

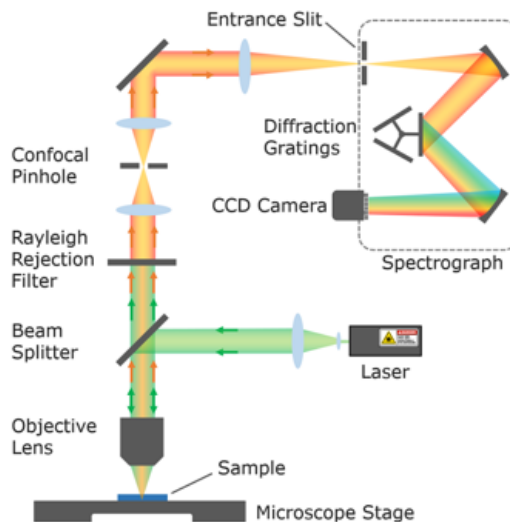


Figure 7 Schematic representation of a confocal μ -Raman set up¹

¹ From [Confocal Microscope | What is Confocal Raman Microscopy? \(edinst.com\)](https://www.edinst.com/Confocal-Microscope-What-is-Confocal-Raman-Microscopy/)

4.3. Relevant parameters for the analysis

Relevant parameters for the μ -Raman analysis can be divided in two main groups. Some are relative to the set-up of the instrument, and are hardly, if ever, changed between the analysis of particles. These include the grating, the pinole and the slit aperture. Other parameters can be varied with more ease. These will be discussed in the following paragraph.

The parameters to choose to perform a μ -Raman analysis of MPs are:

1. Laser wavelength
 2. Laser power
 3. Acquisition time & Number of acquisition
 4. Spectrum post-processing
 5. Library matching method
 6. Libraries choice
-
- 1) The laser wavelength influences the intensity of the Raman scattering, as shown in Eq. 1. Short wavelength, e.g 532nm green lasers, give the stronger Raman intensities, however are also more prone to fluorescence. Longer wavelength instead have a lower Raman signal, but also less problems with fluorescence. The laser can be changed, however it is a timely operation, as alignment and calibration are required at each change.
 - 2) Laser power should be high enough to get a good Raman signal (Eq. 1), but it shouldn't damage the sample. Acceptable values are often indicated between 1 and 10mW (Anger et al., 2018; Schymanski et al., 2018).
 - 3) The acquisition time influences the goodness of the spectrum, as the number of acquisitions. Increasing either or both, won't increase the Raman signal, but it might reduce the noise. Multiple acquisition are needed to cancel the noise due to cosmic rays (Cowger et al., 2020b).
 - 4) Spectra post-processing is needed because the acquired spectrum of a sample is in principle the sum of various components (Renner et al., 2019):
 - Sample (MPs)
 - Matrix (to be removed as much as possible with pretreatments)
 - Noise (all signals detected that do not carry useful information, such as fluorescence, background noise, cosmic rays)

Modern μ -Raman devices automatically correct the Raman spectra acquired e.g with respect to the background noise and cosmic rays. Moreover, it is also often possible to perform other corrections, either automatically or manually. From baseline correction to smoothing, each action might contribute to ease the recognition of a spectrum decreasing the signal to noise (S/N) ratio. However, the modifications run should be very carefully evaluated and performed, not to obtain misleading results (Cowger et al., 2020b).

- 5) The library matching method defines how the correspondence between the acquired spectrum and the reference spectra (gathered in libraries) is evaluated. Of the many methods available, it is often found in literature the “correlation method” (Cowger et al., 2020b), which is demonstrated to be among the most accurate for Raman spectrometry, especially when working with the first derivative of the spectra acquired (Renner et al., 2019). The use of different library matching techniques might lead to different results of the analysis, hence, an accurate evaluation based on literature or experience should be performed.
- 6) The definition of the reference spectra to use, gathered in libraries, is of utmost relevance, especially when dealing with MPs and automatic analysis. Realizing a laboratory-specific library might help in keeping track of the contamination. The higher the number of spectra available in the libraries, corresponding to different plastic polymers, the higher the chance of not missing MPs. The availability of libraries with spectra of weathered MPs is also often recalled as of utmost relevance for a reliable analysis (Renner et al., 2019).

Considering the influence of the cited parameters, it is of utmost relevance to declare all the parameters in use when performing the analysis, to guarantee replicability and the critical evaluation of the results obtained. Strategies and tools are being developed by researchers for researchers, to help increasing comparability and harmonization between methods, and includes guidelines, minimum requirements and open source tools (Cowger et al., 2021, 2020a; Schymanski et al., 2021).

Chapter 5. Development of an analytical protocol for MPs routine analysis in the effluent of drinking water treatment plants (DWTPs)

The development of an analytical protocol for the routine analysis of MPs in DWTPs effluent is described in this Chapter. First, few considerations on the aim and requirements of routine analysis methods are summed, concerning what already mentioned in Chapter 1-2-3. Then, the DXR3 μ -Raman by Thermo Fisher (the instrument made available at the Laboratories of Acea Elabiori) is described in detail as the steps needed to perform the IQC with it. Next, all the steps and aspects needed to define the analytical protocol, from sampling to results reporting, are described and critically commented.

The goal of MPs analysis in effluents is to characterize the phenomenon of MPs pollution, which must then be followed by its monitoring and control. However, the information to be gleaned from the analysis in pursuit of these different objectives (abundance, dimensional/morphological/chemical characterization) may be different, depending on the decisions made by the EU planning and control bodies and the international scientific community. There is no doubt, however, that the first information to be collected from the analysis is the abundance of MPs. However, such information, lacking the first dimensional and then morphological attributes, does not bring any advancement of knowledge about the pollution phenomenon, which is currently necessary for the definition of its monitoring and control. This is because MPs, being defined as particles from 1 μ m (or 100nm) to 5mm, encompass a very wide size range, which includes classes of particles not only with very different effects on humans and the environment, but also with different fates in environmental matrices and thus in water treatment plants.

Another key aspect in the analysis of MPs is the confirmation of chemical composition. In fact, MPs, especially if secondary and/or already circulating in the environment, often have confusable appearance with organic and inorganic solids, and vice versa, as mentioned in Chapter 3. The role of the operator and his experience are of utmost relevance on the outcome of the analysis in the case of simple "visual identification", which no guidelines can reduce and quantify, however detailed they may be. Moreover, the smaller the size range of interest, the higher the chance of error. Chemical characterization is thus crucial, but it cannot be used as a simple confirmation: in fact, a relevant over- or underestimation bias is introduced already in the "visual" selection of the subset of particles to be chemically confirmed. From a theoretical point of view, all particles on the filter should be identified and analyzed. However, an increasing number of particles on the filter, depending on the characteristics of the matrix to be analyzed, the volume filtered and the size range of interest, may result in a undoable or misleading analysis. Indeed, it may not only lead to the occlusion of the filter itself, but also confuse and hide particles due to, for example, overlapping or aggregation phenomena. Particles identification is in these conditions

impossible both for the operator and for the most advanced image analysis software (the latter preferably used for the greater automation, speed and reproducibility of performance in comparison with the operator).

Thus, from these observations, it can be deduced that in order to obtain a representative result, it is a prerequisite not only to have an instrument capable of providing number, morphology and chemical characterization of the particles, but also a filter without an excessive number of particles. This allows on one hand the image analysis instruments to work efficiently, and on the other hand to reduce the analysis time. In fact, considering the case of Raman spectroscopy, it takes c.a 14sec for acquisition and analysis of each spectrum, hence 3000 particles are enough to have an analysis time of c.a 12h, which is not an uncommon case if the whole MPs size range is considered. Thus, it can be guessed how the analysis time expands enormously if reduction and optimization strategies are not applied.

An analytical method for the analysis of MPs must then take into account all these considerations and define appropriate analytical set-ups and pretreatments for an optimal analysis. Moreover, in the context of a water utility and monitoring activity, the analytical method should answer the need of routine analysis and screening, and therefore carefully balance the three pillars time-cost-reliability.

The method for MPs analysis in drinking water was studied and developed at the laboratories of Acea Elabori, society of engineering and services of the Acea S.p.a group.

5.1. The μ -RAMAN DXR3 Thermo-Fisher: description and analytical procedure

The μ -Raman DXR3 Thermo-Fisher, shown in Figure 8, is supplied with a 532nm laser, of 0.1-10mW power, adjustable by 0.1mW. Grating is of 900 lines/cm, pinhole 25 or 50 μ m, and it is equipped with a CCD detector. Moreover, optics of 10x, 20x and 50x were also supplied. Pinhole was set at 25 μ m to improve the spectral resolution, which at 10x is of 4cm⁻¹. The software available for the spectra analysis is OMNIC Spectra whereas the automation of the analysis (including the image analysis) is overviewed by the software OMNIC At μ s.

The DXR3 allows the analysis of the particles in the following modes:

- *point-and-shoot*: analysis of the single particle chosen on the filter by the operator
- *vector map*: a sequence of particles manually selected by the operator
- *map*: particles automatically selected on the filter by an image analysis algorithm. Coordinates are attributed to the particles automatically identified. The laser is then shoot on those coordinates.

The third option is the one of highest interest for MPs analysis in a routine context: it allows the analysis of a high number of particles, reduces significantly the role of the operator (therefore increasing repeatability, strongly impaired when the operator has to choose the particles to be analyzed) and also the time the operator has to spend for each analysis.

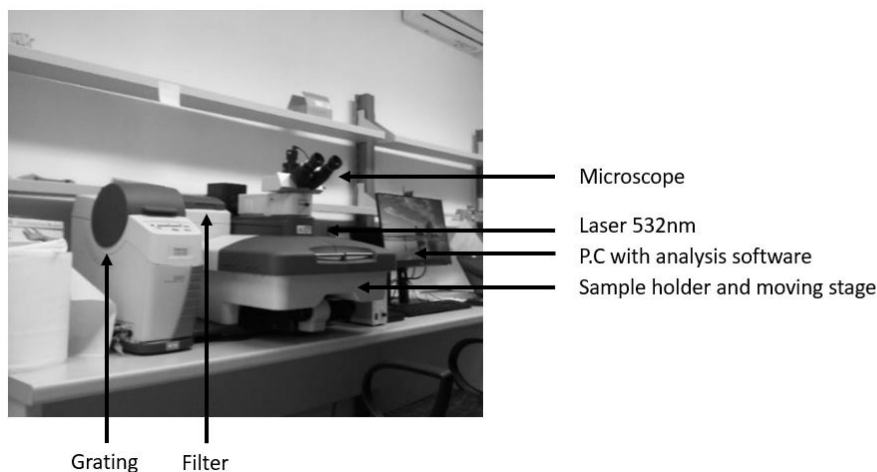


Figure 8 The μ -Raman DXR3 Thermo-Fisher

The automatic MPs analysis procedures with DXR3 therefore contemplates:

1. Image acquisition of the filter (“mosaic acquisition”)
2. Particles selection
3. Coordinates acquisition
4. Spectra acquisition
5. Spectra matching
6. Report of the analysis

In the following paragraph the operation, limitations and solutions corresponding to these steps are described. It is however to be highlighted, how the understanding of the operation, limitation and solutions wasn’t a linear process, and a process often intertwined with the developing of the other aspects of the analysis (e.g pre-treatments), which therefore are here briefly anticipated and deeper described in the following paragraphs.

Mosaic acquisition

Operation: the image of the filter is acquired automatically by the camera set with the microscope and the automated microscope stage. The optics are fixed, whereas the microscope stage moves. The microscope stage minimum step is of $1\mu\text{m}$.

Limitations: two critical points arise with the image acquisition:

- uneven illumination of the image in the field of view
- precision in the mosaic tiles stitching

Regarding the first point, if there are programs that can reduce the effect, however, those are not yet implemented within Atlus, and therefore not much can be done about it.

Regarding the second point, it is relevant to understand the magnitude of the error, in order to define the minimum size automatically analysable with the instrument. The stitching error observed with a 50x is of $1\mu\text{m}$, as shown in Figure 9. This necessarily implies the impossibility to automatically analyse particles with a diameter lower than twice the stitching error. Indeed, the misplacement between the image and the particles impairs the analysis due to the error in the coordinates acquisition. Therefore, the lowest automatically analysable particles size is set at first at $2\mu\text{m}$.

