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The invisible problem of microplastics and microfibres in karst systems and aquifers: a multidisciplinary approach

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I hereby declare that, the contents and organisation of this dissertation constitute my own original work and does not compromise in any way the rights of third parties, including those relating to the security of personal data.

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Turin, October 29, 2025

Summary

Microplastic (MP) pollution is a worldwide concern. MP were found in both terrestrial and aquatic environments, in atmosphere and in different species, raising significant environmental and public health concern. While this kind of pollution has been extensively studied in marine environments, terrestrial ones are less studied, and some habitats are just at an early stage. MP pollution in karst systems is still poorly studied, despite the presence of protected species and habitats, and important water reserves. Indeed, karst aquifers provide 25% of the global potable water, and are particularly vulnerable due to their intrinsic hydrogeological characteristics, which facilitate the direct infiltration and transport of contaminants, including MPs, from surface to groundwater. MPs can adsorb and be vectors for other pollutants, and are rich of additives, which are released into the environment and may be hazardous to habitats and species. Moreover, MPs can be directly or indirectly assimilated by organisms, leading to bioaccumulation and biomagnification. Therefore, understanding the extent of MP pollution in karst environments and associated issues is crucial for assessing risks, developing monitoring frameworks, and implementing effective management and mitigation strategies.

Recently, microfibres (MFs) have emerged as a new and concerning class of micropollutants too, threatening natural environments. Natural, regenerated, and synthetic MFs have been detected across various environmental compartments as well as within organisms. Although synthetic MFs are typically identified in MP analyses, natural and regenerated MFs are often overlooked or incorrectly classified as MPs. Despite being generally considered biodegradable, their degradation pathways and rates within ecosystems remain poorly understood, and their potentially faster degradation could lead to the release of toxic compounds, while their physical properties may contribute to long-term accumulation in the environment. Therefore, comprehending their potential hazards and the impact they may exert on ecosystems is crucial for effective environmental conservation and management.

This research aims to investigate MP and MF pollution in aquifers and karst systems, analysing waters, sediments and fauna, to understand the extent of the

problem with a multidisciplinary point of view, providing new insights into their environmental impacts, and propose correct methods of monitoring and analysis. Monitoring was performed in different Italian Regions.

The following points were specifically addressed:

- Identification of suitable methods for monitoring MPs in karst systems, from sampling to laboratory analysis, microscopy and spectroscopy. In particular, methods for water and sediment analysis were defined.
- Investigations of MP and MF pollution in show caves
    - Investigations of MP and MF pollution in the karst system of Bossea cave, Piedmont, were done with a multidisciplinary approach. Sediments, water and fauna of the system were analysed to understand the extend of the problem. After the sediment analysis, the water of the system was analysed from surface watercourses to springs, developing an extensive sampling inside the Bossea cave, in the speleological and tourist areas. A specimen of *Proasellus franciscoloi*, a stygobiont (i.e. specialist of underground water environments) crustacean, was analysed together with CNR-IRET and Università degli Studi di Firenze. Finally, the presence of bisphenols was investigated in the water of the system, to verify if there could be a correlation with the presence of microplastics. Analysis on these samples were conducted during my period abroad at Universitat de València.
    - The sediments of other two Italian show caves (Toirano and Borgio Verezzi, Liguria) were analysed, making comparison with Bossea cave. A software of image analysis was tasted for MP counting.
    - Dripping waters in different points of the Torri di Slivia cave, Friuli-Venezia-Giulia, were analysed to verify the presence of MPs and other microparticles of anthropogenic origin, suggesting possible source of pollution. This research was conducted together with Università degli Studi di Trieste and CNR-STIIMA.
- Investigations of MP and MF pollution in speleological caves and springs. Karst aquatic environments with protected at EU level stygobionts were investigated in the Italian sector of the Classical Karst, Friuli-Venezia-Giulia. Waters and submerged sediments of different speleological caves and springs were analysed to understand the extend of the problem. This research was conducted together with Università degli Studi di Milano and Charles University of Prague.
- Investigations of MP and MF pollution in unexplored caves. These environments are the last frontier in terms of human exploration. Therefore, several unexplored caves were sampled before the passage of speleologists to observe if microplastics and microfibres were already present, and, if so, assume the source of contamination and the probable transport matrix.
- Investigations of MP and MF pollution in different deep aquifers and springs of the Cuneo province, Piedmont, to check for contamination and identify possible sources. These analyses were carried out as a sort of white, as the waters of certain aquifers are extremely deep, checking the validity of the used methodology.

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I would like to dedicate
this thesis to those who,
despite all the difficulties that
life may reveal to them, never
cease to smile, and continue
to struggle believing in what
they do for a better future…


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Aims of the study

The high vulnerability of karst systems to pollution, coupled with their critical role in natural ecosystems and their function as fundamental freshwater reserves, necessitates targeted actions for the protection of both surface and subterranean environments, as well as the sustainable management of these resources (Balestra et al., 2021; Re, 2019). Therefore, comprehensive investigations are required to assess environmental health status, identify potential pollution, and determine its sources.

Following these purposes, it was decided to investigate MP and other anthropogenic materials, especially MF, pollution in karst systems, analyzing waters, sediments and fauna, to understand the extent of the problem with a multidisciplinary point of view, providing new insights into their environmental impacts, and propose correct methods of sampling, monitoring and analysis.

First step was the identification of suitable methods for monitoring MPs in karst systems, from sampling to laboratory analysis, microscopy and spectroscopy analyses. In particular, tested and adapted methods for water and sediment analysis were developed and described in Chapter 3. An automatic particle counting and characterization software of image analysis was developed and tested too, subsequently made open source for the scientific community (see Chapter 3).

The first case study covered MP investigations along tourist routes in show caves (Chapter 4). The sediments of three Italian show caves (Bossea, Borgio Verezzi and Toirano caves) were analyzed and characterized, testing also a software of image analysis.

The opportunity to explore MP pollution across an entire karst system for the first time, through a multidisciplinary approach, motivated the analysis of sediments, water, fauna, and other micro-pollutants typically associated with MPs in the Bossea karst system, Piedmont, Italy. The water of the system was analyzed from surface watercourses to springs, developing an extensive sampling inside the Bossea cave, in the speleological and tourist areas (Chapter 4). A specimen of *Proasellus franciscoloi*, a stygobiont crustacean living in the Bossea system, was analyzed together with CNR-IRET and Università degli Studi di Firenze (Chapter 4). Finally, the presence of bisphenols, MPs and MFs was investigated in the water of the system, to verify if there could be a correlation with the presence of MPs (Chapter 4). Analysis on these samples were conducted during the period abroad at Universitat de València, Spain.

To complete the investigations in the karst systems and highlight indirect human impact in underground environments, cave dripping waters were sampled and analyzed. This first investigation was done in the Torri di Slivia show cave, Friuli-Venezia-Giulia, in the Italian sector of the Classical Karst Region, to verify the presence of these pollutants and possible sources. This research was conducted together with Università degli Studi di Trieste and CNR STIIMA (Chapter 4).

The next step was to investigate caves and springs of the most famous karst area in the world: the Classical Karst. Investigations of MP and MF pollution were done in karst aquatic environments of the Italian sector of the Classical Karst, Friuli-Venezia-Giulia, diversifying between urbanized and protected environments, underground and surface ones. All analyzed aquatic environments hosted protected at EU level stygobionts. Waters and submerged sediments of different caves and spring were analyzed to understand the extent of the problem and possible sources. This research was conducted together with Università degli Studi di Milano and Charles University of Prague (Chapter 5).

Up to this point, all investigated environments were known, including underground ones. Therefore, together with speleologist friends, unexplored caves were searched to investigate MP and MF pollution in sediments. These environments are the last frontier in terms of human exploration. Therefore, several unexplored caves were sampled before the passage of speleologists to observe if MPs and MFs were already present, and, if so, assume the source of contamination and the probable transport matrix, highlighting the indirect impact in subterranean environments (Chapter 6).

Finally, it was established to investigate MP and MF pollution in waters of different deep aquifers too, to check for contamination and identify possible sources of pollution. These aquifers reach high depths and some of them, such as artesian ones, contain “old” water, thus still possible uncontaminated environments, checking the validity of the methodology used (Chapter 7).

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Chapter 1

Microplastic pollution

Origin of the Chapter
Part of this chapter is adapted from the following peer-reviewed articles, result of this thesis:

- "Preliminary investigations of microplastic pollution in karst systems, from surface watercourses to cave waters" authored by Balestra et al. (2023), published in Journal of Contaminant Hydrology, Volume 252, January 2023, 104117, DOI: **https://doi.org/10.1016/j.jconhyd.2022.104117**.

  The author of this dissertation was responsible for conceptualisation, methodology, validation, formal analysis, investigation, resources, data curation, writing – original draft, writing – review and editing, visualisation.

- "Microplastics in caves: a new threat in the most famous geo-heritage in the world. Analysis and comparison of Italian show caves deposits" authored by Balestra and Bellopede (2023), published in Journal of Environmental Management, Volume 342, 15 September 2023, **118189, DOI: https://doi.org/10.1016/j.jenvman.2023.118189**.

  The author of this dissertation was responsible for term, conceptualisation, methodology, software, validation, formal analysis, investigation, resources, data curation, writing – original draft, writing – review and editing, visualisation.

- "Microplastic pollution calls for urgent investigations in stygobiont habitats: a case study from Classical Karst" authored by Balestra et al. (2024b), published in Journal of Environmental Management, Volume 356, April 2024, 120672, DOI: **https://doi.org/10.1016/j.jenvman.2024.120672**.

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- "The problem of anthropogenic microfibres in karst systems: Assessment of water and submerged sediments" authored by Balestra et al. (2024a), published in Chemosphere, Volume 363, September 2024, 142811, DOI: **https://doi.org/10.1016/j.chemosphere.2024.142811.**

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- “Explorations in the dark continent: did microplastics and microfibres get here before us?” authored by Balestra and Bellopede (2025), published in Science of The Total Environment, Volume 977, May 2025, 179328, DOI: https://doi.org/10.1016/j.scitotenv.2025.179328.

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1.1 Introduction

Microplastics (MPs) pollute natural environments worldwide. The properties of plastics, such as their resistance, durability, and low weight, make them particularly hazardous pollutants in natural environments. The morphology and size of MPs further increase their harmfulness, as these particles can be easily transported over long distances through various environmental matrices (Allen et al., 2019; Liu et al., 2019b). Recently, their presence in human body and negative impacts on health has also been demonstrated too (Barceló et al., 2023; Cox et al., 2019; Kannan and Vimalkumar, 2021). MPs can be assimilated either directly or indirectly by organisms and may be toxic, constituting a serious ecological emergency (Assas et al., 2020; Devereux et al., 2021; Jahan et al., 2019; Romeo et al., 2015). MPs may also contain or adsorb other pollutants, such as pesticides, persistent organic pollutants (POPs), bisphenol A (BPA), chemicals, heavy metals, or antibiotics, which amplifies the environmental risks associated with their presence (Cheng et al., 2023; Rochman et al., 2013; Selvam et al., 2021; Wanner, 2021; Zhou et al., 2019).

MPs have been investigated mostly in marine environments (e.g. De Lucia et al., 2018; Gholizadeh and Cera, 2022; Tsang et al., 2017), however, recently, more attention has been paid to terrestrial ones (e.g. Ballent et al., 2016; Bertoldi et al., 2021; Boyle and Örmeci, 2020; Wong et al., 2020), highlighting the presence of MPs even in remote areas (e.g. Ambrosini et al., 2019; Neelavannan et al., 2022; Zhang et al., 2021). Nevertheless, research on MP pollution in some environments, such as karst areas and subterranean environments, is lagging behind.

The purpose of this chapter is to present an overview of microplastic pollution and related issues, such as the microfibre problem, with particular emphasis on environmental issues related to this PhD thesis, highighting the reasons for my research. Then, the most widely used techniques for sampling, separating and identifying microplastics will be discussed, highlighting the lack of standard procedures for such analyses.

1.2 Definition of microplastic

The first definition of MPs was introduced by Prof. Richard Thompson and his team in 2004 (Thompson et al., 2004). They defined MPs as plastic particles smaller than 5 mm, resulting from the fragmentation of larger plastic (secondary production) or intentionally produced plastic waste with small dimensions (primary production). This definition has been adopted and extended by several scientific and environmental organizations, defining MPs as synthetic particles with a 5 mm - 1 µm dimension (e.g. Frias and Nash, 2019; International Organization for Standardization and European Committee for Standardization, 2020). Moreover, after recent scientific considerations, it is preferable to distinguish between MPs (1 mm - 1 µm) and big MPs (5-1 mm) (International Organization for Standardization and European Committee for Standardization, 2020).

However, the definition of MPs is still much debated. The recent Commission Delegated Decision (EU) 2024/1441 about MPs detection in drinking waters defined MP as “small discreet object that is solid, insoluble in water and is partially or wholly composed of synthetic polymers or chemically modified natural polymers” (European Commission, 2024), and ISO 4484-2 (International Organization for Standardization, 2023) defined MP as “material consisting of a solid polymer containing particles, to which additives or other substances may have been added, and where a weight fraction of ≥1% particles have:

a) all sizes 100 nm ≤ × ≤ 5 mm,

b) for fibres, a length of 300 nm ≤ × ≤ 15 mm and a length/diameter ratio >3

Note 1 to entry: Polymers that occur in nature that have not been chemically modified (other than by hydrolysis) are excluded, as are polymers that are (bio) degradable.

[SOURCE:ECHA, ANNEX XV Restriction Report - Microplastics, 22 August 2019, par 1.2.2.1, modified on lower size recommended dimensions, by Commission Recommendation C/2022/3689 of 10 June 2022 on the definition of nanomaterial (OJ C 229, 14.6.2022, p. 1), modified — "≥1% w/w" was changed to "a weight fraction of ≥1 %"; additional information has been given as a note to entry.]”.

1.3 Characterisation of microplastics

MPs collected in natural environment can have very different characteristics. Moreover, the environmental agents (biological, chemical or physical) can drastically change their appearance and properties. For example, once into the environment, MPs can increase or decrease their density, due for example to weathering or biofouling (Crawford and Quinn, 2016 and references therein) (Fig.1).

MPs can be divided into categories based on their shape, size, colour, fluorescence and typology.

Regarding the shape, MPs are generally categorised in fragments, fibres, pellets/sphere, film and foam (e.g. Crawford and Quinn, 2016) (Fig.s 2, 3). However, there is no pre-established protocol and different classifications can be used. In my research, MPs were characterised according to the Standardised size and colour sorting system (SCS), proposed by Crawford and Quinn (2016). MP characterisation by shape is really important because it suggests the potential origin of MPs. In fact, fragments generally come from larger objects, such as plastic bottles, vials etc., pellets from pre-production industries, spheres from personal care products, fibres from textiles, film from plastic bags and wrappers, and foam from take-away boxes or other packaging. This information is therefore one of the most relevant to be treated in the study of MPs pollution.

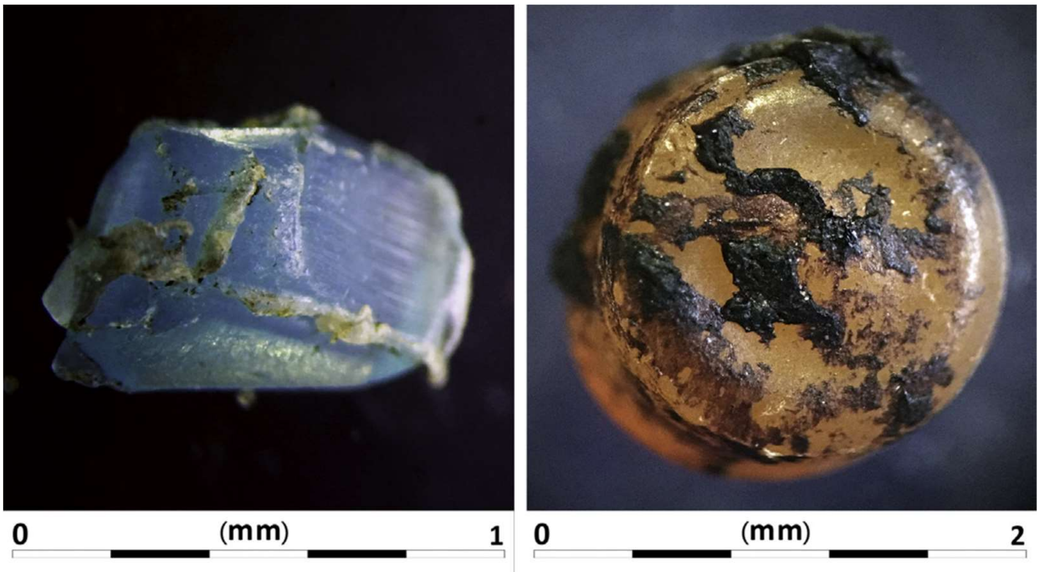

Fig. 1. Biofouled MPs. Image from Crawford and Quinn (2016).

Fig. 2. Microfibre accumulation from sediments in unexplored caves (see Chapter 6). Photo: © V. Balestra.

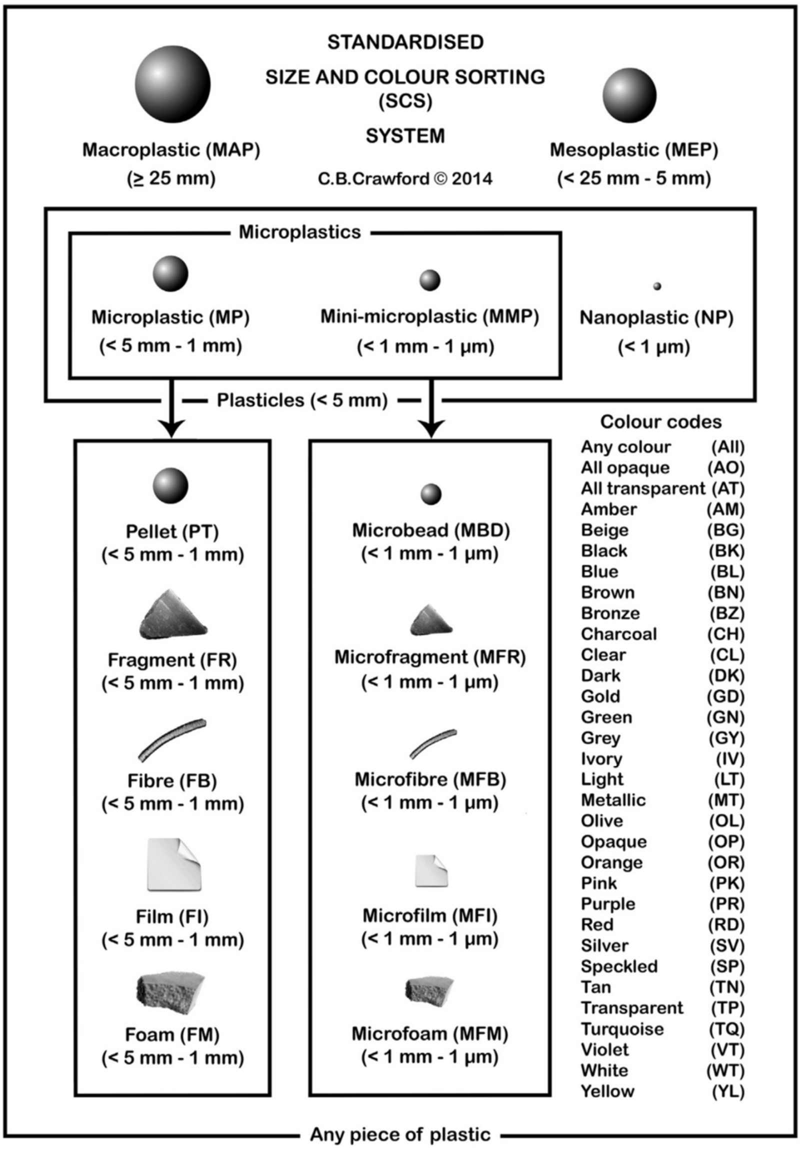

Fig. 3. The Standardized size and colour sorting system (SCS) of Crawford and Quinn (2016) used to categorize plastics, based on size, colour and shape. Image from Crawford and Quinn (2016).

Regarding the size, there is a general consensus on MPs size from 5 mm to 1 µm. However, after recent scientific considerations, today it is preferable to distinguish between MPs (1 mm - 1 µm) and big MPs (5-1 mm) (International Organization for Standardization and European Committee for Standardization, 2020). In my research, I initially followed the definitions given in the SCS for the MP size (Fig. 2); in the last years, I adopted the new separation too.

MP colour can provide information on consumption by organisms (Carpenter et al., 1972; Jahan et al., 2019; Lusher et al., 2013; Romeo et al., 2015; Ugwu et al., 2021), making this step of analysis fundamental for ecological and environmental research. In marine biota, most of MPs particles show a blue colour (32.9%), followed by white (24.7%), black (18.8%) and transparent (16.5%) particles (Ugwu et al., 2021). In fish and marine mammals, the predominant colour of MPs found were blue, black, transparent, and white. Different colours were found in MPs ingested by birds, where transparent colour are the majority (55.6%), followed by blue (33.3%) and brown (11.1%). Some studies identified black plastics as the most often ingested ones by the analysed sea animals (Jahan et al., 2019; Lusher et al., 2013); in Romeo et al. (2015) transparent and white MPs occurred in the stomach of all analysed top sea predators, whereas blue particles and yellow ones were found only in some of them.