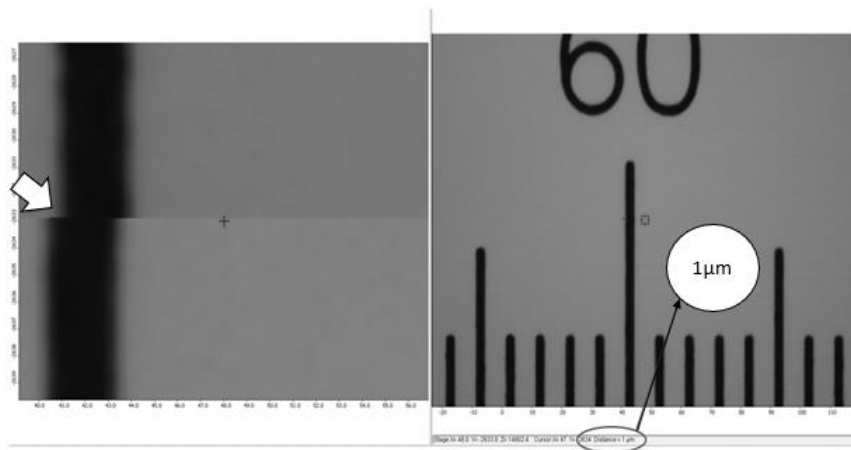


Figure 9 Stitching error during image mosaic acquisition 50x

The same stitching error is shown in Figure 10, on the image acquired at 10x magnification for the mosaic acquisition of a silicon filter with $5\mu\text{m}$ pores width. It is indeed clear the mismatch at the borders of the different tiles, of about $2\text{-}3\mu\text{m}$.



Figure 10 Stitching error for silicon filter with 5µm pore width at 10x magnification (Brightfield). Black squares are the 5µm pores of the filter

Particles selection

Operation: the particles identification on the filter is run based on two parameters:

- Intensity of the pixels
- Size of the particles

The intensity of the pixels is reported in the Image Intensity Histogram. The Image Intensity Histogram, for the 1µm silicon filters, is shown with its components in Figure 11. The intensity of the pixels is reported the X axis of the Image Intensity Histogram as relative intensity (scale 1-100%). On the Y axis there is the number of pixels. The particles are then selected as the brightest pixels when working in darkfield, as the darker spots when working in brightfield. There's also the possibility to define manually the percentage of the threshold that divides particles and background.

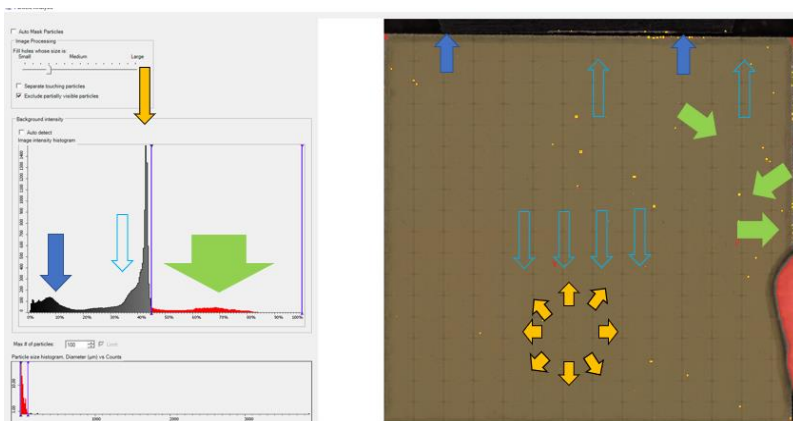


Figure 11 Image Intensity Histogram and its components for a silicon filter with 1µm pore width

The size of the particles can either be selected manually by the operator, or it can be automatically selected based on the number of particles that the operator gives as input.

Limitations: repeatability across samples is not an easy task. Three are the strategies trialed to gain repeatability, which all hold intrinsic limitations that need to be considered and understood:

- Use of automatic algorithm: the algorithm might get confused with very clean filters and chooses as particles of interest the darker spots on the filter (instead of the brighter ones) due to the uneven illumination of the mosaic tiles, as shown in Figure 12. There isn't indeed the option to select "bright particles on dark background" or "dark particles on bright background" as possible with other image analysis software (e.g ImageJ).

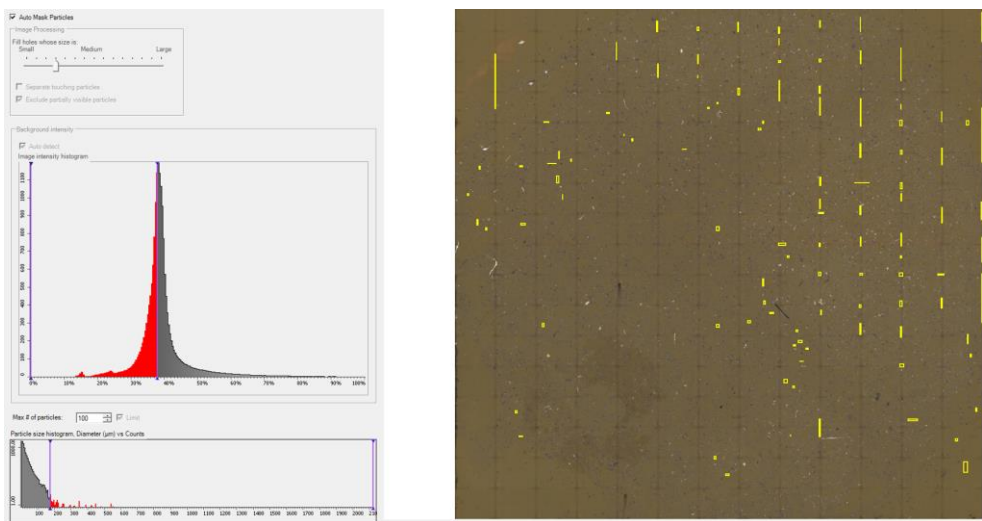


Figure 12 Automatic algorithm choosing the left side (darker pixels) of the image intensity histogram for particles identification

- Fixing a threshold: the filters have different intensity histogram shape and different peak position, depending on the filter background shade and resulting intensities of the particles present.
- Choosing the threshold by fixing a point on the histogram shape (e.g point of inflection): shift of even few percentages, due to the choice left to the operator, might change the number of points to be analyzed by hundreds.

Another limiting aspect, with respect to MPs analysis, is the subsampling modes available. Indeed, it is not possible to define the particle to analyze with the image algorithm and then choose a random subset. If this aspect might not be too relevant when dealing with a relatively low number of particles on the filter (c.a 1000), it gets relevant when the number of particles increases, as to say when high volumes are analyzed or particles in a low size interval (<20 μ m). It is however possible to analyze sub-areas of the filters, but it is again not possible to indicate to the program the coordinates of those areas all at once and expect them to be analyzed automatically. Hence, the time the operator spends on the analysis is expected to rise significantly.

Solutions: the solution found for particles identification on the filter, considering the limitation until now described and other that will be addressed in more detail in the next paragraph, is the use of silicon filters with 5 μ m pores. These filters have indeed a darker and less reflective background that allows for more contrast and less relevance of the uneven illumination of the tiles. Moreover, their background is more even among filters and among batches, which is another term of consistency compared to the 1 μ m filters (which might appear greyish or brownish according to the filter batch they belong, more than on other video source parameters). Therefore, the automatic algorithm works consistently. If repeatability might not be assured between samples, due to the inner operation of the algorithm which is based on relative pixel intensity (and therefore the results change according to the intensity of the darkest and brightest spot present on the filter), it is anyway assured in terms of analysis procedure and mode. The role of the operator is indeed irrelevant at this point for the particles choice.

Regarding the subsampling limitation, no solution is foreseeable, if not in the optimization between the number and size of the subareas to analyze and the possible uncertainty linked to the estimate of MPs based on subsampling. This optimization should be run by both modeling and practical trials, based for example on the tools provided by Schwaeferts et al. (2021) (see Chapter 8. Conclusion and Future perspectives) (Schwaeferts et al., 2021).

Coordinates acquisition

Operation: once the pixels of interest (bright particles) are identified on the filters, the centre of the pixel group is chosen as reference coordinates for each particle.

Limitations: a perfect correspondence between coordinates and particle is of utmost relevance. The stitching error previously mentioned is however a first source of error, which increases the lowest size of the particle automatically analysable. Moreover, another source of error was found, although not expected: the filter itself was moving within the filter holder. It is now clear how even a movement of 20-100 μm impairs heavily the reliability of the analysis due to the mismatch between the acquired coordinates and the particle below. This issue was also discovered only after few months of trials, as the filters were getting cleaner and the understanding of the operational aspects of the analysis were getting clearer. Indeed, it was noticed how often the spectrum acquired by the DXR3 was the same as that of the filter. Therefore the doubt rose of issues between the coordinates acquired and those of the laser shoot. It is however not intuitive how to get the coordinates of the particles identified to be shown with a blue cross not only on the filter mosaic acquired but also on the live image from the camera, as shown in Figure 13. This is indeed the most straightforward and reliable way to understand possible shifts. At the beginning of the research they were indeed quite randomly appearing or not, without any clear consequentiality. Indeed, this behaviour was lately discovered as a bug of the software, which was however systematized to have the coordinates shown every time on both images after the analysis.

It was then verified a severe inconsistency between the coordinates acquired from the mosaic image and the actual coordinates of the particles. This inconsistency seemed to increase with the number of movements done by the filter stage. Upon verification by the technician of Thermo-Fisher, it was indeed concluded that the filter was moving within the filter stage. Once a solution was found (described in the next paragraph), another observation was still to be made: there is a residual movement of the particles on the filter, as already mention (only) by Thaysen et al. (2020) (Thaysen et al., 2020). This movement is of about 2 μm , hence setting a lowest size analysable automatically to 5 μm seems reasonable. This movement might be caused either by the summing of the stitching error and the precision of the stage movement, by the inertial movement of the particles on the filter, or both. The fact is however that it is there, and that it should be accounted for.

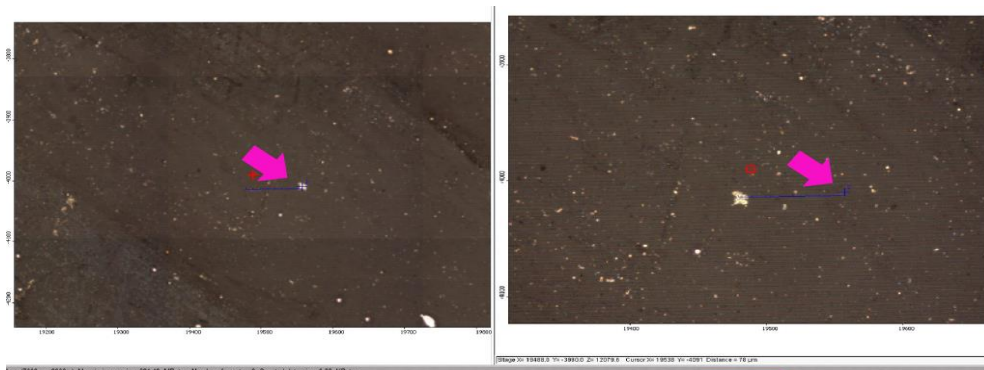


Figure 13 Movement of filters in filter stage and coordinates displacement (78 μ m), 20x magnification

Solutions: the filter was first fixed on a glass sample slide with tape, which however is clearly not the best option for MPs analysis. Therefore, once the movement of the filters was confirmed, different trials were run, and the final choice was a water-soluble adhesive, Raman neutral. The spectrum of the adhesive was acquired and saved, but most importantly, it was verified the ability of the DXR3 to recognize particles even under a layer of adhesive. The test was passed as shown in Figure 14: if it is noticed a slight increase in the baseline, but no other peaks are registered if not those typical of PE, which was the polymer of the fragments placed under the adhesive layer. Its use is recommended both for 1 μ m filters but better suited for the 5 μ m filters, due to their higher thickness (150 μ m to 500 μ m respectively). Indeed, if properly spread, the adhesive won't pass through the pores. However, the lower the thickness of the filter, the higher the chance of that happening.

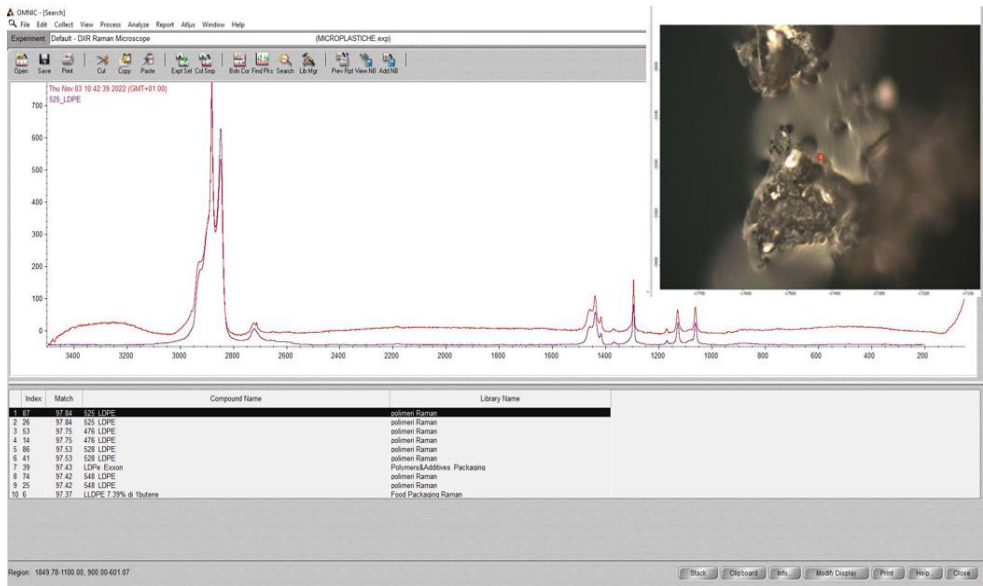


Figure 14 Spectrum of PE particle under water-soluble adhesive

Spectra acquisition

Operation: the laser is shoot on the coordinates of all particles and the spectra and their coordinates registered in a “map”. The acquisition parameters are set just once by the operator. It is possible to define the time and number of acquisitions, or the desired Signal to Noise Ratio (S/N). It is also possible to define the laser power (from 0.1 to 10mW with 0.1mW steps).

Limitations/Solutions: no limitations have been found at this step of the analysis, which is actually well automated. The map is saved, and all the spectra acquired are also saved with it.

Spectra matching

Operation: once all the spectra in the map have been collected, those are matched with spectra of known materials. The input to give to the software are:

- Spectra libraries, where known spectra are stored
- Region of search
- Matching method

This step is also highly automated, and fast. Only few seconds are required to match one spectrum with those present in numerous libraries, which is a noticeable advantage of the DXR3.

Limitations/Solutions: in this case, it is probably more correct to discuss critically the potential, more than defining the limitations to this step. The spectra libraries and the search regions are choices to be made independently from the operation of the analysis step and they will be discussed further in the next paragraph on the method development. The matching method instead is strictly linked to the options available by the Omnic software. The options offered by the software for the automatic analysis are named as:

- Correlation
- Absolute difference
- Squared difference
- Absolute derivative
- Squared derivative

These methods cannot be modified in any way. It is also not available on the software manual the description of the formula used by the algorithm to calculate the matching score (in percentage). Methods to use for the spectra matching have been discussed already by the literature, and the options offered by Omnic refer to the three main methods used (correlation, differences, derivatives) (Renner et al., 2019; Schymanski et al., 2021).

Report of the analysis

Operation: the acquired spectra coordinates, the matched library spectra, the matching score (0-100%), area, length and width of the particles are all reported in the report. It is possible to save the report in .pdf or .tsv for an easier data management. This step is also highly automated and fast.

Limitations: logically, only the matched spectra with the highest score is reported. However, with peculiar ambiguous spectra, there might be a difference of only few decimal percentages, and the highest score might not be the right matching.

Solutions: the solution found is not automated, but it is based on a double-check by the operator, as described in detail within the paragraph dedicated to the method development (paragraph 5.2).

5.2. Method development: preliminary evaluations

Before beginning the trials to frame the advantages and pitfalls of the method and instrument, some preliminary evaluations are due, to define the starting set-up:

- Magnification to use
- Sample volume to try out
- Matrix to analyze

Indeed, these are essential aspects from which all subsequent choices regarding the structuring of the method derive. Of course, the development of a method is not linear and foresee feedbacks changes in its course.

With regard to magnification, the choice was to proceed by successive refining steps, using 10x magnification for the evaluation of the first results in order to obtain rough indications of the direction to be pursued. Subsequently, having obtained these first indications, the magnification is increased by refining the results. The ultimate goal is to be able to exploit the instrument to its full potential, that is, getting to analyze particles as small as 1 or 5 μm in size. Among the many differences between $\mu\text{-FTIR}$ and $\mu\text{-Raman}$, in fact, there is the latter's ability to go well below 10 μm , the lower limit of FTIR. This greater power of analysis is also critical: indeed, literature indicates that most MPs entering and leaving drinking water treatment plants are in the 1-10 μm range (Pivokonsky et al., 2018; Z. Wang et al., 2020), and it is known how MPs <20 μm can access into organs (Yuan et al., 2022).

The practical differences in analyzing different size fractions, as well as in using different magnifications, however, are not negligible. These, in fact, range from the control of environmental contamination (just think of how air quality parameters are PM10 and PM2.5) to the time required for microscope image acquisition. In this regard, an estimate of the filter integral image acquisition times is shown in Table 9. It is here to consider how autofocus (AF) may not be necessary at 10x, but essential at 20x and 50x for analysis of comparable areas.

Table 9 Time required for mosaic acquisition of a 1cmx1cm filter for different magnifications with and without Autofocus

Magnification	No Autofocus	With Autofocus
10x	4min	20min
20x	8min	2.25 h
50x	43min	8-9 h

For these reasons, it was therefore decided to seek preliminary but indicative results at the most practical magnifications, and then to continue by adding complexity at higher magnifications. On the basis of these practical considerations, with a view to optimizing modes and times of analysis, MPs analyses were structured by assuming three packages of increasing complexity, based on the size range of interest:

Package A: >20 μm , 10x magnification

Package B: 5-20 μ m, 10x or 20x magnification

Package C: 1-5 μ m, 50x magnification

On the other hand, with regard to the volume to be sampled, the volume initially devoted to gaining confidence with the instrument and obtaining preliminary results was 250mL, given the available filter equipment and an initial discussion with the instrument's application technician. Later, as the knowledge of the international scientific community progressed and more practice was gained in the filtration and analysis steps, it was increased to 1L, commonly considered the minimum amount to be analyzed for MPs in drinking water given the size ranges that include the smaller particle sizes (packages B and C).

Different water sources were evaluated to choose the trial matrix, specifically tap waters, from taps inside the laboratory or from private homes. However, due to issues of practicality in acquiring samples and stability in water quality, water from a drinking fountain inside the perimeter of Acea Elabiori's laboratories was chosen for the preliminary trials.

5.3. Pretreatment choice

From the first filtrations performed, it was immediately clear that pretreatment of the sample was necessary, even though it was water intended for human consumption. Initially in fact, from literature, pretreatments seemed to be necessary only for wastewater or surface water samples. However, if the size of interest goes down to 1 μ m, the need for pretreatment is undeniable even for drinking water samples, as confirmed by our experience, supported then also by literature data that were getting richer year by year (as previously shown in Chapter 3). Indeed, Figure 15 shows the filter image of unpretreated samples and the resulting automatic particle recognition result on the filter. It is clear and evident the crowding of particles on the filter, albeit for a small volume such as 250 ml, leading to the identification of thousands of particles already for sizes >50 μ m. As mentioned above, the analysis of thousands of particles is not feasible in practical terms of laboratory operation, nor is the analysis of such a crowded filter desirable: the background noise and aggregation phenomena might result in misleading dimensional and morphological definition by the automatic particle identification algorithm. The theory behind the pretreatment choice has already been detailed in Chapter 3. The reagents trialed were EDTA, Acetic Acid (AA) and Hydrochloric acid (HCl). EDTA was available as powder, whereas AA and HCl 37% were purchased from Carlo Erba.

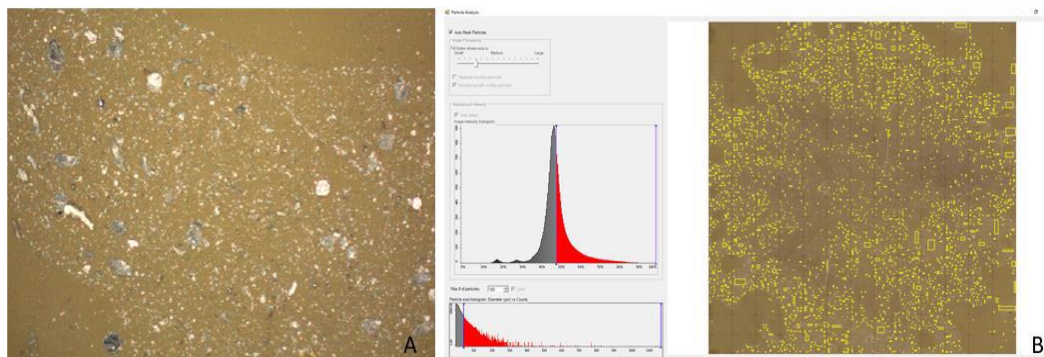


Figure 15 Un-pretreated drinking water sample; A zoom on filter, B filter image analysis (particles individuation)

An EDTA solution was prepared with concentration of 0.25M, prepared with ultrapure water produced with ARIUM VF PRO (Sartorius).

- EDTA was added in a concentration equivalent to the carbonates present. The solution was filtered with 0.22 MCE filters
- HCl was added to the sample to reach a pH of 2.5-3
- AA was added to reach a pH of 4

A sum up of the trials run for pretreatments are shown in Table 10.

Table 10 Experimental conditions for pretreatments trials

Reagent	Concentration/ set pH	Temperature	time
EDTA	Equimolar to carbonates	rt	Few minutes - day
HCl	pH ~ 4 - 1	rt-50°C	Few minutes - day
AAG	pH ~ 4	rt	Few minutes - day

The trials were first evaluated based on the number of particles found on the filter. The results of the trials between un-pretreated water and treated water with EDTA are shown in Figure 16, for particles $>1\mu\text{m}$, evaluated with a 10x magnification on $1\mu\text{m}$ pores silicon filters. It wasn't however possible to compare pretreatments methods directly, due to the similar effect and the therefore relevance of the manual thresholding (used at the beginning of the research) that biases the results. However, the trials run allowed the consideration of some practical aspects:

- To speed up the filtration on $1\mu\text{m}$ filters, few mL of ethanol are filtered previous the sample: its interaction with the pretreatments reagents however is relevant. Indeed, it seems that Acetic Acid interacts forming some kind of darker films on the filter, whereas EDTA seem to precipitate on the filter.
- EDTA is slowly soluble. The management of the solution as its dose introduces the use of more glassware that might increase the contamination of the sample.

- HCl is the easiest reagent to manage in terms of dosage, use and interaction with ethanol
- No significant differences are qualitatively noticed between increasing time, dose and temperature for pretreatments with HCl.

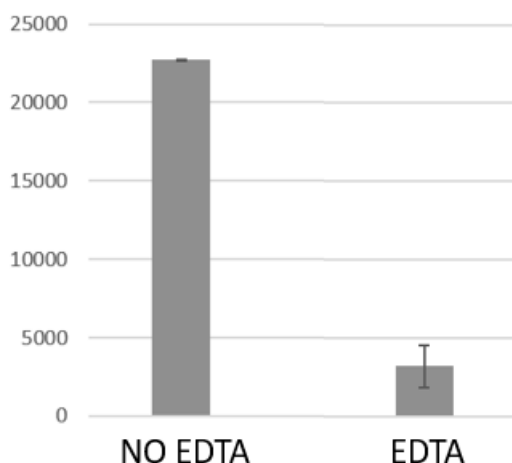


Figure 16 Effect of EDTA pretreatments on particles > 1µm

To try to obtain results that were quantifiable, the subsequent step for the pretreatments choice was the evaluation at higher magnifications. The exploitation of the higher contrast between the background and the particles at higher magnification allows indeed the use of the automatic algorithm, and the unbiased quantification of the particles on the filter. The evaluation at higher magnification were run counting the particles not on the entire filters, but on a subarea. The subarea was composed from various windows with the length and width of the field of view at the magnification used and located randomly on the filter. The magnification used was 50x and the windows size was 4x FOV (field of view of 50% is 153x108µm). The number of windows is 25. Managing the illumination of the FOV, it was indeed possible to use the automatic algorithm almost in every occasion. In order to calculate the coordinates for the random location of the windows, the R code made available by Schwaferts et al (2021), was adapted and used (Schwaferts et al., 2021). The results for pretreatments with HCl and EDTA are shown in Figure 17. The pretreatments with EDTA consisted in adding EDTA equimolar to the carbonates, leave it overnight and analyze the particles the day after. The test with HCL were two: HCl_1 consisted in lowering the pH to a value <2 and heat the sample to 50°C overnight, whereas HCl_2 consisted in lowering the pH at c.a 3 and leave it overnight at room temperature.

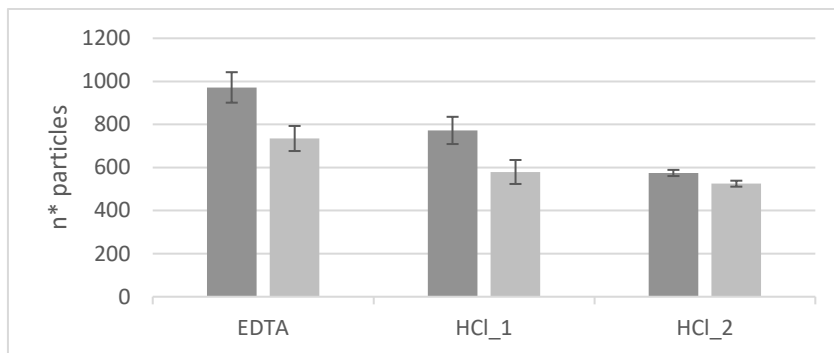


Figure 17 Pretreatments results of two trials (blue and orange respectively), average number of particles >1µm for 4xFOV at 50x. Colors represent the duplicates

The results seem coherent and informative, however, there is a reason for having only two replicates instead of the more traditional three replicates: the container. Indeed, the samples were pretreated in volumetric flask, with glass cap. If the first two trials resulted coherent, the third one showed incoherent results. This incoherent result led to the discovery of another relevant practical aspect. Upon analysis, many of the particles on all the trials resulted with these characteristics, demoting the results obtained:

- Absence of significant Raman signal (signal equal to that of the background)
- Insoluble
- Untouched by acids, temperature or EDTA
- Size between 2-4µm

After some thoughts it was hypothesized their provenience from the ground-glass stopper of the volumetric flask. Indeed, blanks were then evaluated the same way but using a conical bottle and ultrapure water produced by Arius VF PRO, resulting in a much lower average number of particles per window and a definite lower presence of the described particles.