In addition, MP colour can provide indication about the chemical pollutants which can bind to them by contaminating them (e.g. Frias et al., 2010; Karapanagioti et al., 2011). In marine environments, high levels of pollutants were found on yellow and black particles (Frias et al., 2010; Karapanagioti et al., 2011).

Different plastics contain Fluorescent Whitening Agents (FWAs) (Qiu et al., 2015), therefore, many MPs can be easily detected under an ultraviolet (UV) light (Ehlers et al., 2020; Klein and Fischer, 2019; Qiu et al., 2015). Consequently, MP fluorescence could provide information on consumption by organisms too. However, different organic and inorganic materials are fluorescent under a UV light and not all plastics contain fluorescent additives. Therefore, preliminary screening analysis have to be done.

The type of plastic can provide us information on the origin of the material, its possible permanence in the environment, and the additives which it could contain and that may have spilled into the environment. While shape, size, color, and fluorescence can be determined under a microscope, the type of plastic is evaluated with spectroscopic techniques. The most common type of polymer in different water environments in Europe, the Mediterranean area and China is polyethylene (PE), followed by polypropylene (PP) and polystyrene (PS) (Schwarz et al., 2019). These polymers are commonly used in many everyday products, so much so that together they represent about 42% of the plastic produced globally in 2023 (Plastics Europe, 2024).

1.4 Microplastics, additives, and interactions with other pollutants

Often, plastics contain different additives which are added during the manufacturing process to modify polymer properties, enhance stability and durability, and protect against environmental or biological degradation. Additives are also used to facilitate the manufacturing processes themselves. Most common used additives are plasticisers, colourant, filler, lubrificants, biological protection, or flame retardant. Plastic material release tonnes of chemical additives in the environment every year, and, after organism ingestion, it is possibile that additives could leach out of the plastics and directly diffuse into biological tissues, inducing toxicity (Crawford and Quinn, 2016 and references therein).

MPs can be sources and vectors for other pollutants too, such as pesticides (Wanner, 2021), persistent organic pollutants (POPs) (Koelmans et al., 2013), bisphenol A (BPA) (Cheng et al., 2023), chemicals (Rochman et al., 2013), heavy metals (Li et al., 2019; Selvam et al., 2021; Zhou et al., 2019), BPA(Martín-Gómez et al., 2024), or antibiotics (Li et al., 2018). When plastic degrade in the environment, these pollutants can be released into surronding media (water, soil, organisms) amplifying the environmental risks associated with their presence.

1.5 Biological impacts of microplastics

MPs may be assimilated, directly or indirectly, by organisms. Due to their small dimensions, MPs exhibit high bioavailability, and their widespread occurrence across ecosystems renders them potentially hazardous contaminants (Bergami et al., 2020). This raises concerns because organisms can mistake MPs for prey or ingest them incidentally with food (Andrade et al., 2019; Expósito et al., 2022) (see Paragraph 1.3). The adverse effects of MP pollution are further amplified by their capacity to act as vectors for other pollutants, including bisphenol A (BPA) and heavy metals (Cheng et al., 2023; Selvam et al., 2021) (see Paragraph 1.4). Additionally, plastics contain various chemical additives, such as plasticizers and UV stabilizers, which can leach into the environment or be transferred through trophic interactions, posing significant health risks (Anbumani and Kakkar, 2018) (see Paragraph 1.4). Once directly or indirectly assimilated by organisms, MPs lead to bioaccumulation and biomagnification. An example of the mechanism by which contaminated MPs can introduce contaminants into the food web is reported in Fig. 4. Microplastic ingestion may lead to physical blockages, reduced feeding efficiency, oxidative stress, and internal injuries (Crawford and Quinn, 2016 and references therein).

Despite existing research highlighting the impacts of MPs in some organisms, especially marine ones (Ugwu et al., 2021 and references therein), there is a lack of evidence regarding their ingestion and effects in different organisms, such as terrestrial and subterranean ones. Therefore, understanding the extent of MP pollution in the environments and associated organisms is crucial for assessing risks, developing monitoring frameworks, and implementing effective management and mitigation strategies.

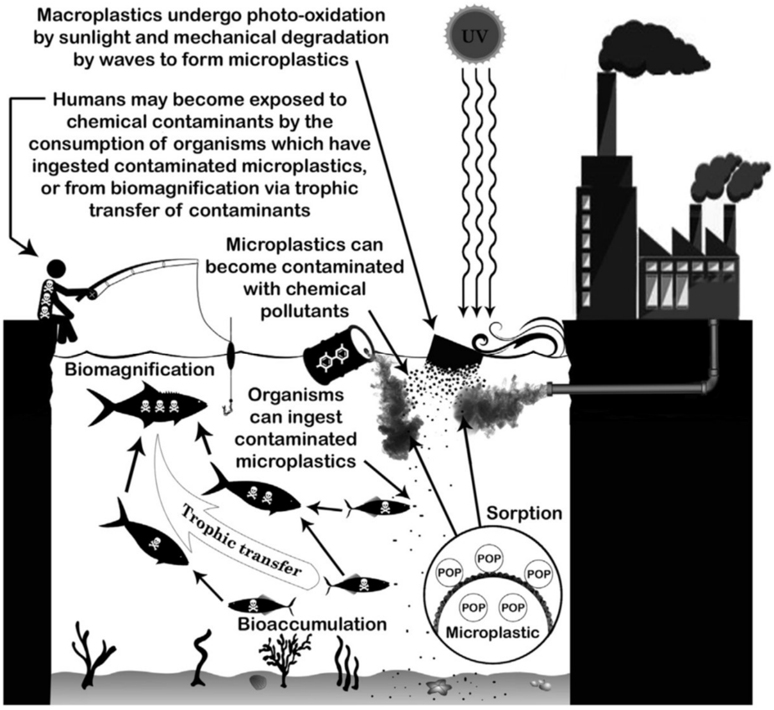

Fig. 4. The mechanism by which contaminated microplastics introduce contaminants into the food web. Image from Crawford and Quinn (2016).

1.6 Microplastics and microfibre pollution

Recent studies highlighted concern for other pollutants that impact natural environments: the microfibres (MFs) (Athey and Erdle, 2022; Dris et al., 2016; Finnegan et al., 2022; Hasenmueller et al., 2023; Liu et al., 2022a; Okyere et al., 2025; Stanton et al., 2019; Suaria et al., 2020a). The definition of MF is still debated, depending especially on the field of application (textile industry, engineering, environmental pollution, etc.). Over the past decade, MFs have emerged as a new class of pollutants of global concern, representing the most prevalent form of MPs detected in the environment (Miller et al., 2017). The first general definition of MF, taking into account both size and diameter and all previous MF definitions as environmental pollutants, was described in Liu et al. (2019a): "Microfibres are any natural or artificial fibrous materials of threadlike structure with a diameter less than 50 μm, length ranging from 1 μm to 5 mm, and length to diameter ratio greater than 100". In the MP definition of ISO 4484-2 (International Organization for Standardization, 2023) fibre were described with "a length of 300 nm ≤ × ≤ 15 mm and a length/diameter ratio >3".

MFs originated from textile production throughout their entire life cycle, cigarette filters, personal care products like face masks and wet wipes, and other manufactured fibrous products (Athey and Erdle, 2022; Liu et al., 2019a) (Fig. 3). Primary MFs means original natural or synthetic fibres employed in the textile and clothing industry without subsequent modification. Secondary microfibres result from the fragmentation of larger textile materials derived from primary MFs during manufacturing, use, maintenance and disposal processes (Liu et al., 2019a).

Anthropogenic fibres are divided in natural, regenerated (or man-made cellulosic, or artificial), and synthetic (Finnegan et al., 2022; Stanton et al., 2019). Natural fibres derive from the treating of plant (cellulosic) and animal (proteinaceous) fibres, such as cotton or wool (Fig. 5). Regenerated fibres are disbanded from cellulose material, such as rayon/viscose. Natural and regenerated MFs are an accessible source of carbon for microorganisms (Zambrano et al., 2019), and biodegradation processes of regenerated MFs are similar to cotton ones (Park et al., 2004), therefore, natural and regenerated fibres should not be considered MPs. Synthetic fibres are made of plastic polymers, therefore, MFs less than 5 mm can be considered MPs following the original definition of Thompson (Fig. 6). Moreover, different fibres used in textile production are copolymer, a mix of different plastics or of cellulosic and synthetic materials, making difficult to recognize them in the three main categories.

Recent studies raised concerns about natural and regenerated MFs too (Athey and Erdle, 2022; Dris et al., 2016; Finnegan et al., 2022; Hasenmueller et al., 2023; Stanton et al., 2019; Taptiklis et al., 2025). These MFs have been likely included in MPs monitoring by hundreds of research in the past, because they are processed industrially and coloured (Obbard et al., 2014; Woodall et al., 2014), increasing MP concentrations (Wesch et al., 2016). However, non-synthetic fibres can now be included in the definition of MPs as well, as expressed in recent standards and regulations (European Commission, 2024; International Organization for Standardization, 2023).

Since regenerated and natural materials of anthropogenic origin are not limited to MFs, but also occur in other forms, the literature has begun to refer more generally as anthropogenic microparticles (AMPs) (e.g. Balestra et al., 2024c; Hasenmueller et al., 2023)

Fig. 5. Blue cotton fibre from sediments of Italian unexplored caves (see Chapter 6). Photo: V. Balestra.

Fig. 6. Grey synthetic fibre from waters of Trebiciano cave, Friuli-Venezia-Giulia, Italy (see Chapter 5). Photo: © V. Balestra.

While originally the most used fibre was cotton, synthetic fibres dominated the textile market since the mid-1990s, representing nearly 65% of worldwide output by 2021, especially with the polyester production, which represent alone the 54% of the global total fibre production (Textile Exchange, 2022). Plant fibres had a combined market share of around 28% of the global fibre market, regenerated of around 6.4%, while animal fibres represent only the 1.6% (Textile Exchange, 2022). Cigarette filters are made of cellulose acetate with plasticized additives (Belzagui et al., 2021), and common commercially non-flushable wipes are usually made of polyethylene terephthalate (PET), polypropylene (PP), or combined PET and cellulose, while flushable ones are generally a mixture of PET and cellulose or cellulose alone (Ó Briain et al., 2020). As well as these materials, different fibres used in textile production are copolymer or a mix of cellulosic and synthetic materials, making difficult to clearly recognize MFs in the three main categories.

Currently, a large number of fibres are discharged from washing machines and industrial processes (Akyildiz et al., 2022; Cesa et al., 2017), and enter into the environment through wastewater (Xu et al., 2018), air deposition (Allen et al., 2019), and the application of contaminated sludge for agriculture (Zubris and Richards, 2005). Fibre degradation in natural environment can reduce their dimension over time, making this pollutant more easily transported by different matrices, and more dangerous for ecosystems. Cigarette filter is generally composed of >15,000 fibres strands and in water can release even 100 MFs/day, with most MFs less than 0.2 mm. (Belzagui et al., 2021).