Everything considered, the pretreatment to be chosen for MPs analysis in drinking water is therefore: dosing HCl until a pH 2-3 is obtained, leave to rest for 15-30min. Care should be given to the sampling container choice, avoiding glass stoppers if possible.

5.4. Filtration

All the filtration activities are to be run under a laminar flow cabinet. Chemical fume hoods are indeed to be avoided due to the aspiration of laboratory air within the cabinet. The filtration set up is shown in Figure 18. The filtration set up includes: vacuum filtration system, conical flask, sintered glass base, filtration funnel, rubber gaskets and squared silicon filters of 1cm x 1cm (Smartmembranes).

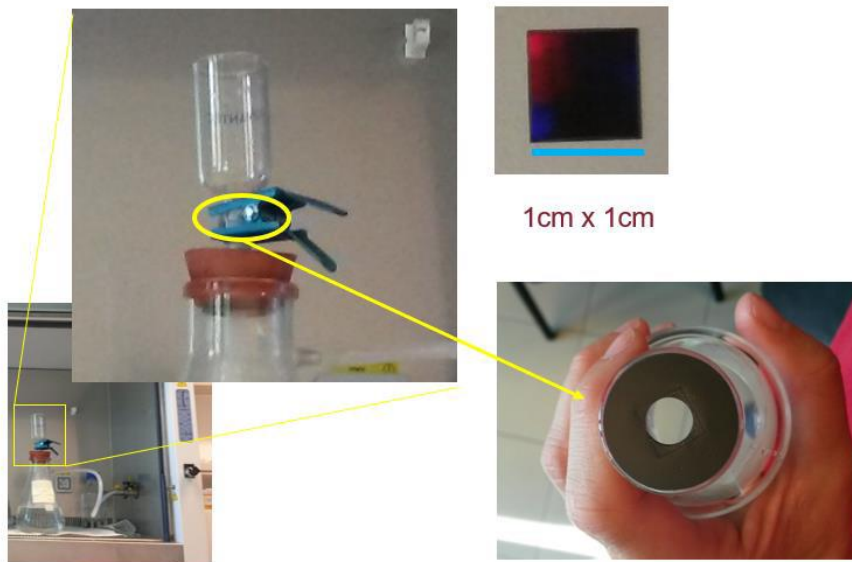


Figure 18 Filtration set-up

The time needed by $1\mu\text{m}$ to filter 1L of water can be sped up by filtering first few mL ethanol: indeed, thanks to the lower surface tension, the ethanol substitutes the air within the filter pores with more ease than water, opening up the way for water itself to pass. In this way, less than half hour is needed to filter 1L of water. However, ethanol might interact with pretreatment and increase contamination. Another issue that was noticed during filtration with $1\mu\text{m}$ filters, was the formation of bubbles on the filter surface. The bubbles formation is linked to the shifted balance in the carbonates equilibrium at pH 3: most carbonates are present as carbonic acid, which is also in equilibrium with the dissolved CO_2 . Increasing the carbonic acid concentration, increases the CO_2 which starts to form microbubbles that coalesce on the filter surface. The bubbles slow down the filtration procedure reducing the area available for filtration. Furthermore, the particles disposition on the filter is not uniform anymore, leading to agglomeration and superposition. The not uniform distribution of particles on the filters impairs also the analysis of small particles on subareas, making almost impossible the definition of representative subareas (see Chapter 8 Conclusion and future perspectives).

All these issues however are not present when using $5\mu\text{m}$ filters. The wider pore width allows for faster filtration, there is no formation of bubbles as the coalescence is impeded, there is no need for ethanol.

The downside of using $5\mu\text{m}$ filters is however in the need to adjust the filtration set up. Indeed, the higher thickness of the $5\mu\text{m}$ compared to the $1\mu\text{m}$ filters ($500\mu\text{m}$ and $200\mu\text{m}$ respectively) makes the gasket used for the $1\mu\text{m}$ filters not efficient. Preferential fluxes

between the filter and the gasket are therefore created, where the water passes avoiding filtration.

The solution found was the use of a modified silicone gasket, to reduce the thickness of the filter, as shown in Figure 19.

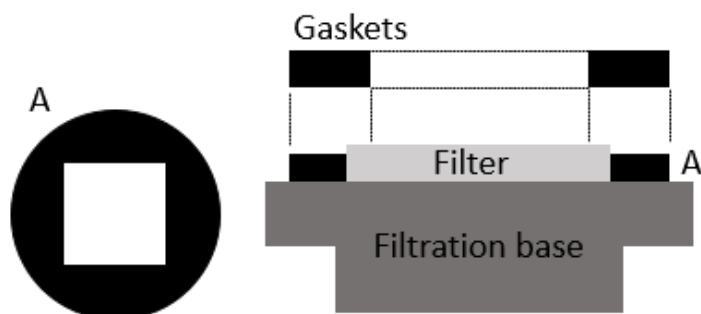


Figure 19 Home-made gasket (A) and filtration set up for 5µm silicon filters

5.5. Image analysis

The image analysis issues and solutions have already been discussed in paragraph 5.1. The 5µm pores silicon filters are therefore chosen for the protocol under development due to their undeniable advantages in many stages of the analysis: from faster filtration to easier and more efficient image analysis.

The area of the filter that is scanned for analysis is equal to square of 9x9mm, being the internal diameter of the gasket used for filtration equal to 8mm. To be precise, the area analyzed is it not perfectly a 9x9mm, but the closer to 9x9mm higher multiple of the 10x FOV (720x540µm). Analyzing the whole filter is indeed counterproductive due to the higher number of spots selected by the algorithm, which includes all the imperfection of the filter at its borders. This highly luminous spots moreover have also influence on the performance of the automatic algorithm, increasing the highest intensity. Therefore, it was deemed wiser to analyze only the area of the filter where the actual filtration happens.

5.6. Spectra Acquisition

The parameters that are set for the automatic spectra acquisition are the following:

- 2 acquisitions
- 5 seconds per acquisition
- 5mW laser power

Two acquisition are indeed the minimum requirement for the correction of cosmic rays. These parameters allow a first screening of the particles on the filter. After the automatic screening the operator will double-check the dubious results setting the signal-to-noise ratio at 300 instead of setting as parameters the number of acquisitions and time. The double-check will indeed be run by the operator on all those spectra which are recognized as MPs but with a match score <60%. The whole map is not acquired with such mode, due to the long time it might be required e.g for fluorescent particles. The spectra acquisition is run with the 10x. The 10x has indeed a depth resolution lower than the higher magnification, allowing radiations from a bigger volume to reach the detector, therefore reducing the difficulties linked to poor focus. Moreover, many libraries present a spectral resolution equal to 4cm^{-1} , which is the one reached by the μ -Raman DXR3 with the set up described in paragraph 5.1.

5.7. Library matching

The libraries chosen for the matching are all but one commercial libraries. Specifically, the commercial libraries chosen are:

- Food Packaging Raman
- HR-FT Raman Polymer library
- HR-FT Raman polymer library addendum
- Pigmenti Raman
- Polymers&additives_Packaging

All made available with the Omnic software. The home-made library is

- MP_ACEA

which comprises spectra from the plastic item anyway present in the laboratory and other spectra often found.

The search regions were chosen in order to avoid the peaks typical of the silicon filter. The Raman shift range recorded is $3500-50\text{ cm}^{-1}$ and the search region are $3500-1100$, $900-600$. The spectral resolution indicated is of 4 cm^{-1} . The matching threshold is set at 60% as suggested by the literature (Cowger et al., 2020b).

The matching method chosen is “correlation”. Indeed, not only it is the most mentioned in the literature among those available in the software, but it was also determined to be the best performing. The spectra of a map were indeed matched with all the methods available and double checked by the operator. The reliability score was set to three levels as follow:

- REL_3: highest reliability score (three points), the plastic is individuated within the right polymer group and a match percentage >60%
- REL_2: middle level of reliability (two points), the particle is individuated as plastic and either the polymer name is right or the matching is >60%
- REL_1: lower level of reliability (one point), the particle is individuated as plastic, but with low matching score and wrong polymer identification
- ERR: the particle is identified as plastic even if it is not, or plastic identification is missed

The trial was run on 100 spectra and each spectrum was evaluated singularly. The results are shown in Figure 20. The method “correlation” (referred as CORR in the graph) shows indeed the highest number of spectra identified with the highest score of reliability and the lowest error. Moreover, the error was quantified in conservative terms, as it is here below explained.

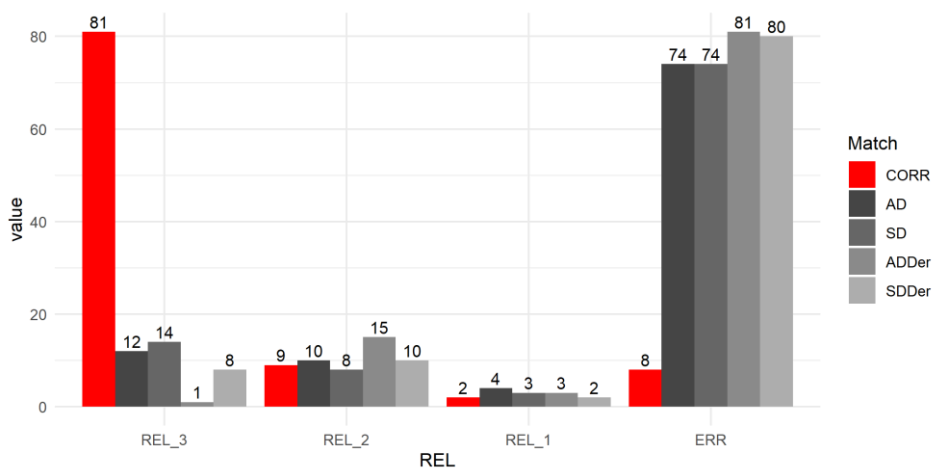


Figure 20 Matching method comparison. CORR correlation, AD absolute difference, SD squared difference, ADDer, absolute derivative, SDDer squared derivative

A spectrum with distinct fluorescence and only few peaks visible, might go as “unidentified” by all the matching methods available in the software. However, if postprocessing in terms of baseline correction and smoothing is applied, it might turn out as a plastic according to the correlation matching method, as shown in Figure 21. This misidentification was considered as an error in the previous matching method evaluation, in order to be conservative in terms of MPs identification. However, it is debatable the identification of MPs with only few distinct and representative peaks.

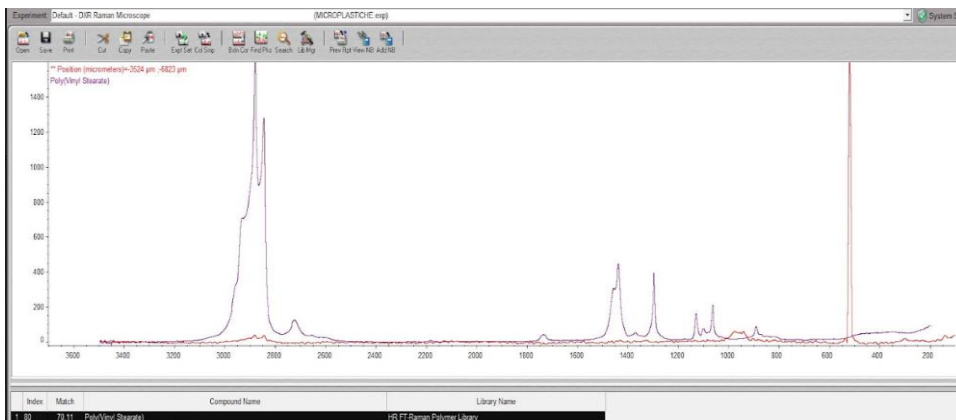


Figure 21 Particle spectrum identified as PVS with 70% matching score after baseline correction and smoothing

The effect of baseline correction is also shown in Figure 22. It is shown as a particle is recognized as plastic thanks to a spectrum present in the custom-made Library, but with matching <60%. As soon as the baseline correction is applied, the matching increases to a 77% and the particle is undeniably identified as plastic, specifically Polyethylene, even if the peaks are barely distinguishable.

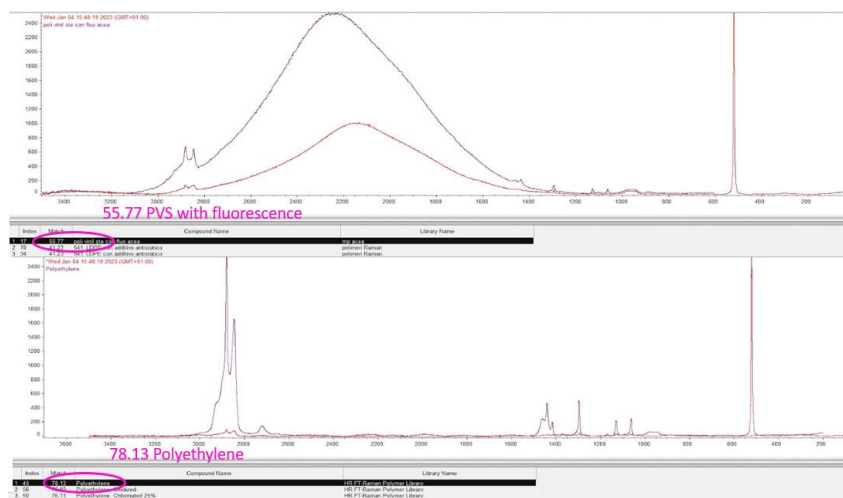


Figure 22 Effect of baseline correction on a spectrum with fluorescence

Another topic linked to the matching method has already been mentioned: the software reports only the highest matching in the final report. However, when the matching score difference among results is lower or close to 1-2%, the unambiguous identification cannot be assured. This outcome is not only associated with spectra belonging to the same polymer group (e.g polyethylene and polyethylene oxidized), but also between polymers

groups, as often between polyethylene and propylene glycol monostearate, as shown in Figure 23.

The only solution to solve the ambiguity, considering the option offered by the software, is through the intervention and judgment of the operator.

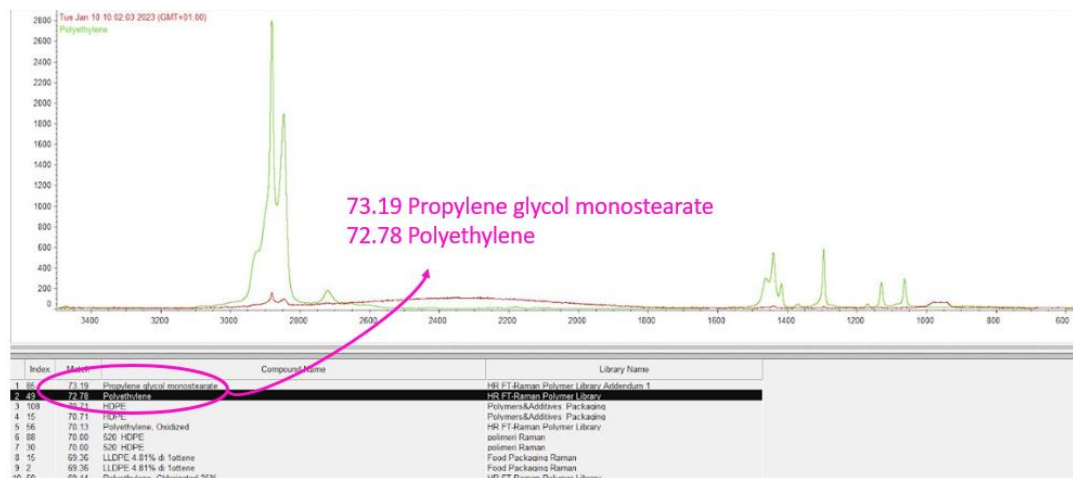


Figure 23 Ambiguous identification of a MP particle

This aspect is taken into consideration also in the reporting, as it will be described in detail in paragraph 5.9, by grouping the polymers in the following major groups: "PE", "PE-C" (polyethylene chlorinated), "PP", "PS", "PVC", "PC", "PA", "PLA", "PMMA", "ABS", "PP-C" (polypropylene chlorinated), "PET", "PU", "PTFE", "PVS", "other", "UnID". The group "others" comprises all the synthetic polymers non included in the other groups, such as additives and pigments.

5.8. QA/QC: quality parameters for the method

MPs are a solid pollutant, characterized according to size, shape, and chemical composition categories. For each of these categories, MPs can be defined as "polydisperse", as already discussed in Chapter 1: the size range spans multiple orders of magnitude with power law distribution (Kooi and Koelmans 2019), the chemical composition is multiple and equal to polymers in production (and decommissioned from mass production, given their persistence), and the shape is variable depending on the processes that led to the formation of the MPs themselves. Given the recent development in the field of MPs research, to date there are no representative reference materials available that can be used to define the quality parameters characteristic of each analytical method: precision, accuracy, limit of detection, limit of quantification, recoveries.

Therefore, in order to define quality parameters for the method, it must be considered the international reference context with respect to the advancement of protocols for the analysis of MPs (for comparison), the ultimate goal of the analysis, and the main critical issues of the analysis that could affect its reliability:

- regarding protocols for analysis of MPs in drinking water, multiple examples from scientific literature exist, but only the California Water Board has defined, very recently, an official analysis protocol for MPs in the >20 μ m size range (Wong, Charles and Coffin, Scott 2022, 1).
- the ultimate goal of the protocol definition is to provide a tool for water utilities to be able to perform a routine MPs analysis, i.e., one that mediates the time-cost-reliability needs typical of routine, differentiating itself from research that may often think to prioritize reliability and reduce costs even at the expense of available time.
- the main critical issues in the analysis concern the presence of contamination of samples, how to choose particles and recognize spectra.

Thus, the relevant scientific literature can provide methodological guidelines, reinforced and defined in the method offered by the California Water Board. These guidelines must then be adapted and implemented according to the conditions, opportunities and peculiarities of the laboratory in which the analyses are conducted. In order to have a reliable international standard as reference (i.e. the Californian guidelines), the quality parameters are determined for package A (package for the analysis of MPs >20 μ m). First, recovery rates will be discussed, then the limit of detection and limit of quantification.

5.8.1. Recovery rates

The lack of a representative reference material for the contaminant present in the matrix of interest greatly limits the information power of the recoveries. Or rather, recoveries in the context of analysis for MPs cannot also be defined as they are for other types of analysis. In fact, recoveries, necessarily made with spike material that is not representative, are useful for comparing the method in use with what is happening internationally and for understanding and thus what may be the possible causes of analyte loss. Strong hypotheses are instead to be made to correct the data obtained based on the results of such recoveries.

The reference material used to make the recoveries are green fluorescent polyethylene spheres, with an average size of 100 μ m, (interval 90-106 μ m) produced by Cospheric, as used in the work by Nizamali et al. (2023) and Bauerlein et al. (2022) (Bäuerlein et al., 2022; Nizamali et al., 2023).

The usefulness of making recoveries was evident in the case of the 5 μ m filters: these are in fact thicker than the 1 μ m filters (500 μ m vs. 150 μ m). Therefore, the filtration set up is not suitable; more specifically, the gaskets do not hold (as described in detail in paragraph 5.4).

In fact, only filtering tap water with spikes of such particles revealed that the gaskets did not hold: some green particles were found on the bottom surface of the gaskets (the one in contact with the filter base and the filter itself) (Figure 24 A). To solve this critical issue, an ad hoc gasket was created to reduce the relative height of the filter, as previously described in paragraph 5.4. Thanks to this escamotage, the polyethylene spheres weren't found anymore at the bottom of the gaskets.

However, there is still particles loss due to their attachment to the inner diameter of the gaskets, as shown in Figure 24 B. This behavior is probably linked to the hydrophobicity of the reference material. Further hypothesis is that the sealing of the gaskets is still not optimal, but from the filtrations carried out there does not seem to be evidence of lateral leakage. Solution to the issue is still under evaluation.

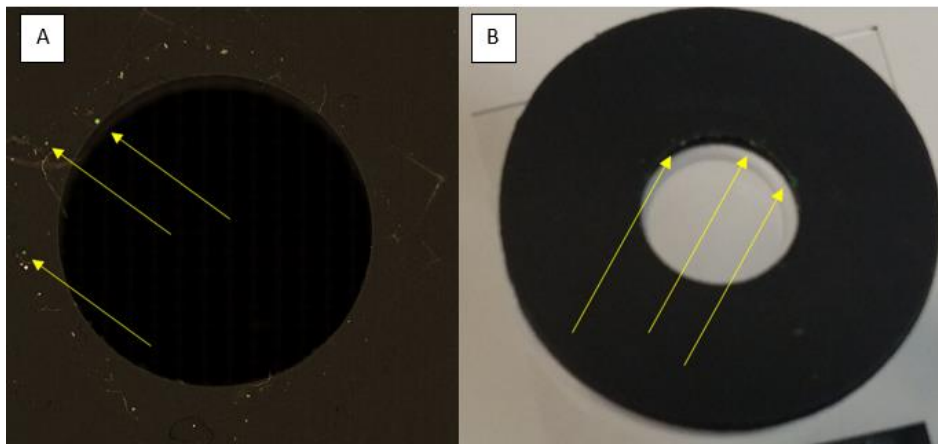


Figure 24 Particles loss on filtration gaskets: A below the gasket, B on its inner diameter

The recovery rates should be run as below described:

- a. fix a 5 μ m filter pore on a glass microscope slide
- b. cover the filter with a thin layer of water-soluble adhesive
- c. with a needle, distribute a few green particles on the filter
- d. acquire the image of the filter
- e. count the green particles
- f. transfer the green particles to the container used for sampling, filled with volume representative of the analysis, detaching the particles with a spray

bottle. Care should be taken not to lose the filter (just hold it to the slide with tweezers)

- g. Pretreat and filter the sample as per the procedure
- h. Acquire image of the filter and count the particles again
- i. (For completeness, also count the particles left on the surrounding of the seal)

5.8.2. Limit of Detection (LOD) and Limit of Quantification (LOQ)

LOD and LOQ have already been discussed in Chapter 3. Contamination control should be implemented inasmuch filtration procedures must be run under a fume hood, all glassware stored under aluminum foil, the use of plastic within the laboratory reduced to a minimum and glassware cleaning must follow a specific procedure. To this purpose tap water further refined (referred to as Acea water) was used, since it was demonstrated by preliminary trials its low contamination of particles and plastics. The glassware cleaning should be run as follows: glassware must be cleaned three times with methanol and Acea water in volume 1:1 and then rinsed three times 0.45 filtered water. If the cleaning procedure results not satisfying, a previous step with soap might be added. LOD and LOQ are to be evaluated as indicated in Chapter 1, specifically referring to the work of Bäuerlein et al. (2022) (Bäuerlein et al., 2022). Indeed, the choice of the 95% confidence interval as LOD and the double of the upper limit of the 95% interval seems to the author as a reasonable compromise. The strategy proposed indeed by Rodland et al (2020) implies the evaluation of the LOD as the upper limit below which 99% of the dataset lie, under the hypothesis of a normal distribution. The approach used by Johnson et al. (2020) is instead often used for dissolved pollutants, which is not the case of MPs. In these terms, the approach suggested by the California Water Board stirs interest: it is indeed said that the formula for determining the MRL (minimum reporting value) looks at the Poisson-normal approximation for low background. However, considering the will to maintain a cautious approach, the approach proposed by Bäuerlein is more conservative as it considers often lower values for both LOD and LOQ. All polymers not detected in blanks have an LOD and LOQ equal to 1 MP.

5.9. Data analysis and reporting

The data analysis is developed mainly on the open-source software R. Data are to be analyzed based on LOD and LOQ and the comparison of the abundances of MPs between blanks and samples. MPs are deemed to be present in the sample if their number is higher than the LOD, and they are to be quantified if higher than the LOQ. The results are to be reported summarized based on main polymer groups and in detail, supplying the report output of the DXR3- μ -Raman. MPs size is also to be reported, with the shape descriptor Aspect ratio (Major dimension/ Minor dimension). Whenever possible, density function

should be introduced, as mentioned in Chapter 1. All the parameters chosen for the μ -Raman analysis are to be reported as the quality parameters for the method. Polymer grouping is preliminary organized according to: "PE", "PE-C" (polyethylene chlorinated), "PP", "PS", "PVC", "PC", "PA", "PLA", "PMMA", "ABS", "PP-C" (polypropylene chlorinated), "PET", "PU", "PTFE", "PVS", "other", "UnID". However, further experience with samples and monitoring might supply further material for refining and modify the grouping.

5.10. Analysis costs

Final remarks are to be linked to the analysis costs, which are hereby briefly summed up:

- Cost of silicon filter (24-36€)
- Cost of laser operation (c.a 6€/h. Depends on the n° of particles on the filter and acquisition parameters used, i.e. 14 seconds per particle for screening)
- Cost of reagents for pre-treatments and cleaning, including filtered (MilliQ) water (depends on the water source mostly. Estimated c.a 20€ per sample if the water is filtered in-house)
- Cost of operator time (depends on the operator wage, the time expected of operator engagement in the analysis is of 2-3h per analysis on effluents / drinking water)
- Amortization of the analytical tool acquisition: in case of the μ -Raman 10 years can be considered due to the fact that it can be used for many analyses and its relatively low technological turn-over

Considering all these aspects, the cost for the analysis of 1L water for human consumption falls reasonably between 150-170€ per sample, which is in line with the cost of other CECs.