As MPs, MFs have been detected in different environments and matrices (Finnegan et al., 2022; Stanton et al., 2019; Suaria et al., 2020a; Suaria et al., 2020b), as well as in animal (Le Guen et al., 2020; Remy et al., 2015; Zhao et al., 2016) and human organs (Pauly et al., 1998). Recently studies have shown prevalence of natural and regenerated MFs into the environment compared to MPs (e.g. Balestra et al., 2024a; Stanton et al., 2019), and in the gastrointestinal tract of some animals (e.g. Remy et al., 2015; Zhao et al., 2016), suggesting that these particles can be poisonous for ecosystems as well as MPs, due to the dyes, additives, and chemicals used in their manufacturing (Athey and Erdle, 2022; Kim et al., 2021a; Lusher et al., 2013). Under laboratory conditions, negative effects on animal health have been also observed (Jemec et al., 2016; Watts et al., 2015).

Although there is a general consensus on the reduced dangerousness of the not-synthetic fibres in the environment, being considered biodegradable, little is known about natural and regenerated fibre degradation in natural ecosystems and their effects on biota (Athey and Erdle, 2022). Moreover, their potential faster degradation in comparison to synthetic fibres could be the route for the release of toxic compounds into the environment (Ladewig et al., 2015). Natural and regenerated fibres are often processed and coated with a wide range of chemicals, such as resins, dyes, softeners, and flame retardants (Athey and Erdle, 2022), which may considerably slow their degradation in the environment (Li et al., 2010), persisting from months to decades, up to withstand more than 130 years in a deep-ocean environment (Athey and Erdle, 2022; Chen and Jakes, 2001). Moreover, the specific density of some cellulosic material is higher than different synthetic polymers, such as polyester, polypropylene, and nylon/acrylics, and is greater than seawater. Therefore, these fibres could sink in the environment. In addition, natural and regenerated textiles release more fibres than the synthetic ones during laundering (Rathinamoorthy and Raja Balasaraswathi, 2021). All these factors may explain a long-term accumulation of natural and regenerated fibres in the environment over time. Some research found a prevalence of natural and regenerated fibres in the gastrointestinal tract of animals (e.g. Remy et al., 2015; Zhao et al., 2016), suggesting that these particles may be toxic for ecosystems as well as synthetic polymers, due to dyes and chemicals used during their production (Athey and Erdle, 2022; Kim et al., 2021a; Lusher et al., 2013).

Consequently, analyzing MPs and MFs in environmental research is becoming increasingly important. Despite the recent surge in studying these micropollutants in natural environments, especially MPs, while sea environments have been extensively researched over the years (e.g. Cutroneo et al., 2020; Leistenschneider et al., 2021; Tsang et al., 2017), several environments, such as karst areas, remain poorly studied despite their high pollution risk, and research in this areas is at the initial stage.

Chapter 2

Microplastic pollution, karst systems and deep aquifers

Origin of the Chapter
Part of this chapter is adapted from the following peer-reviewed articles, result of this thesis:

- "Microplastic pollution in show cave sediments: first evidence and detection technique" authored by Balestra and Bellopede (2022b), published in Environmental Pollution, Volume 292, Part A, 1 January 2022, 118261, DOI: **https://doi.org/10.1016/j.envpol.2021.118261.**

  The author of this dissertation was responsible for conceptualisation, methodology, validation, formal analysis, investigation, resources, data curation, writing – original draft, writing – review and editing, visualisation.

- "Preliminary investigations of microplastic pollution in karst systems, from surface watercourses to cave waters" authored by Balestra et al. (2023), published in Journal of Contaminant Hydrology, Volume 252, January 2023, 104117, DOI: **https://doi.org/10.1016/j.jconhyd.2022.104117.**

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- "Microplastics in caves: a new threat in the most famous geo-heritage in the world. Analysis and comparison of Italian show caves deposits" authored by Balestra and Bellopede (2023), published in Journal of Environmental Management, Volume 342, 15 September 2023, 118189, DOI: **https://doi.org/10.1016/j.jenvman.2023.118189.**

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2.1 Karst systems

Karst areas are characterized by typical geological formations resulting from the dissolution of carbonate rocks (Fig. 7). These landscapes exhibit unique surface and subsurface features such as sinkholes, dolines, disappearing streams, and extensive cave systems. However, karst phenomena can also occur in gypsum, halite and sometimes in quartzites (Wray and Sauro, 2017).

Fig. 7. Karst area of Ellero Valley, Piedmont, Italy. Photo: V. Balestra.

Within the complex three-dimensional landscapes of karst systems, caves are the principal voids accessible to humans (Fig. 8). Karst caves have an exceptional scientific value and are a precious archive that must be protected from damage, allowing paleo-environmental and paleo-climatic reconstructions and preserving paleontological and archaeological finds (Balestra et al., 2021; Cigna and Forti, 2013; Romano et al., 2019; Zunino et al., 2022).

Fig. 8. Costacalda cave, Piedmont, Italy. Photo: © V. Balestra

Karst systems have a high environmental value, due to the particular geological and hydrological conditions. The subterranean waters of karst areas constitute some of the major water reserves of terrestrial environments (Moldovan et al., 2020) and host specific complex ecosystems that contribute to the functioning of above-ground landscapes (Canedoli et al., 2022; Dossi et al., 2007). Subterranean karst habitats support fragile ecosystems and species, such as troglobiont (i.e. specialist of underground environments) and stygobiont (i.e. specialist of groundwaters) (Culver and Pipan, 2019; Mammola, 2019) and are considered one of the most fragile natural environments of the world (Kurwadkar et al., 2020).

Due to the open nature of karst systems, they are very susceptible to environmental changes (Balestra et al., 2023; Gillieson, 2011; Kurwadkar et al., 2020; Ruggieri et al., 2017; White, 1988), therefore, subterranean environments are often protected. However, these environments are often subjected to contamination by surface pollution, because of the surface and subterranean environment connections (Chiarini et al., 2022).

2.2 Karst caves

Caves are the most important geological heritage worldwide (Cigna and Forti, 2013; Piano et al., 2022), rich in speleothems and minerals (Hill and Forti, 1997), extremely peculiar habitats hosting organisms with interesting ecological adaptations (Culver and Pipan, 2019; Mammola, 2019), and custodians of important drinking water reserves (Moldovan et al., 2020) (Fig. 9). Indeed, over the past decades, the interest in subterranean karst environments has grown remarkably, from a scientific and economic viewpoint, emphasizing the importance of conservation and sustainable management actions (Chiarini et al., 2022; Cigna, 2013; Cigna, 2016).

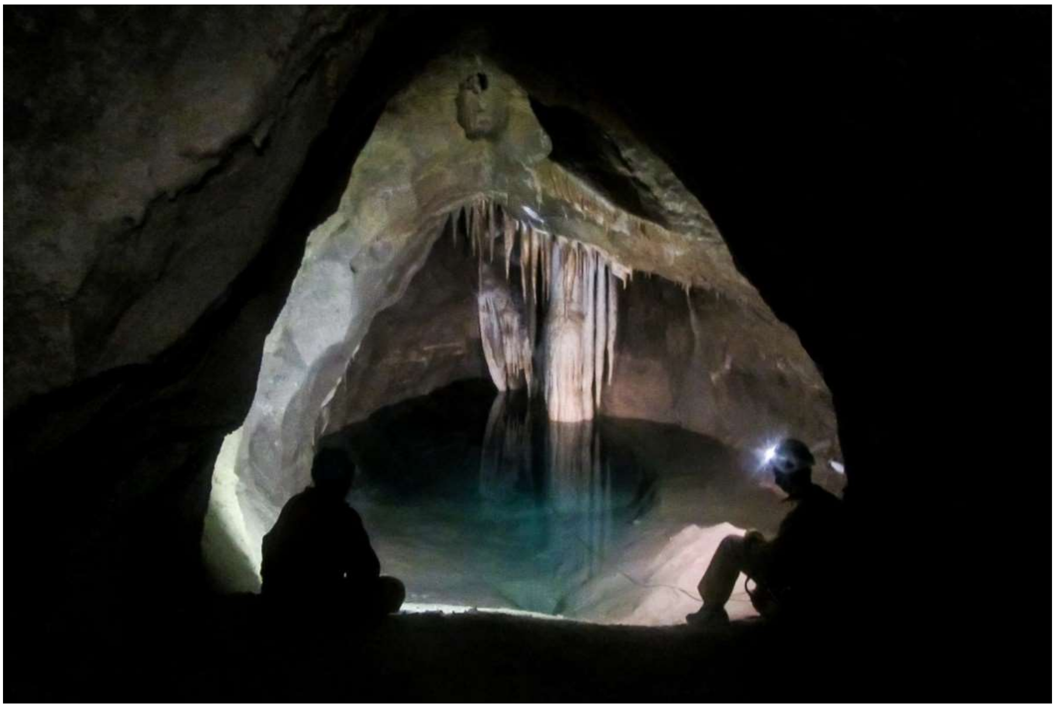

Fig. 9. Speleological area of the Bossea show cave, Piedmont, Italy. Photo: © V. Balestra.

Thank to their relatively stable environmental conditions, caves can be considered "conservative environments" (Chiarini et al., 2022), able to preserve information for a long time such as paleontological and archaeological remains (Fig. 10). However, these characteristics make caves vulnerable environments too, easily damaged by climate variations and pollution, causing an irreparable loss of scientific information and natural habitats (Chiarini et al., 2022; Gillieson, 2011). The open nature of karst systems causes water and air masses exchanged between the external environment and the internal one (Badino, 2010), making them vulnerable also to contamination by surface pollutants, which can be transported through the rock fractures and caves (Balestra et al., 2023; Ruggieri et al., 2017; White, 1988).

Fig. 10. Bones of Ursus spelaeus *in Toirano show caves, Liguria, Italy. Photo: © V. Balestra.*

When cavities are transformed in show caves, an additional impact is produced (Calaforra et al., 2003; Cigna and Forti, 2013), making it important to follow strict management rules to safeguard their values (Cigna and Burri, 2000; Watson et al., 1997). The installation of lighting systems, the construction of path infrastructures and the passage of people can increase the energy balance of the cave, modify the cave atmosphere and microclimate (Lang et al., 2015a; Lang et al., 2015b), cause the soiling and the corrosion of speleothem surfaces, and introduce alien materials, such as lint, dust, pollutants, spores and other organic materials (Balestra and Bellopede, 2022b; Chelius et al., 2009; Christman, 2019), creating favorable environments for lampenflora growth and bacteria activity (Burgoyne et al., 2021; Havlena et al., 2021; Mulec, 2012; Piano et al., 2021).