Overall, the higher the number of particles to be analysed on the filter, the higher the costs. The higher the number of particle on which to run the mentioned "double check" (paragraph 5.6) after the screening, the higher the time required to the operator. The higher the volumes analysed the higher the MPs encountered, the higher number of particles on which to run the double-check. This is therefore another aspect to take into account when evaluating the volume to be sampled and analysed, especially in case of routine monitoring.

Reducing costs can be therefore linked to two main aspects:

- cleaner filter: the use of reagent and the engagement of the operator for pre-treatments operation has however to be taken into account;
- careful evaluation of the volume to be sampled: define the question to be answered (characterization, routine monitoring...) and reduce the volume to be sampled to the minimum possible

Chapter 6. Case study

The case study consisted of the effluent of a DWTP located in Italy which was sampled for two weeks to validate the method defined in Chapter 5 and discover and underline possible pitfalls. The DWTP under the spotlight treats water from one of the Lazio Region major river; hence the water source is classified as surface water.

6.1. Materials and methods

6.1.1. Sampling, pretreatments and MPs identification

Sampling was conducted during six different days in the first half of January 2023. The inflow turbidity was within 32-90 FNU, indicating a quite stable water quality, and hence plant operation. Sampling was conducted with one-liter glass bottles covered with aluminum foil. The samples were pretreated and analyzed the same day. Glassware was cleaned three times with water and methanol in 1:1 volume ratio, rinsed three times with water and then one time with 0.45 μ m filtered water. Pretreatments consisted in lowering the pH to the interval [2-3] with HCl 37% acquired from Carlo Erba. The time for the pretreatment is of 15 min at room temperature. The samples were then filtered under a fume hood on silicon filters of 1cmx1cm area with 5 μ m pore width (Smartmembranes). The MPs Identification-Quantification-Characterization was run with the μ -Raman DXR3 Thermo-Fisher, exploiting the potential of the automatic algorithm for particles selection (software Atl μ s) and the software Omnic for the acquired spectra analysis and matching. The whole filtration area of the filter was analyzed (9x9mm). The size interval selected comprises all particles with length in [20-150] μ m. The automatic analysis is run with 10mW laser power (green laser 532 nm), 2 acquisition and 5seconds per acquisition. Double check is run manually with S/N set at 300, for all the particles that resulted as MPs during the scanning but with a matching score <60%. The spectra acquired are matched against the commercial libraries "Food Packaging Raman", "HR-FT Raman Polymer library", "HR-FT Raman polymer library addendum", "Pigmenti Raman", "Polymers&additives_Packaging" and the home-made library MP_Acea (which includes the spectra of plastic elements anyway present in the laboratory and of often found spectra). The matching method used is correlation, the search regions are 3500-1100cm⁻¹ and 900-600cm⁻¹. A particle is considered as plastic if its matching score is >60%.

Data are reported by summarizing the results based on main polymer groups: "PE", "PE-C" (polyethylene chlorinated), "PP", "PS", "PVC", "PC", "PA", "PLA", "PMMA", "ABS", "PP-C" (polypropylene chlorinated), "PET", "PU", "PTFE", "PVS", "other", "UnID". The group "others" comprises all the synthetic polymers non included in the other groups, such as additives and pigments. The group "UnID" comprises all the particles not identified as

plastic polymers. The Aspect Ratio of the particles, calculated as Length/Width, is used as shape parameter. The Aspect Ratio can indeed be directly calculated from the μ -Raman DXR3 output. R and R Studio are used for data analysis.

6.1.2. QA/QC

Contamination was limited by filtering the samples under a fume hood; only glassware and glass tools were used, if not for the caps of the reagent bottles and the gaskets used for filtration (black rubber). All the glassware and tool were covered with aluminum foil to reduce the contamination by laboratory air. Contamination was evaluated through laboratory blanks, where 1L of tap water filtered at 0.45 μ m was treated following the same procedures of the sample: from the sampling with the glass bottle covered with aluminum foil to the identification and quantification procedures. Laboratory blanks in number of six were acquired, and LOD and LOQ calculated from those, following the recent literature indications. Specifically, results were corrected following B auerlein et al (2022) (B auerlein et al., 2022). Positive controls were also run, in number of four, with green polyethylene fluorescent microspheres (Cospheric, size 90-106 μ m). The samples were spiked with known amount of microsphere which were then counted on the filter after pretreatments and filtration. LOD and LOQ results are reported only for the polymer groups detected in the blanks, for all the others it is assumed a value of 1.

6.2. Results

6.2.1. Results: QA/QC

The results of the recovery rates assessment are reported in Table 11. Particles lost in the bottle or on the filter system are generally well below 10 and in numbers consistent among trials. The recovery that is achieved is about 70% on average, as shown in Table 11. It is moreover to be noticed how the number of particles lost on the gaskets and in the container is fairly constant. Using a higher number of particles to calculate the recovery rates leads therefore to higher recoveries. This information however is not always reported in the literature.

Table 11 Recovery rates. n $^\circ$: number of particles

Matrix	n$^\circ$ spiked	n$^\circ$ gaskets	n$^\circ$ filter	Recovery	n$^\circ$ lost
A Tap water	39	13	21	54%	4
B Tap water	71	6	63	89%	0
C Tap water	89	17	66	74%	5
D Tap water	67	20	41	61%	5

The recoveries are comparable or slightly lower than the literature data (Bäuerlein et al., 2022; Dronjak et al., 2022; Frond et al., 2022; Nizamali et al., 2023; Tsering et al., 2022), putting the method in line with the international research context. The values of the recovery rate is due to the attachment of the spiking spheres at the inner diameter of the gaskets, and not to the loss of particles on the glassware. The results obtained are not corrected based on the recovery rates, since the reference material is not representative of the mixture of microplastics polymers analyzed. Recovery rates are indeed deemed useful for the setting of the methodology within the international setting of the advancement in MPs research and to understand possible dynamics during analytical procedures.

The results of the negative controls (blanks) are reported in Table 12. On average, 5 MPs were found in negative controls. Only PE, PET, PP, PTFE have been detected in blanks among the main polymer groups. Polymers detected within the group “others” are: polydimethylsiloxane (probably from the rubber gaskets), propylene glycol monostearate (personal care products or plastic additive)² and pigments.

The size range of the particles recovered with the blanks is [20-76] µm, with median of 26 µm and mean 32 µm. Only fragments have been detected.

Table 12 LOD and LOQ by polymer group. 1* refers to the value of 1 given to polymers not identified in blanks

Polymer	ABS	PA	PC	PE	PE-C	PET	PLA	PMMA	PP	PP-C	PS	PTFE	PU	PVC	PVS	other
LOD	1*	1*	1*	3.4	1*	2.9	1*	1*	1.3	1*	1*	2.1	1*	1*	1*	3.1
LOQ	1*	1*	1*	6.8	1*	5.8	1*	1*	2.6	1*	1*	4.2	1*	1*	1*	6.2

6.2.2. Results: MPs abundance in DWTPs effluent

The results of the analysis are reported in Table 13. On average 9 MPs were identified in samples. Only PE, PET, PTFE and PVS have been identified in the samples among the major polymer groups. Within the group “other”, the most identified are once again propylene glycol monostearate and pigments. However, only PE and PTFE have been detected and only PE quantified in the samples.

The blank correction was run as follows: the average value of the blanks was subtracted to the value detected in the effluent samples. If the concentration values was higher than the determined LOD then MPs were considered detected, if higher than the LOQ, quantified. The strategy was applied according to the polymer composition, not yet according to MPs

² [Substance Information - ECHA \(e https://echa.europa.eu/substance-information/-/substanceinfo/100.013.959 uropa.eu\)](https://echa.europa.eu/substance-information/-/substanceinfo/100.013.959)

size or shape. MPs resulted as detected in each sample but one and quantified only in one sample (sample of 05/01/2023). As the concentration difference is quite relevant however, the suspicion of contamination arises. The blank run during that day however does not record significant contamination. Considering the randomness of such contamination though, it still cannot be completely excluded. The mean concentration calculated including the results of the 05/01/2023 is of 2MPs/L, which is slightly higher than what reported in the literature: Bäuerlein et al. (2022) indicates an average concentration of 0.5MPs/L, Wu et al (2022) of 0.3MPs/L. In both studies, MPs>20µm were analyzed (Bäuerlein et al., 2022; Wu et al., 2022). If the sample of the 05/01/2023 is not included, MPs result as never quantified, which is coherent with the results obtained by the literature, which report a concentration often lower than 1MPs/L. The detection of PTFE also is to be considered carefully, being PTFE rarely detected in water according to literature data (Oßmann, 2021).

The size range of the MPs identified in the samples is of [20-140] µm, with median of 25 µm and mean of 31 µm. Only fragments have been identified.

Table 13 Detection and quantification of MPs in the DWTP effluent

Date	Polymer	MPs n°	Detected	Quantified
02/01/2023	PE	3	<LOD	<LOQ
02/01/2023	PET	1	<LOD	<LOQ
02/01/2023	PTFE	3	2.5	<LOQ
02/01/2023	PVS	1	<LOD	<LOQ
02/01/2023	other	1	<LOD	<LOQ
05/01/2023	PE	12	10.7	10.7
05/01/2023	PTFE	3	2.5	<LOQ
05/01/2023	other	2	<LOD	<LOQ
09/01/2023	PE	5	3.7	<LOQ
09/01/2023	PVS	1	<LOD	<LOQ
09/01/2023	other	3	<LOD	<LOQ
12/01/2023	PE	3	<LOD	<LOQ
12/01/2023	other	3	<LOD	<LOQ
13/01/2023	PE	1	<LOD	<LOQ
13/01/2023	PTFE	2	<LOD	<LOQ
13/01/2023	other	7	5.5	<LOQ

6.3. Discussion

The results obtained from the monitoring of the effluent of a DWTP are coherent with what reported so far by the literature. However, this coherence is strongly dependent on the contamination control and the data correction procedures applied to evaluate the MPs presence in the samples.

The interest of the water utility is to ascertain that the water they provided is safe in terms of MPs pollution. Strategies for risk evaluation in terms of human health cannot be put in place yet due to the lack of risk assessment data. However, it is demonstrated by this work that, with a simple method applicable in every laboratory (for what regards sample acquisition and treatment, whereas I-C-Q requires a μ -Raman as analytical tool), it is possible to ascertain the very low level of contamination of drinking water by MPs in size $>20\mu\text{m}$. Another consideration that can be derived from this experience, is that the environmental sources of MPs might be more relevant than the water we drink: the contribution to the MPs count of the environmental contamination, evaluated by blanks, is indeed overshadowing the contribution linked to the MPs in the sample. It is however interesting to notice how in almost every sample there is at least one polymer group with an abundance $>\text{LOD}$.

Moreover, one of the questions posed in Chapter 1 is if MPs get rounder with their decreasing size. This aspect is investigated by observing the relation between Length and Aspect Ratio (ratio between length and width).

The Length and Aspect Ratio are therefore plotted. The linear relation between Aspect Ratio and Length is shown in Figure 25 for MPs in the size interval $20\text{-}90\mu\text{m}$. Indeed, only two MPs have $\text{Length}>90\mu\text{m}$, however, their weight on the definition of the regression line is significant. Therefore, it was decided to reduce the interval to the more representative subset of the sample.

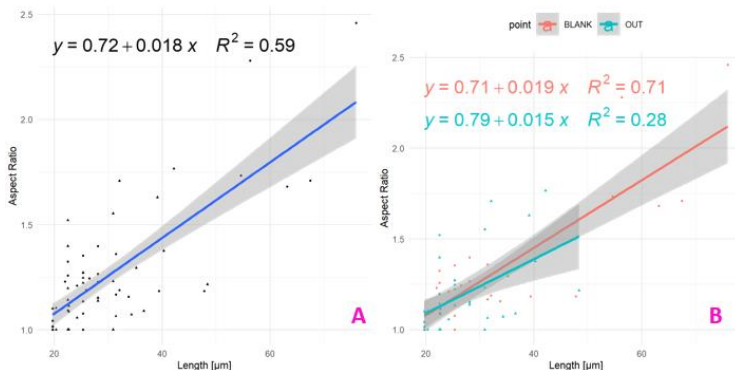


Figure 25 Length and aspect ratio relation for $20\mu\text{m}<\text{MPs}<90\mu\text{m}$. Grey areas: 95% confidence interval for regression lines

The graph plotted suggests the possible existence of a linear relationship between the two variables. This correlation seems to be more relevant for the MPs identified in the blank samples compared to that in the effluent. The relatively low number of values (33 blanks, 44 effluent) however might influence the outcome. The correlation among the two variables was next evaluated as its significance. The Spearman correlation coefficient was calculated for the whole dataset and it results of 0.67. The correlation is also significant ($p.value < 0.05$). This result suggests therefore that MPs main dimensions, length and width, becomes more similar with decreasing size. It is not possible to properly evaluate the relation also based on polymer group, due to the low number of particles present per polymer group.