2.3 Karst aquifers

Karst aquifers are groundwater environments constituting about 25% of the global drinking water sources (Panno et al., 2019) (Fig.s 8, 11). The hydrodynamic regime of these aquifers is affected by the geology and the rock cluster fracturing, the karstification degree and the local meteorological conditions (Braun, 1984; Chauve et al., 1990; Hottelet et al., 1993; Moindrot et al., 1988).

Fig. 11. Speleological area of the Bossea cave, Piedmont, Italy. Photo: © V. Balestra.

Considering the drainage system, three basic conceptual aquifer models have been described: systems with dominant drainage (absent or reduced phreatic zone, high permeability), systems with interconnected drainage (extensive phreatic zone, high permeability) and systems with dispersive circulation (highly extensive phreatic zone, middle–low permeability) (Balestra et al., 2022a; Ford and Williams, 2013; Ford and Williams, 1989; Vigna, 2007; Vigna and Banzato, 2015; White, 1969). Various intermediate situations exist, which can be described using these models considering hydro-chemical data and spring hydrography (Banzato et al., 2011).

The open nature of karst aquifers makes them vulnerable to contamination by surface pollutants, which can be transported through the rock fractures, soil pores, or can directly access the karst systems through some caves, especially tourist ones, increasing the pollution risk (Balestra and Bellopede, 2022b, 2023; Balestra et al., 2021).

2.4 Karst systems and subterranean fauna

Subterranean environment of karst systems host peculiar organisms adapted to these unique habitat, characterised by darkness, high relative humidity and stable temperatures.

Karst systems represent the most sensitive aquatic environment worldwide (Dossi et al., 2007). These habitats host organisms with interesting ecological adaptations, such as stygophiles (aquatic, facultative subterranean environment-dwelling organisms) and stygobionts (aquatic organisms that are obligate cave-dwelling) (Fig. 12) (Culver and Pipan, 2019; Mammola, 2019). However, the particular biology of these species, and the diversity of the aquatic environment in karst areas (groundwater, ponds, pools, stream and lakes), even if interconnected to each others, pose several challenges to conservation (Pipan et al., 2010).

Fig. 12. The olm, Proteus anguinus*, an aquatic salamander endemic to the underground waters of the Dinaric Alps. It is the only exclusively cave-dwelling chordate species in Europe and is notable for its adaptations. Photo: © V. Balestra*

Subterranean waters are usually strongly depleted of trophic resources and local species richness of species communities is low (Barzaghi et al., 2017; Zagmajster et al., 2010). Subterranean aquatic environments do not host complex food webs, which makes predation risk limited for some organisms. Hosted communities are not very resilient and can be easily impacted by the variations of environmental conditions (Hose et al., 2022). However, in spring habitats subterranean and surface water features interplay, leading to the formation of complex environments (Cantonati et al., 2020), in which many species exploit both ecosystems during their life cycle (Barzaghi et al., 2017). Different animals provide ecosystem services in these habitats, especially invertebrate, whose movements helps to remix sediments, oxygen and organic materials (Mermillod-Blondin et al., 2023).

2.5 Microplastics and karst systems

MP pollution in karst habitats can be linked to direct and indirect human activities, and is strictly related to the hydrodynamic regime of the aquifer, the geology, the karstification degree and the local meteorological conditions. MP contamination in these environments include litter, wastewater, soil and surface water pollution, and athmospheric deposition. MP presence in precipitation was detected in different areas (e.g. Allen et al., 2019; Liu et al., 2019b), and this kind of pollution is strongly related to the soil contamination (Zhou et al., 2021). Into the soil, MPs can be transported, travelling throughout the soil pores and rock fractures, and accumulate in subterranean waters and environments (Chia et al., 2021; Fahrenfeld et al., 2019; Frei et al., 2019; Lwanga et al., 2017; McGechan, 2002; Viaroli et al., 2022; Wanner, 2021).

2.5.1 Microplastics in karst water environments

Before the starting of this research in 2022, only Panno et al. (2019) investigated MP pollution in waters of a karst system, monitoring springs and wells under low-flow conditions. Today, studies on MP pollution in cave waters, as well as in surface and underground waters of the same karst area, are still lacking. The potential contamination of groundwater is often only mentioned, and a limited number of studies focused on groundwater MP pollution (Hoang et al., 2025; Lee et al., 2024; Sumam et al., 2025). The invisibility of the subterranean karst system structure prevents the study of such systems, even if they preserve a precious asset such as groundwater.

Sources of MPs to groundwater include wastewater, litter, atmospheric deposition and soil and surface waters pollution. Atmospheric transport favors the distribution of MPs over long distances in relation to wind direction and intensity, rain/snow precipitation intensity and duration (Allen et al., 2019). In the hydrogeological cycle, atmospheric water contribution is the main aquifer recharge. The presence of MPs in precipitation was detected in cities and remote areas (Allen et al., 2019; Liu et al., 2019b), highlighting the omnipresence of this contaminant. Therefore, MP contamination in soil is strongly related to precipitation, facilitating MP infiltration (Zhou et al., 2021). When MPs are deposited on the ground, they can be transported vertically into the subsoil, travelling over long distances throughout the rock fractures and accumulating in the groundwater system (Chia et al., 2021; Wanner, 2021). Previous studies highlighted the downward movement of microparticles through soil pores and cracks for smaller MPs (Fahrenfeld et al., 2019; McGechan, 2002; Viaroli et al., 2022), and vertical and horizontal migration in soil matrices could be also be related to bioturbation (Lwanga et al., 2017). The hyporheic zone, the zone between surface and groundwater, is an important accumulation zone for MPs of small dimensions (Frei et al., 2019). The hydrogeological setting of the basin and seasonal trends must be taken into account, as well as the flow regime of streams and riverbeds. Therefore, hydrogeological information and aquifer types are decisive in choosing the sampling approach and detection method.

The vulnerability of groundwater resources to pollution, anthropogenic pressure and climate change, as well as their important role in natural ecosystems and as a fundamental drinking water reserve, require actions targeted at underground environment protection and sustainable resources management (Balestra et al., 2021; Re, 2019). Therefore, investigations are required for understanding the system dynamics and monitoring the state of the health of the environment, detecting the possible pollution and its sources.

2.5.2 Microplastics in caves

Before this research, only few preliminary works on lint (organic and synthetic fibres from clothing carried by tourists) had been done in show caves (Baker and Roberts, 2015; Chelius et al., 2009; Christman, 2019; Jablonsky et al., 1993; Pate, 1999).

Show caves are the most attractive natural features of geo-tourism, allowing more than 50,000 visitors/year in the major show caves of the world (Cigna, 2016; Cigna and Forti, 2013). Human presence in these caves has led to several environmental issues such as pollution. Visiting the cave, tourists transport dust and lint into caves, covering and damaging speleothems and walls (Fig. 13) and providing an artificial food source for different species (Baker and Roberts, 2015; Chelius et al., 2009; Christman, 2019; Jablonsky et al., 1993; Pate, 1999). Lint can damage speleothems indirectly by providing nutrients for acid-producing organisms that can dissolve limestone (Jablonsky et al., 1993) and can be incorporated into the cave formation growth. Some lint fibre analyses gave a synthetic fibre content between 30 and 75% (Christman, 2019; Jablonsky et al., 1993). Therefore, MP monitoring had to be explored.

Fig. 13. Microplastics on speleothems in Vento cave, Tuscany, Italy. Photo: © V. Balestra.

2.5.3 Microplastics in stygobionts

Groundwater fauna provide essential ecosystem services (Deng et al., 2014). Many species feed on viruses and bacteria, thereby contributing to the removal of pathogenic microorganisms from water destined for human consumption (Deng et al., 2014). They play a pivotal role in the recycling of organic carbon within subterranean environments and, through their locomotion and fecal deposition, facilitate sediment mixing and enrichment (de Sá et al., 2015). Such activities maintain open interstitial spaces, enhancing water flow, sediment oxygenation, and pollutant dilution. Moreover, their movement promotes the dispersal of bacteria into previously not yet colonized areas (Deng et al., 2014).

Although existing research has demonstrated the impacts of MPs on aquatic benthic organisms, including reduced feeding activity, impaired growth, and the induction of toxicity and oxidative stress (Deng et al., 2014; Jeong et al., 2016), there is a significant lack of evidence concerning their effects on groundwater fauna. Only recently researchers started studying these animals (Kokalj et al., 2025; Sforzi et al., 2024), including part of this work, the first at international level (Sforzi et al., 2024) (Fig. 14). Even the basic phenomenon of MP ingestion by groundwater organisms remains largely uninvestigated. This knowledge gap is particularly concerning given the crucial role these organisms play in the recycling of organic carbon within groundwater ecosystems. Should they replace their natural diet with MPs, it could severely compromise this fundamental ecosystem function, ultimately threatening the health and stability of groundwater environments.

Fig. 14. Proasellus franciscoloi*, a stygobiont crustacea from Rio dei Corvi cave, Piedmont, Italy. Photo: © V. Balestra.*

2.6 Deep aquifers

Growing evidence suggests that MPs infiltrate and accumulate in groundwaters (Hoang et al., 2025 and references therein). These particles may enter through recharge zones, preferential flow paths, or via leakage from overlying contaminated strata (Hoang et al., 2025 and references therein). Once introduced, MPs can be transported through interconnected pore spaces or fractures, depending on their size and characteristics (Hoang et al., 2025 and references therein). However, deep and confined aquifers are understudied.

Deep waters and confined aquifers are often used for human consumption, therefore, MP pollution can raise concerns for water quality, as conventional filtration systems may not remove them.

Confined aquifers are groundwater reservoirs bounded above and below by low-permeability layers, which isolate them from direct surface influence. Water within these systems is under hydrostatic pressure, often exceeding atmospheric levels. When a well penetrates such an aquifer, the internal pressure may cause water to rise naturally, and, in some cases, to flow freely at the surface, forming artesian wells. These artesian conditions result from recharge occurring at higher elevations, where the permeable layer is exposed. Even though artesian waters are often very old and protected, they are not immune to seepage, especially where geology is complex or human activity modified the natural stratigraphy. Confined and artesian aquifers are particularly vulnerable to persistent contaminants, as their limited exchange and slow flow rates can favor long-term accumulation.

At the conclusion of this thesis, it was decided to sample several deep waters in Piedmont, including several artesian aquifers, to verify MP and MF pollution, thus testing the methodology used and the possibility of infiltrations in certain areas.