Regarding size distribution there is a clear prevalence of MPs within the size range 20-40 μ m. The database is however deemed for the moment too scant to evaluate properly a density distribution for its main morphology metrics (length, width and area). However, the adamant prevalence of smaller MPs confirms once more the higher abundance of smaller MPs.

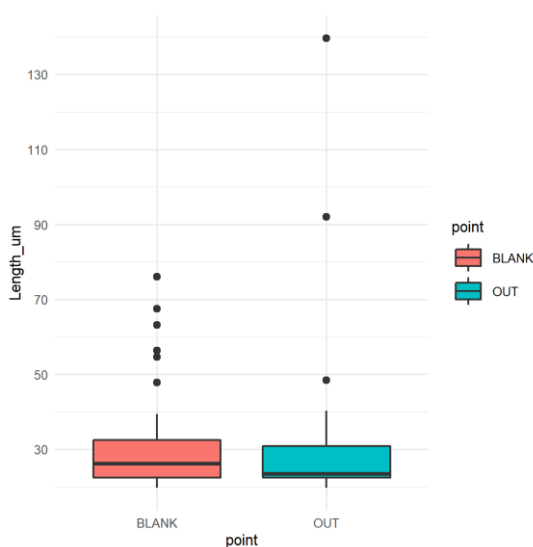


Figure 26 Size distribution of the MPs identified in the samples

6.4. Conclusions

The method applied was demonstrated to fit the purpose. The effluent of a DWTP was screened to investigate the MPs presence. The results revealed the scarce pollution of the matrix by MPs $> 20\mu$ m. The results obtained are in line with what observed by the scientific community for MPs in the same size range. It is therefore confirmed how the MPs presence

drinking water is probably to be related mostly to MPs <20 μ m, for which further investigations are required. There is still room for improvement of both the analytical method and data reporting: environmental contamination might be further reduced as polymer cluster grouping refined. However, keeping in mind the need of time-cost-reliability, the analysis workflow here proposed is already deemed suitable for the screening of MPs in DWTPs effluents.

It is hereby also confirmed that, if the goal of the analysis is not the screening of the effluent, or the evaluation of a method, but it is the complete characterization of the MPs pollution of the effluent (continuous distributions for the morphological metrics, polymer groups relative abundance), samples of higher volume are required. The acquisition of higher volumes grants indeed the chance of an easier discrimination between MPs due to environmental contamination and those in the matrix, due to the higher number of the latter in the sample. The higher volume acquired will also result in a more representative sample, especially for MPs>20 μ m.

Chapter 7. Experience abroad: the MoVE project

*Presented at the MICRO2022 conference - Lanzarote, Spain, as
“Adding depth to Microplastics”*

Authors: Margherita Barchiesi, Merel Kooi, Albert A. Koelmans

7.1. Introduction

The work presented in this chapter was developed at the Aquatic Ecology and Water Management Group of the Wageningen University and Research under the supervision of Professor Albert A. Koelmans and with the collaboration of Merel Kooi during the period 03/2022-08/2022. The goal was to evaluate the strategies until now available for the estimation of MPs volume from 2D morphology information (**M**orphology to **V**olum**E** project).

Spectroscopic techniques are among the most used for MPs analysis, as already discussed in Chapter 2. Spectroscopic techniques however provide data “only” on MPs chemical identification and 2D morphology parameters (size, area, aspect ratio et cetera). Other metrics relevant for environmental and health risk assessment, as for the understanding of all the processes that involve MPs, are missed: volume, mass and surface area are indeed information that cannot be derived by the analysis of MPs with spectroscopic techniques.

Among these metrics, volume is a parameter of utmost relevance due to its implication for ecotoxicology, transport processes, mass balances.

In the urban water cycle context, volume is relevant for a better understanding of MPs fate in water treatment plants, especially if coupled with density for mass estimation, source apportionment and environmental risk evaluation.

The lack of information due to the analytical technique applied is usually superseded by the use of models.

However, only few studies have been conducted on the reliability of such models. Indeed, only few papers evaluated the goodness of fit for volume or mass models, and in most cases focus was on the single particle (even if overall conclusions were driven also on the bulk) (Medina Faull et al., 2021; Tanoiri et al., 2021).

Aim of this work is to develop an empirical approach to estimate the collective volume of a mixture of microplastic particles, and to further develop and validate mathematical models required for this conversion.

The approach applied is innovative inasmuch the volume estimate is not evaluated based on the single particle but on the collective mixture. Three reasons stand behind this approach:

- MPs are found as mixtures in the environment
- Often, it is not of interest the volume/mass of the single particle, but that of the mixture (e.g food dilution, mass balances)
- The inaccuracies in the model estimate for the single particle might cancel out when considering the bulk
- More analytical methods for volume measurement are available when considering the bulk and not the single particle.

7.2. Materials and methods

7.2.1. Models applied for volume estimation from 2D microplastic features

The available models found in literature for volume estimates from 2D information are reported in the following table (Table 14):

Table 14 Models for volume estimate form 2D information

Model	Formula
Medina model (Medina Faull et al., 2021)	$V_M = \frac{4}{3} \times \pi \times \left(\sqrt{\frac{A}{\pi}} \right)^3$
Cozar model (Cózar et al., 2014)	$V_C = k_C \times M^3, k_C = 0.1$
Isobe model (Isobe et al., 2019)	$V_I = k_I \times M \times \pi \times \left(\frac{M}{2} \right)^2, k_I = 0.4$
Simon model (Simon et al., 2018)	$V_S = \frac{4}{3} \times \pi \times \left(\frac{M}{2} \right) \left(\frac{m}{2} \right) \left(\left(\frac{m}{M} \right)_{median} \times \left(\frac{m}{2} \right) \right)$
Tanoiri model (Tanoiri et al., 2021)	$V_{T, Fragments} = \frac{4}{3} \times \pi \times \left(\frac{M}{2} \right) \left(\frac{m}{2} \right) \left(k_{T,S} \times \left(\frac{m}{2} \right) \right), k_{T,S} = 0.372$ $V_{T, Pellets} = \frac{4}{3} \times \pi \times \left(\frac{M}{2} \right) \left(\frac{m}{2} \right) \left(k_{t,P} \times \left(\frac{M}{2} \right) \right), k_{t,P} = 0.565$
V=volume, A= area retrieved from 2D image analysis, M= major axis of the best fit ellipse, m minor axis of the best fit ellipse, k = calibrating parameter.	

Up to 2022 however, only the paper by Tanoiri et al. (2021) has tried an evaluation and comparison of the different strategies for volume evaluation.

7.2.2. Microplastic mixture used

The MPs mixtures were collected in Singapore (Changi beach) and in the Netherlands (Hoek van Holland beach). The plastic was sorted visually in the laboratory of Wageningen University using forceps, and organic matter was removed. Expanded polystyrene (EPS) was also removed through flotation in ethanol. MPs were then divided by sieving in two size categories: 1-2mm and 2-5mm. Moreover, MPs were divided in three groups based on their shape (pellet and round MPs, fragments and fibers, referred to as “primary”, “secondary” and “fibers” respectively). Three samples for each group were subset. The subgroups trialed are therefore:

- Primary MPs in size range 2-5mm ‘P25’
- Secondary MPs in size range 2-5mm as ‘S25’
- Secondary MPs in size range 1-2mm as ‘S12’
- Fibers

To understand the relevance of MPs number in the assessments, small samples composed by few MPs (3-5 MPs) were also subgrouped, named according to size and shape group, as follows:

- P25_few
- S25_few
- S12_few

7.2.3. Volume measurement

Volume of MPs mixtures was measure with a pycnometer (Ultracyc 1500e by Quantachrome) and with a newly developed method for volume measurement. Indeed, the pycnometer measurement chamber allow the volume measurement of only few MPs in the size range 2-5mm. Therefore, for the proper assessment of volume estimate for mixtures, a new method is proposed. The new method is based on weight difference, as follows:

- The weight of the plastic to evaluate is measured W_{MPs}
- The weight of a volumetric flask filled with exactly 25.0 ml (V_1) of ethanol was measured (W_1).
- In the same but empty flask, MPs with known weight (W_{MPs}) were added. Then, ethanol was added until a total (MPs plus ethanol) volume of 25.0 ml is reached (V_2).
- The weight of the flask with MPs and ethanol is measured (W_2).

The volume of MPs hence is calculated from:

$$V_{MPs} = \frac{(W_1 - (W_2 - W_{MPs}))}{\rho_{et}}$$

ρ_{et} is the density of ethanol. Ethanol ($\rho_{et} = 0.81\text{g/cm}^3$, ethanol 96% acquired from VWR) has a density lower than most MPs, which therefore sink to the bottom of the volumetric flask, allowing for the precise filling of the volumetric flask to 25.0 mL. Furthermore, ethanol has a lower surface tension compared to water, which eases the release of air bubbles from the particles.

The weights were measured with an analytical balance of $\pm 0.0001\text{g}$ precision at constant room temperature. The weight of the volumetric flask filled with ethanol and MPs was taken after sonication for 15min, to get rid of all the air bubbles possibly embedded within the MPs. The volumetric flask was then left at rest for 30min to return to room temperature (same temperature as the previous W_2 weight measurements), and the volume adjusted (if needed) to the 25.0 mL.

The reliability of the new volume measurement protocol was checked by testing the replicability of the volume measurement, to validate the underlying hypothesis that $V_1 = V_2$. The weight of a 25mL volumetric flask filled with ethanol was measured ten times, each time emptied and refilled. Moreover, the collective volume of glass spheres of 2mm diameter was also measured both with the pycnometer and with the new protocol developed for particle volume measurement. These measurements were run in triplicates. The standard deviation of the 10 replicates of the weight measurements for the replicability of the volume acquisition was 0.016%, which is considered very good for the scope of this study. The difference between the glass spheres' volume measurements run with the pycnometer and with the developed protocol, was less than 2%.

7.2.4. 2D information acquisition

The 2D data were acquired from the image analysis of high resolution pictures of the MPs. The pictures were taken with a 24.2 megapixel high resolution camera (Nikon D3200), positioned on a tripod, with a 35mm or 105mm focal length Nikon lens (depending on MPs size), at ISO 200, using exposure times and aperture selected to accommodate the different light conditions during the day. Lens distortions, if any, were automatically corrected in-camera. The MPs were scattered on a black carton. To be able to analyze reproducibility, three “throws” were run for each batch. A “throw” is one deposition of MP on the black carton followed by Image analysis. Throws can lead to different results if the particles end up lying on the carton on a different side at each throw. NX Studio, program openly available by Nikon, was used for picture postprocessing

The pictures were then analyzed with ImageJ (Schneider et al., 2012). A new macro was developed to integrate the image thresholding plug-in with the image analysis plug-in, in order to retrieve the 2D parameters. Image quality was monitored by including a ruler and a reference object as an internal standard in each image. The image analysis process was modified for the analysis of fibers, to include the use of the plug-in “ridge detection”(Steger, 1998; Wagner et al., 2017). The results of the image analysis were used to retrieve the MPs dimensional parameters (Area, Major and Minor axes, Aspect Ratio, Circularity) required for the volume estimate. For all the other parameters included in the models (k_i ; Table 1), the best-fit parameters obtained by the different studies presented in section 7.2.1 were used. For particle “length”, the Major axis of the best-fit ellipse was used, following Tanoiri et al. (Tanoiri et al., 2021).

The results of the automated image analysis were considered acceptable if the min-max difference of the measurement of the Area of the reference object was less than 1%. In all other cases, each image was processed singularly. The resolution was 18.6 – 19.2 pixels/mm for the 35mm focal length and 0.042-0.043px/ μm for the 105mm focal length. All the data were processed using R studio or Microsoft Excel. The triplicates are from now on indicated by the index “i”, and throws (T) by the index “j”: e.g the results from the “jth” throw of the “ith” replicate (batch) for group P25 is indicated as $T_{i,j}^{P25}$.