Chapter 3

Sampling, separation and identification techniques

Origin of the Chapter

Part of this chapter is adapted from the following peer-reviewed articles, result of this thesis:

- "Microplastic pollution in show cave sediments: first evidence and detection technique" authored by Balestra and Bellopede (2022b), published in Environmental Pollution, Volume 292, Part A, 1 January 2022, 118261, DOI: **https://doi.org/10.1016/j.envpol.2021.118261.**

  The author of this dissertation was responsible for conceptualisation, methodology, validation, formal analysis, investigation, resources, data curation, writing – original draft, writing – review and editing, visualisation.
- "Automated method for routine microplastic detection and quantification" authored by Giardino et al. (2023), published in Science of the Total Environment, Volume 859, 2023, 160036, DOI: **http://dx.doi.org/10.1016/j.scitotenv.2022.160036.**

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3.1 Introduction

Since there are several methods to sampling, analyze and quantify MPs in different environments, there is no established standard to harmonize all procedures and measures. However, there are some general guidelines and standards for specific sectors or matrices, e.g. ISO/TR 21960:2020, ISO 4484-2:2023 and ISO 5667-27:2025 (International Organization for Standardization, 2023, 2025; International Organization for Standardization and European Committee for Standardization, 2020). Moreover, many existing methods are expensive and require the use of specific instruments, and the lack of standard procedures leads to difficulties in comparing results.

Nevertheless, monitoring concentrations of MPs in the environment is essential to better understand their sources, where they can be transported and the impacts they have on species and habitats (Huang et al., 2021; Prata et al., 2019; Wong et al., 2020; Wright et al., 2013). Finally, MP monitoring is necessary to understand potential risks for humans (Barceló et al., 2023; Henry and Klepp, 2018; Kannan and Vimalkumar, 2021).

Despite the diversity of existing methods, for each matrix, the processes can be divided into three main steps: sampling, separation and identification.

Techniques of sampling are very different from environment to environment and from a matrix to another one (Sharma et al., 2024). Generally, samples can be selective (direct extraction of items from the environment), bulk (volume of sample extracted from the environment) or volume-reduced (the volume of bulk sample is reduced during sampling) (Hidalgo-Ruz et al., 2012).

Separation can be done directly during sampling (selective samples) or with laboratory analyses (bulk and volume-reduced samples). During lab sample processes, separation from the matrix and filtration are done, depending on the matrix, volume sample type, and specific research goals (Hidalgo-Ruz et al., 2012; Sharma et al., 2024). Density separation for MPs is one of the most common and is generally done with a concentrate NaCl solution (~1.2 g/cm^3), to balance between recovery of particles, impacts on environment, and cost for process (Zhao et al., 2022). However, it not cover all kind of plastics, therefore, to increase the density are often used also other less eco-friendly solutions, such as sodium polytungstate (1.4 g/cm^3), calcium chloride $CaCl_2$ (1.3-1.35 g/cm^3), zinc chloride $ZnCl_2$ (1.5-1.7 g/cm^3) or sodium iodide NaI (~1.8 g/cm^3) (Zhao et al., 2022). MP can be separated from samples also by sieving, using different mesh sizes or other methods (Hidalgo-Ruz et al., 2012; Sharma et al., 2024). Filtration is essential to separate MP to the sample matrix or the solution and is generally done using a filter and a vacuum pump. Different kinds of filters are used in MP analysis, depending on the matrices, samples, and lab equipment for analysis and identification.

MP analysis can be quantitative, qualitative, or both. Quantitative analyses are fundamental to understand MP pollution into the environment and are generally defined as quantitative concentration (number of MPs) or mass concentration. The first one is the most used and can be acquired with visual analysis or spectroscopy. Qualitative analyses provide morphological and chemical information about analyzed MPs.

Identification and characterization of MP are generally made by microscopy or spectroscopy. Optical microscopy is one of the most used methods for MP identification and physical characterization (Huang et al., 2023 and references therein). Visual inspection offers benefits such as simplicity and low cost, however, it requires a lot of work and time, does not provide information on the polymer composition and is generally limited by low precision and efficiency (Huang et al., 2023 and references therein). Scanning electron microscopy is another important method for MP morphology analysis. Regarding spectroscopy, at the moment, Fourier Transform Infrared (FTIR) and Raman spectroscopy are the most used techniques for providing chemical composition information on MPs. These methods are very precise and provide material's spectra which can be compared with known polymers ones in spectra libraries, determining microparticles chemical composition (Huang et al., 2023 and references therein). Besides, the sample pretreatment procedure to obtain adequate filters and particles is not always simple. To a lesser extent, mass spectrometry is also used for MP identification, providing important information about polymer structure, main functional groups and their structure, molecular weight, and degree of polymerization (Huang et al., 2023 and references therein). Thermal analyses can be used too for MP identification.

With the rapid growth of analytical sciences, new methods and tools for MP analysis will be developed. Image analysis and artificial intelligence for particle counting and characterization are also promising (Guo et al., 2024; Jin et al., 2024; Zhang et al., 2023b; Zhao et al., 2024).

Research on AMPs is challenging, and the analysis of natural and regenerated MFs in environmental matrices is more difficult compared to synthetic ones, because the methodologies used to detect and characterize MFs were originally designed for MPs (Athey and Erdle, 2022; Liu et al., 2022a; Liu et al., 2019a).

In the following paragraphs, the methodologies used in this research were described in more depth. Analyses were conducted on sediments, waters and fauna, pointing out that before these, there was no method for the analysis of MPs and other AMPs, such as MFs, in underground environments.

3.2 Contamination control

To avoid MP and MF contamination during sampling, laboratory analysis and characterization, different precautions must be taken.

Whenever possible, plastic equipment was substituted with glass and metal alternatives, even if more difficult to use during sampling in not touristic caves.

Nitrile gloves were worn throughout all sampling, while during all lab procedures, in addition to nitrile gloves, were worn white cotton lab coats.

To prevent MP and MF contamination, all open containers, laboratory glassware, and equipment were covered with aluminum foil during all analyses.

All work surfaces, laboratory materials, and glass jars for sampling were thoroughly cleaned with bi-filtered ethanol and MilliQ water.

All analyses were conducted within a fume hood.

Initially, blank controls were performed on 30% H_2O_2 (Merck), absolute ethanol (VWR Chemicals), MilliQ water, and a NaCl solution (NaCl (Carlo Erba) + MilliQ water), to assess potential MP and MF contamination from the chemicals and water used. All chemicals and MilliQ water were then filtered and analyzed using the same method applied to the samples.

Moreover, possible air contamination and contamination by operator clothes during sampling and laboratory activities (Munno et al., 2023; Scopetani et al., 2020; Shruti and Kutralam-Muniasamy, 2023) were monitored, posing a glass jar near the operator during sampling, and an open glass Petri dish near the operator during lab analyses, for all the time. After, the glass jar and the Petri were closed until analyses. Subsequently, they were cleaned with bi-filtered MilliQ water and filtered through a glass vacuum pump, following the same method used for the other analyzed samples.

The blank correction method applied assesses the results of unknown samples by subtracting the blank contribution (Shruti and Kutralam-Muniasamy, 2023 and references therein) (chemicals and MilliQ water + air contamination). The pollutants detected in the tested chemicals and MilliQ water were summed to air contamination and then subtracted from the total amount found in the samples. This method is common and easy to use, however, it only allows for the correction of data about abundances, without the ability to completely distinguish between particles related to contamination and those originally present in the sample. Consequently, data about MP and MF characteristics cannot be totally corrected.

Subsequently, chemicals, MilliQ water and solutions were filtered twice through a 47 mm 1.2 µm pore size glass fibre filter (whattman, or Phenomenex, or Enrico Bruno srl) before using. Therefore, they were considered without contamination during analyses. The blank was then performed only for air and operator contamination during sampling and analyses.

3.3 UV light

Fluorescent whitening agents (FWAs) are often used in the production of plastic (Qiu et al., 2015), for example to brighten colours and mask the natural yellowing of plastics. Therefore, plastics with FWAs can absorb ultraviolet light (300–400 nm) and radiate purple (400–450 nm) or blue (450–480 nm) fluorescence (Qiu et al., 2015). Although MPs with FWAs can easily be detected under a fluorescence microscope, such equipment cannot be afforded by many laboratories. Therefore, a low-cost and easy to use ultraviolet (UV) flashlight can be used to irradiate MPs (e.g. Ehlers et al., 2020; Klein and Fischer, 2019).

However, a lot of organic and inorganic things could be fluorescent under a UV light, therefore, the use of this kind of flashlight has to be combined with the microscopy for MPs detection. Different kind of flashlights can be used, depending on the size of the surface to observe and on the comfort of the working environment.

A 365-nm (Alonefire SV10) and 395-nm (Youthink 100 LEDs) UV flashlights were tested on cave sediments and on filters in the laboratory (Fig. 15).

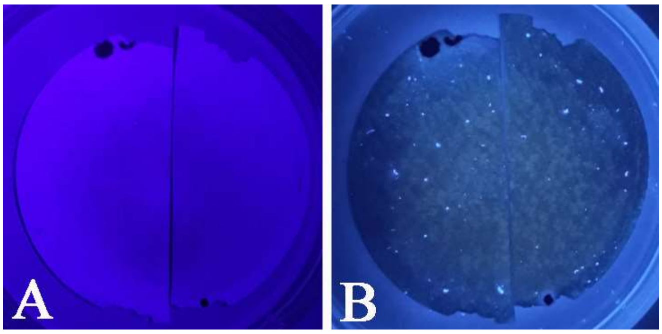

Fig. 15. UV flashlights tests on filter. A: the 395 nm UV flashlight did not evidence the presence of any microplastics. B: the 365 nm UV-light highlighted the fluorescent microplastic particles. Image from Balestra and Bellopede (2022b).

The UV flashlight tests on filters revealed fundamental information. On the same filters and on the same areas of the cave, the 395-nm UV flashlight did not evidence the presence of any MPs, whereas the 365-nm UV light perfectly highlighted all fluorescent particles (Fig. 15). The use of 365-nm UV light yielded excellent results for MP enumeration on dried filters, therefore, this wavelength was selected for application throughout this research.

Fluorescent dyes and brighteners are commonly used in the textile industry too (Barker, 1975; Christie, 2011; Tang et al., 2019), and in a wide range of different products, therefore, UV light can be also used for MF detection and other non-fibrous AMPs.

3.4 Sampling

Sampling is the first fundamental step to analyse MP in the environment, which determines the types of MPs to be separated and identified. There are no universally accepted methods for MP sampling in the different matrices, however, accurate sampling is the key to obtaining reliable and representative results for monitoring programmes regardless of the matrix analysed (water, sediment, soil, air or biota).

Different sampling protocols for diverse matrices were published in the last decades (Hidalgo-Ruz et al., 2012; Sharma et al., 2024). However, at the onset of this research, no previous studies had addressed MP pollution in subterranean karst environments. Therefore, following the research carried out in springs (for water) and in muddy environments (for sediment), efforts were made to develop adequate sampling protocols for waters and sediments in tourist caves and, subsequently, in speleological ones. Finally, methods were adapted to enable the analysis of karst systems along their continuum, from surface watercourses to cave waters, and ultimately to springs and deep aquifers.

Different studies on MPs in marine sediments used bulk samples when MPs cannot be easily identified visually, for example when they are too small for a naked eye identification or when their abundance is low (Hidalgo-Ruz et al., 2012). Due to the peculiarities of the environments monitored in this research, it is not possible to easily observe the presence of microparticles on fields, therefore, bulk samples are fundamental. However, for a preliminary on-field survey, it is possible to use UV light directly on some matrices, such as sediments or speleothems, to observe the presence of some particles of anthropogenic origin (see Paragraph 3.3)

Although it is acknowledged that a low volume of samples could not be representative of sampling stations, in particular and sensitive environments, such as the subterranean ones, the availability of materials per site could be limited, and it is always better to apply the precautionary principle, making attempts to damage habitats and species as little as possible.

Moreover, spatial and temporal monitoring is always recommended, although often due to time and funding problems it is not possible. However, in such little-studied environments, even preliminary data are fundamental, and further monitoring can be explored in depth later.

3.4.1 Sediment sampling in show caves

Due to the subterranean environment peculiarities, it is not possible to easily observe the presence of particles on fields, therefore, bulk samples were fundamental.

After a preliminary survey, five superficial bulk sediment samples (upper 5 cm) were collected near the tourist paths, in different 1x1 m areas of the cave (Fig. 16). As a sort of blank, one sample was taken in a non-touristic cave zone, visited only by researchers and speleologists (Fig. 16).

About 300 g of superficial (first 5 cm) sediment for each sampling area were collected with a metal spoon and placed in pre-cleaned glass boxes (see Paragraph 3.2 for information about contamination control). The metal spoon was cleaned every time with ethanol. The samples were stored in the fridge at 6°C until laboratory analysis.

Although it is acknowledged that a low volume of sediment could be not representative of sediments at individual sampling stations, cave environment and concreting of cave sediments limited the volume of sediment that was available per site. Moreover, in sensitive and protected environments such as caves and subterranea habitats, the precautionary principle applies, and attempts are made to damage habitats and species as little as possible.

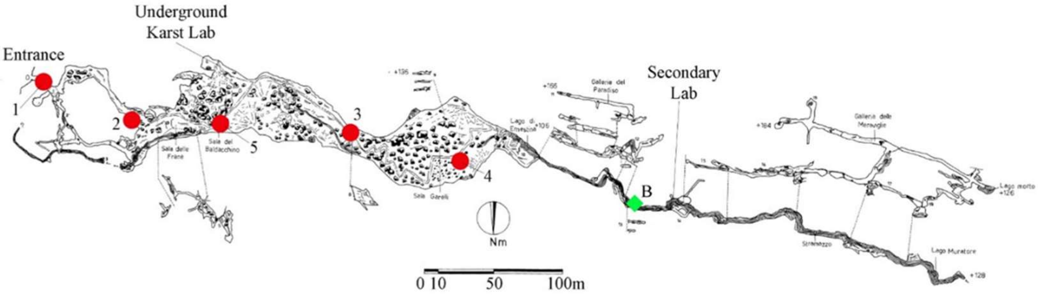

Fig. 16. Example of sediment sampling in the Bossea show cave, Piedmont, Italy (see Chapter 4). Image from Balestra and Bellopede (2022b).

The caves examined in these studies have not particularly developed in length tourist paths, therefore, five sampling points along the tourist route were considered representative of the environment, analyzing the areas of greatest interest. For more developed caves or with different peculiarities and problems it is strongly recommend adding more sampling points. An increased number of sampling points enhances the knowledge on MP distribution along the tourist routes.

For more details about the studies on MP pollution in show cave sediments see Chapter 4.

3.4.2 Sediment sampling in speleological and unexplored caves

Caves and underground environments are typically extreme, which makes it challenging to gather large sample volumes. Moreover, they are sensitive and protected habitats, therefore, it is necessary to follow the precautionary principle and evaluate the environmental characteristics of the studied cave. In particular, unexplored caves present more logistical difficulties due to the unknown cave morphology and speleological challenges.

The limited availability of sediment for analysis, coupled with the complexities of sample collection and transport, can create significant hurdles. Nevertheless, subterranean environments remain understudied, and unexplored caves have not yet been sampled. As a result, any sampling and subsequent analysis are crucial for advancing knowledge about these ecosystems' health and the indirect effects of human activities.

Bulk samples are fundamental in this peculiar environments (Hidalgo-Ruz et al., 2012). Following the precautionary principle and evaluating the environmental characteristics of the studied caves, the amount of collected samples was always limited to about 600 g each, according to the availability. Sediments were collected from the upper 5 cm. All samples were collected with a metal spoon and put inside pre-cleaned glass jars. The metal spoon was cleaned every time with ethanol (see Paragraph 3.2 for information about contamination control) (Fig. 17).

The number of samples per cave depended on the characteristics of the examined cavities, the type of study carried out, and the availability of funds. The greater the number of sampling points, the more detailed will be the knowledge on the MP pollution in the cave.

Generally, samples in caves were directly collected during personal speleological activities. When it was not possible, before sampling, other speleologists were trained on methodology and sampling through training courses and the drafting of a protocol (see Chapter 6).

Fig. 17. Sediment sampling in caves. Photo: V. Balestra

The jars were limited and coated with anti-impact material to transport them safely in the speleological bags (Fig. 18).

Samples were stored in the fridge at 6 ◦C until analysis in the laboratory.

For more details about the studies on MP and other AMP pollution in cave sediments see Chapters 5 and 6.

3.4.3 Water sampling in karst systems and groundwaters

MPs are commonly found in most water bodies, however, sampling strategy used depends on the aquatic environment typology, and on the MP type to be sampled. In fact, depending on their properties, such as density, shape or chemical composition, as well as the extent of biofouling, MPs can float on surface waters, be suspended in the water column or in the depths of the water body.

Sampling in water bodies can be done punctual, horizontal, along the water surface, or vertical, along the column of water (Crawford and Quinn, 2016 and refernces therein). However, not all water bodies can permit all kinds of sampling. Spot sampling is always possible, and it is often used for sampling in small water bodies and groundwaters, but, in some environments, it could be not representative. For horizontal sampling, it is possible to use nets or manta ray with different mesh. For vertical sampling, it is possible to use Schindler-Patalas plankton trap or similar methods.

The subterranean sites generally require the use of caving equipment (Fig. 18). In these environments, especially caves, it is not always possible to collect large volumes of samples for analysis, due to lack of material, and the difficulty in collecting and/or transporting samples. Moreover, it is necessary to damage these environments as little as possible. Hence, only small amounts of samples were collected in this research. However, these environments are poorly studied, therefore, any kind of sampling and analysis is crucial to better understand the status of the analyzed ecosystems and their conservation.

Fig. 18. Me, sampling in 214 cave, in the Italian sector of the Classical Karst, Friuli-Venezia-Giulia, Italy. Photo: M. Galbiati.

Spot sampling, from surface watercourses to springs, was used in this research, as well as in caves. Water bulk samples (Hidalgo-Ruz et al., 2012) were collected using pre-cleaned glass jars and nitrile gloves (see Paragraph 3.2 for information about contamination control).

The number of samples and sampling points depended on the characteristics of the examined karst areas and cavities, the type of study carried out, and the availability of funds.

For each sampling area, a quantity between 200 and 1150 mL of surface waters were collected, depending on the water availability, the aim of the study, and funds availability. In the subterranean sites, the jars were limited and wrapped with anti-impact material to transport them safely in the speleological bags (Fig. 18).

Samples were stored in the fridge at 6 ◦C until laboratory analysis.

For more details about MP and other AMP pollution in karst system waters and groundwaters see Chapters 4 and 5.

3.4.4 Water sampling in deep aquifers

Spot sampling was used in this research for deep aquifer water monitoring. Water bulk samples (Hidalgo-Ruz et al., 2012) were collected using pre-cleaned glass jars and nitrile gloves (see Paragraph 3.2 for information about contamination control).

The number of sampling points depended on the characteristics of the examined areas, the type of study carried out, and the availability of funds. The greater the number of sampling points, the more detailed will be the knowledge on the MP pollution in deep aquifers. In this research, twenty-eight water samples were collected in twenty-eight different deep aquifers in the Cuneo province, Italy.

Waters were sampled directly from the mouth of the water intakes, therefore, blank control during sampling was not carried out. Once sampled, glass jars were immediately closed with the cap. For each sampling point, a quantity between 200 and 750 mL of water was collected.

Samples were stored in the fridge at 6 ◦C until laboratory analysis.

For more details about MP and other AMP pollution in deep aquifer water see Chapter 7.

3.4.5 Biota

MP analysis in animals depends on the species and their ecology. However, no stygobiont animal were analyzed before this research.

The species *Proasellus franciscoloi,* a stygobiont crustacea living in the Bossea cave system, was analyzed to have a comprehensive overview of the MP problem in this system, together with other specimens of different species collected in caves and springs of Tuscany by other researchers.

The specimens in the Bossea cave were punctually collected with brushes in cave waters, in the undersaturated karst habitat, and carefully placed in glass containers, filled with groundwater, wood fragments, and sediment from the collection site. The specimens were placed in an artificial pool inside the cave, directly connected to the running water of the karst system, until they were transferred. During transportation, specimens, groundwater, wood fragments, and sediments were placed in glass containers and stored in a temperature-controlled environment. The day after collection, the specimens were transported to the laboratory strictly following the procedures outlined in Castaño-Sánchez et al. (2021). In the laboratory, deceased individuals were isolated and stored in glass vials filled with 96% ethanol.

For more details about MP pollution in stygobiont crustacea see Chapter 4A.

3.5 Separation

3.5.1 Filtration

Filtration is essential to separate MPs to the sample matrix or the solution and is generally done using a filter and a vacuum pump. Different kinds of filters are used in MP analysis, depending on the matrices, samples, and lab equipment for analysis and identification.

During the years of PhD, Ø 47 mm glass fibres (Whatman, or Phenomenex, or Enrico Bruno srl, mm, 1.2 µm), paper (Whatman, 11 µm), ANODISC (Cytiva, 0.2 µm) and silver (GVS Life Sciences, Membrane, 0.8 and 1.2 µm) filters were tested. In general, after testing some considerations can be made:

- Glass filters are very good for microscopy, but they cannot be used for spectroscopic analysis
- Paper filters are good for microscopy, however, they can be easily deformed on the surface, making difficult to analyze particles; they are not good for spectroscopy, especially for natural and regenerated AMPs
- ANODISC filters are good for spectroscopy, but they are very thin and break easily
- Silver filters are very good for spectroscopy and microscopy

Therefore, following the initial analyses conducted with glass fibre filters, only ANODISC and silver filters were subsequently employed in this research.