7.2.5. A new model for volume estimation

All the models proposed and described in section 7.2.1 theoretically require calibration, except for the Medina and Simon model. All the parameters of the Medina and Simon model can be retrieved from the 2D images, whereas for the other models a “best fit” evaluation with measured volume data is required. However, this is not always possible or practical. Moreover, it has been reported that the Simon model consistently overestimates MPs volume or mass, especially for larger particles (Primpke et al., 2020; Tanoiri et al., 2021). The Simon model includes particle asymmetry for the major dimension (length, width and height). However, there is a surface irregularity that remains unaccounted for, that might affect also the estimate for the 3rd dimension (particle height). It is here assumed that this irregularity might be quantified by the ratio between the best fit ellipse perimeter, computed by the first Ramanujan approximation (Ramanujan, 1914), and the actual perimeter of the MPs observed in the image, and as provided by ImageJ. However, being the particle a 3D object, this unitless correction factor should be applied to the three space dimensions, and therefore is calculated to the power three. The more the particle is regular, the less relevant is the correction factor. When the particle 2D area is a perfect ellipse or circle, the correction factor is equal to 1. This model is referred to as the Barchiesi model and the volume estimate can be summarized as $V_B = C_f^* V_{\text{Simon}}$. The model equation is shown in equation 2, where ‘M’ and ‘m’ are major and minor axis respectively and P_{MP} is

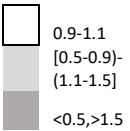
the perimeter of the MPs. Here, the first term refers to the correction factor (Cf) that accounts for surface irregularity, whereas the other terms represent the volume of an ellipsoid as proposed by Simon et al. (2019) (Table 14).

$$V_B = \left(\frac{\pi \times \left[3 \times \left(\frac{M}{2} + \frac{m}{2} \right) - \sqrt{\left(\left(3 \times \frac{M}{2} + \frac{m}{2} \right) \times \left(\frac{M}{2} + 3 \times \frac{m}{2} \right) \right)} \right]}{P_{MP}} \right)^3 \times \frac{4}{3} \times \pi \times \left(\frac{M}{2} \right) \left(\frac{m}{2} \right) \left(\left(\frac{m}{M} \right)_{median} \times \left(\frac{m}{2} \right) \right) \quad (2)$$

7.3. Results

The results of the volume estimate evaluation are shown as the ratio $((\bar{V}_{estimate,i}(T_{ij}))/V_{measured,i})$ averaged per group (Table 15). Overall, the best performance is recorded for the *Barchiesi* model (present study), followed by the Tanoiri model, and then the Simon model. The ellipsoid as the best shape for MPs is therefore confirmed for the size range under investigation, albeit with the additional surface heterogeneity correction which only is available in the *Barchiesi* model. The Simon model overestimates particle volume up to 56%, which is in agreement to earlier findings (Primpke et al., 2020; Tanoiri et al., 2021). The *Barchiesi* model performed very well for the groups P25, S25, and S12, showing a $V_{estimate}/V_{measured}$ ratio in the interval [0.99-1.05]. The performance of the *Barchiesi* model was also good for the samples with a lower amount of particles (MPs n° 3-4 for P25_few, S25_few, and 26-33 for S12_few), with a $V_{estimate}/V_{measured}$ ratio in the interval [0.9-1.2]. The Tanoiri model performed very well for all the MPs for samples, with a $V_{estimate}/V_{measured}$ ratio in the interval [0.98-1.02]. However, the accuracy dropped substantially for secondary MPs samples with few MPs, showing a $V_{estimate}/V_{measured}$ ratio in the interval [0.76-0.87]. It is of utmost interest however to notice how the use of parameters calibrated on MPs from a very different setting results anyway in a volume model with good performance. The good performance of the *Barchiesi* model, however, encourages pursuing a strategy for MP volume exploiting only the information retrievable by 2D images.

Table 15 Ratio between estimated and measured volume for MPs mixtures

	P_2-5	S_2-5	S_1-2	P_25_few	S_25_few	S_12_few	
Medina	1.65	3.56	3.86	1.56	2.14	3.12	 0.9-1.1 [0.5-0.9]- [1.1-1.5] <0.5,>1.5
<i>Simon</i>	1.25	1.56	1.55	1.09	1.20	1.48	
Cozar	0.42	2.10	3.26	0.42	0.75	1.54	
Isobe	1.30	6.60	10.23	1.31	1.90	4.84	
Tanoiri	1.02	0.98	0.99	0.98	0.76	0.87	
<i>Barchiesi</i>	1.05	0.99	1.04	0.92	0.94	0.92	


Information on the relevance of the side (orientation) of the MPs can be derived from observing the standard deviation among throws for the volume estimate. The standard deviation for the $V_{\text{estimate}}/V_{\text{measured}}$ ratio among throws ($SD_{g,i}(T_{ij})$) is usually higher when few particles are analyzed than when a higher number is included (Table 16).

The ($SD_{g,i}(T_{ij})$) is always lower than 1.1% for primary microplastics for batches with many MPs, whereas it is higher for batches with only few MPs (small samples). The situation is more blended for secondary MPs, where the ($SD_{g,i}(T_{ij})$) shows lower variability.

It can be concluded that the side where the MPs end up laying may be of lower relevance when many MPs are analyzed. Estimating volume on a higher number of particles is hence more reliable also with consideration to the different sides the MPs might end up on.

Table 16 Standard deviation in % among throws for the two best performing models. Bi indicates the batch, f indicates samples with fewer MPs (small samples).

Group	Model	B1	B2	B3
P25	Tanoiri	0.62	1.03	0.80
	Barchiesi	0.69	0.11	0.61
S25	Tanoiri	1.19	0.99	0.55
	Barchiesi	3.01	0.96	0.87
S12	Tanoiri	2.06	2.12	0.92
	Barchiesi	1.56	2.13	1.50
<i>P25few</i>	<i>Tanoiri</i>	<i>2.02</i>	<i>0.76</i>	<i>2.15</i>
	<i>Barchiesi</i>	<i>6.49</i>	<i>2.20</i>	<i>10.39</i>
<i>S25few</i>	<i>Tanoiri</i>	<i>0.52</i>	<i>1.81</i>	<i>1.81</i>
	<i>Barchiesi</i>	<i>2.70</i>	<i>5.35</i>	<i>0.86</i>
<i>S12few</i>	<i>Tanoiri</i>	<i>2.67</i>	<i>1.66</i>	<i>0.77</i>
	<i>Barchiesi</i>	<i>3.37</i>	<i>2.05</i>	<i>1.60</i>


 primary MPs
 secondary MPs

Regarding fibers, the model proposed by Simon et al. (2018) performed very well for two of the three samples, with a ratio of $V_{\text{modeled}}/V_{\text{measured}}$ volume of 1.07 and 1.1. The third sample showed an overestimation of about 45%.

It should be mentioned that the measured volume of the fibers is much lower than the calibration volume for the pycnometer (0.01 vs 0.08 cm³), which might impact the reliability of the measure. However, the results are satisfying and promising for future development of methods for fibers volume estimate.

7.4. Conclusions

The main outcomes of this work are:

- New protocol for the volume measurement of large MPs samples. Good reliability based on comparison with pycnometer data
- Soundness of the new approach to MPs volume estimate: estimating volume on the bulk instead of pursuing estimates for individual particles. This approach is indeed more reliable, stable and less prone to inaccuracies linked to the 2D image analysis. The study of models reliability is moreover facilitated, especially with decreasing size
- The best model for MPs volume estimate are those that assume an ellipsoidal shape for MPs, at least for the size under study [1-5mm].
- The new Barchiesi model showed remarkable goodness in the size range studied. This might lead to the conclusion that the irregularity of MPs should be taken into account, and that it could be done based on 2D images. Further validation is however recommended, especially for smaller size classes.

The new and improved methods to measure the volume of MPs and to estimate it based on 2D image analysis data can therefore be considered as valid tools to improve the understanding of fate effect and risk assessment for MPs.

Chapter 8. Conclusions and future perspectives

The main take out points of this work are:

- MPs analytical methods are getting stronger thanks to the development of quality assessment and control parameters, even for an elusive pollutant as MPs.
- μ -Raman is an effective analytical technique for MPs analysis, although there is still room for improvements and its analytical limits should be evaluated based on the automation required.
- MPs monitoring for water utilities is possible, although the time required and the cost of the analysis are still high.
- The μ -Raman analysis packages for MPs should be better defined based on the size range of interest, as the optimal protocol and instrument set up may vary
- More is needed to be known in terms of health hazard from MPs to define appropriate monitoring strategies for MPs in drinking water
- Sampling of few liters of water, might be used for the screening of DWTPs effluents, but not for the full characterization of the MPs pollution (which is needed for the understanding and definition of the related risk).
- The use of models to estimate MPs volume from 2D images of MPs has been proven feasible and reliable, when applied to MPs mixtures. Further validation of the approach is recommended for MPs < 1mm.

MPs are continuously fed into the urban water cycle by WWTPs discharge, urban and agricultural runoff and atmospheric deposition. Every time surface water is used as water source, MPs are then recycled into the urban water cycle. Scant evidences are until now available on groundwater sources. MPs pollution in water cannot be studied or evaluated if not framed in the context of the water cycle, more so for the urban water cycle.

The presence of MPs in every environmental compartments is not anymore in doubt. The knowledge gap to be filled is therefore its time-space dynamics, processes, influencing factors and effects. To gain information on these aspects, the quality of the analytical protocol is of utmost relevance. MPs analytical methods are improving with time in terms of time-cost-reliability. QA/QC are nowadays to be found quite complete (positive and negative samples, full description of analytical method and parameters used) in almost all outputs offered by the scientific community. This trend in higher transparency will help the results comparison and the knowledge gathering on MPs pollution. The quality parameters for MPs analysis are to be carefully interpreted due to the peculiar nature of MPs as pollutant: solid particles suspended in the matrix, characterized by at least three categories (size, shape, chemical composition). The making of reference material, which has to be representative of the outcome of the categories, is not an easy task and it is at the moment

not available. However, the evaluation of quality parameters (LOD, LOQ, recovery rates) even with unrepresentative reference material, allows a better comparison between methods, gives indication on the method performances by comparison with other methods, and might anyway be informative to improve the method procedures.

μ -Raman is a powerful technique for MPs analysis, especially for routine monitoring for water utilities. However, its analytical limits in automatic mode should be carefully evaluated with respect to the instrument operation: the precision of the microscope stage is indeed relevant to define the minimum MPs size analyzable in automatic mode. Another relevant aspect to be considered, especially when reporting the data, is the polymer clustering of the matched spectra: it is possible that some particles might be identified as plastics but their spectra might be ambiguous in terms of chemical composition.

The use of μ -Raman for MPs analysis in drinking water is advisable, due to the possibility of automatizing the procedure, the independence of the analysis from the perfect drying of the sample and the higher spatial resolution reachable. However, depending on the volume sampled and the number of particles analyzed, the time and cost might increase significantly. The size range to be analyzed will be defined by the control organisms at the European and national level; however, it is advisable to divide the MPs analysis packages based on their size. Indeed, MPs abundance is a function of their size. The analysis protocol, the protocol quality parameters and the optimal instrument set up may vary with changing size ranges. The automatization of the analysis depends on the performance of an automatic algorithm for image analysis and spectra matching, whose performance should be fully understood. Automatization is of utmost relevance as it reduces the time of the operator needed for the analysis, the time needed from the operator and the relevance of the operator on the results obtained. Therefore, the results obtained are replicable and comparable. The volume to be chosen for the analysis depends on the scope: for effluents screening, reduced volume could be justifiable, whereas for the complete characterization of the MPs pollution, higher volume are required. The volume definition depends also on the size range analyzed. It is also recommended to report all the parameters and operation run for the analysis, to improve results comparison: it has indeed been demonstrated how each parameter has its role in determining the final output of the analysis.

Future perspectives might include:

- Understanding of the MPs pollution influencing factors, accounting for different time-space scales
- MPs characterization along the DWTPs layout to understand their removal processes
- Knowledge improvement with regards to MPs effect to define the monitoring needs

- Improvement of methods for polymer clustering for reporting data

Of specific interest for the water utilities are:

- Definition of a method for the screening analysis of the influent to DWTPs
- Definition of a method for the analysis of MPs < 20 μm
- Evaluation of the DWTPs performance towards MPs with changing inflow characteristics

Regarding these points, the proposals of the research group are:

- First the definition for the analysis of MPs > 20 μm also in the influent. The possibility to define a statistically significant difference between inflow MPs and outflow MPs should help in quantifying the DWTPs performance towards MPs. The method might include the density separation by ZnCl₂, and the digestion of the organic matter by H₂O₂ or KOH. This reagent choice should allow for the recovery of the entire density spectrum of MPs, except for PTFE (density > 2 g/cm³). The analysis procedure with the μ-Raman is to be implemented as described in this work.
- MPs analysis < 20 μm is to be performed at 20x or 50x magnification on a subsample of the particles on the filter, due to the otherwise too high number of particles. Considering the μ-Raman Thermo-Fisher, direct subsampling of particles is not possible, hence the subsampling is run on subareas. The assessment and quantification of the uncertainties linked to the subareas size and placement is to be run on the basis of what proposed by Schwaferts et al. (2019) (Schwaferts et al., 2019). In this case, it is to be underlined the need of an homogeneous distribution of the particles on the filter.
- To evaluate the consistency of the plant performance of the DWTP towards MPs with changing inflow characteristic is of utmost relevance for the water utilities for two main reasons: to understand the plant behavior towards MPs and to evaluate possible improvements through operation for MPs removal. The goal might be better achieved with sampled volume in the order of hundreds of liters, especially for MPs > 20 μm. However, preliminary results might be achieved also with the design proposed by this work. Sampling should be run on the effluent according to the variation in inflow turbidity. The minimum samples to achieve a representative series is set at 10 per each turbidity interval.
- The design of a sampling device for MPs with the aim of complete characterization of MPs at the outflow and inflow of DWTPs is foreseen in the near future. The characteristics required to the sampling device are: low contamination (achieved by reducing as much as possible the plastic components of the device), low

particles fragmentation (to be evaluated), high throughput (obtained by cascade filtration), easy operation (assembly and disassembly should be manageable in the laboratory, in a controlled environment, to prevent environmental contamination).

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