3.5.2 Sediments

Different tests were done on sediment samples to verify the best method for MP separation. The main questions concerned the organic matter removal (OMR) and the kind of filter to use.

OMR is a necessary step. Hydrogen peroxide (H_2O_2) digestion is the most commonly used method for MP and MF analysis (Athey and Erdle, 2022), but it can alter the mechanical properties and IR spectra of the samples. The H_2O_2 concentration of solution used for OMR in previous research in different environments varied from 15 to 30% (e.g. Mathalon and Hill, 2014; Prata et al., 2019; Zhang et al., 2019). According to Nuelle et al. (2014), 30% hydrogen peroxide solution could damage MP particles and dissolve smaller ones, reducing also MP fluorescence intensity under the microscope. However, for samples rich in organic material 15% H_2O_2 could be not enough.

First step regarded the OMR as pre- or post- treatment. Consequently, OMR was tested after filtration directly on filters (methods 1 and 2), or before filtration directly on sediments (method 3). Moreover, it was used 15% and 30% H_2O_2 (Table 1).

Method 1

Samples were weighted before and after drying process. The sediments were placed in an aluminium box, covered with aluminium foil and dried into an oven at 40°C to constant weight.

For each dried sample, three sub-samples of 10-20 g were selected via coning and quartering and poured in precleaned glass beakers. Sand and minerals were then removed by density separation. For each beaker, 100-200 ml of NaCl solution (200 g NaCl/0.6 l, density 1.22) was added, and the mixture was stirred with a magnetic mixer for 2 minutes. After settling for 24 hours, the supernatant (30 ml) was then extracted with a glass pipet and filtered by a vacuum pump.

Filters were placed on glass petri dishes covered with aluminium foil and dried in an oven at 40°C for 2 h. Dried filters were subjected to OMR through the application of 0.5 ml of 15% hydrogen peroxide solution, left to react for 30 minutes and dried again for 1 hour at 50°C.

Method 2

Samples were weighted before and after drying process. The sediments were placed in an aluminium box, covered with aluminium foil and dried into an oven at 40°C to constant weight.

For each dried sample, three sub-samples of 10-20 g were selected via coning and quartering and poured in precleaned glass beakers. Sand and minerals were then removed by density separation. For each beaker, 100-200 ml of NaCl solution (200 g NaCl/0.6 l, density 1.22) was added, and the mixture was stirred with a magnetic mixer for 2 minutes. After settling for 24 hours, the supernatant (30 ml) was then extracted with a glass pipet and filtered by a vacuum pump.

Filters were placed on glass petri dishes covered with aluminium foil and dried in an oven at 40°C for 2 h. Dried filters were subjected to OMR through the application of 0.5 ml of 30% hydrogen peroxide solution, left to react for 30 minutes and dried again for 1 hour at 50°C.

Method 3

Sediments were initially placed in an aluminum box, covered with aluminum foil, and dried into an oven at 40° C to constant weight.

Dried sediments were pre-treated through the application of 1:1 30% H_2O_2 solution, left to react for seven days under natural conditions, and dried again at 40 ◦C to constant weight.

In relation to the quantity of dry sediments, trying to use as much material as possible, three sub-samples of 10-20 g were selected for each sample via coning and quartering, and put into beakers with 100-200 ml NaCl solution (200 g NaCl/0.6 L, $\rho = 1.2$). The mixture was stirred with a magnetic mixer for 2 min and settled for 24 h. Subsequently, the supernatant was extracted with a glass pipet and filtered by a vacuum pump.

Filters were placed on glass petri dishes, covered with an aluminum foil and dried in an oven at 40° C until completely dry.

Based on the analysis carried out on the sediments of the examined show caves, the different steps used for the OMR have led to make some important considerations for future research on cave sediments. The use of H_2O_2 solution is not recommended as post-treatment on filter (Table 1), because it makes the surface of the filter less uniform and bubble, making more difficult the characterization under microscope and the creation of good images for an automated counting by a software. A part of organic material in the cave sediments is clearly visible at naked eye, however, a large micro-components amount is present in all samples and promote the creation of bad filters, both for MP characterization with visual counting under microscope and image analysis, therefore, the use of 30% H_2O_2 as pre-treatment must be preferred to 15% one (Table 1). Using the OMR as pre-treatment, pouring a volume of 1:1 30% of hydrogen peroxide solution directly on sediments (Table 1), the samples resulted much cleaner and uniform making easier MP separation from sediments with NaCl solution. Above all, the filters obtained using the pre-treatment had uniform and little dirty backgrounds, perfect for image analysis.

Table 1. Comparison between the different steps for sampling preparation and analysis. Table from Balestra and Bellopede (2023).

Step	Bossea cave method	Toirano caves improved method	Borgio Verezzi cave improved method
Drying of the samples	40°C to a constant weight	40°C to a constant weight	40°C to a constant weight
Pre-treatments	-	-	Organic matter removal 1:1 30% H_2O_2, left to react for 7 days under natural conditions, dried at 40°C to constant weight
Sub-samples though coning and quartering	10 g	10 g	20 g
Density separation with NaCl solution (200 g NaCl/0.6 L, density 1.22), mixing for 2 min, 24 h at rest. Extraction of the supernatant.	100 ml of NaCl solution	100 ml of NaCl solution	200 ml of NaCl solution
Filtration by vacuum pump	1.2-μm pore size glass microfibre filter (Whatman, Ø 47 mm)	1.2-μm pore size glass microfibre filter (Whatman, Ø 47 mm)	1.2-μm pore size glass microfibre filter (Whatman, Ø 47 mm)
Drying of the filters	40°C for 2 h	40°C for 2 h	40°C for 2 h
Post- treatments	Organic matter removal 0.5 ml 15% H_2O_2, left to react for 30 min under natural conditions, dried at 50 ◦C for about 1 h	Organic matter removal 0.5 ml 30% H_2O_2, left to react for 30 min under natural conditions, dried at 40 ◦C for about 2 h	-

After testing these three methods, Method 3 resulted the best, with more homogeneous, flat and clean filters, ideal for microscopy and spectroscopy.

Subsequently, for each research, the quantity of subsamples was chosen in relation to sample availability, maintaining the 1:10 proportion with NaCl solution. Filters used were chosen according to subsequent analyses (see Paragraph 3.5.1) and, after filtration, put in a glass Petri dish, covered with an aluminum foil, and dried in a fan oven at 40°C until completely dry.

One of the most used procedures to separate MPs from sediments is density separation with NaCl solution, to balance between recovery of particles, impacts on environment, and cost for process (Zhao et al., 2022). Accordingly, NaCl was employed in this study to separate MPs from sediments. The specific density of MPs can vary considerably depending on the type of the polymer, the manufacturing process and the environmental factors as biofouling (Hidalgo-Ruz et al., 2012). Density values for concentrated saline NaCl solution is 1.2 g/cm^3, consequently, denser polymers could be excluded. However, the densities of materials in natural environments may differ from those of newly produced materials. Due to factors like increased porosity, degradation, biofouling, or organic matter adsorption, the density of these materials can either increase or decrease over time in natural environments (Kaiser et al., 2017). Indeed, it is possible to observed materials with reported densities greater than 1.2 kg/L (Horton et al., 2017). However, it is important to consider that some materials with higher densities may remain in the sediments due to their inability to be separated by this method.

For more details about MP pollution in cave sediments see Chapters 4, 5 and 6.

3.5.3 Water

Water samples were processed according to the method for cave sediments, adapted for the liquid matrix and improved to determine the particles using the IR spectroscopy.

Initially, the OMR was done with the application of 2 mL of 15% hydrogen peroxide solution directly on filters, left to react for 30 min at room temperature and dried again for 2 h at 50°C (see Chapter 4).

Subsequently, with new tests and information regarding the methods, OMR was done as pre-treatment with 1:1 or 1:2 15% or 30% hydrogen peroxide, left to react for minimum 7 days at environmental conditions, or left to react at 45 °C for at least 2 hours in a shaker bath (Stuart Scientific Bibby SBS30). After OMR, samples were directly filtered. Filters were placed on glass petri dishes covered with aluminum foil and dried in an oven at 40°C until completely dry.

For more details about MP pollution in waters see Chapters 4, 5 and 7.

3.5.4 Biota

Analysis on stygofauna were done by CNR-IRET and Department of Chemistry “Ugo Schiff”, University of Firenze laboratories. Detailed information about the specific methodology used for stygofauna are directly reported in the dedicated Chapter 4A.3.

3.6 Identification

Identification and characterization of MPs, and subsequently MFs, are generally made by microscopy or spectroscopy.

Optical microscopy is one of the most used methods for MP identification and morphological characterization, being simple and economic. However, visual analysis can’t provide information on the chemical composition of MPs and is a laborious method (Huang et al., 2023 and references therein). Moreover, when MP particle size is too small, visual analysis method alone is not recommended (European Commission, 2013; Hidalgo-Ruz et al., 2012; Song et al., 2015). However, it allows to better see the surface morphology of MPs and other AMPs, and some of their important characteristics such as color. A preliminary screening step under microscope can be useful to distinguish between synthetic materials and natural and regenerated ones, especially for MFs.

Spectroscopy is useful to identify material composition and particles with micro and nano size, however, this method is very time-consuming and requires expensive equipment and specialized researchers. Moreover, surface particles collected in natural environments are often covered by other materials and impurities, and/or contaminated by other pollutants, consequently, MP and MF spectra are difficult to match with high percentages with spectra libraries (Song et al., 2015).

A combination of several methods is the optimal choice to identify MPs in natural environments (Song et al., 2015), therefore, in this work MPs and other AMPs were analyzed by means of microscopic and spectroscopic techniques.

In the first research on MP pollution in sediments on show caves, an automated software of image analysis was tested too (see Chapter 4).

Identification and characterization on MPs found in stygofauna were done by CNR-IRET and Department of Chemistry "Ugo Schiff", University of Firenze laboratories. Detailed information about the specific methodology used for are directly reported in the dedicated Chapter 4A.3.

3.6.1 Microscopy

Particles on filters were observed under a Leitz ORTHOLUX II POL-MK microscope equipped with a DeltaPix Invenio 12EIII 12 Mpx Camera. Microparticles were counted at 2.5× magnification, enlarging to 10× or higher magnifications for identification and characterization; each filter was observed with and without a UV flashlight (Alonefire SV10 365 nm UV flashlight 5 W) (Fig. 19). Since the accuracy in visually identifying big particles is more reliable than with little ones (Hidalgo-Ruz et al., 2012; Song et al., 2015), it was established to analyze MPs and other AMPs up to maximum 0.05 mm. Particles not clearly identifiable as AMPs were not taken into consideration. Observed microparticles were characterized according to the Standardised size and colour sorting system (SCS) (Crawford and Quinn, 2016).

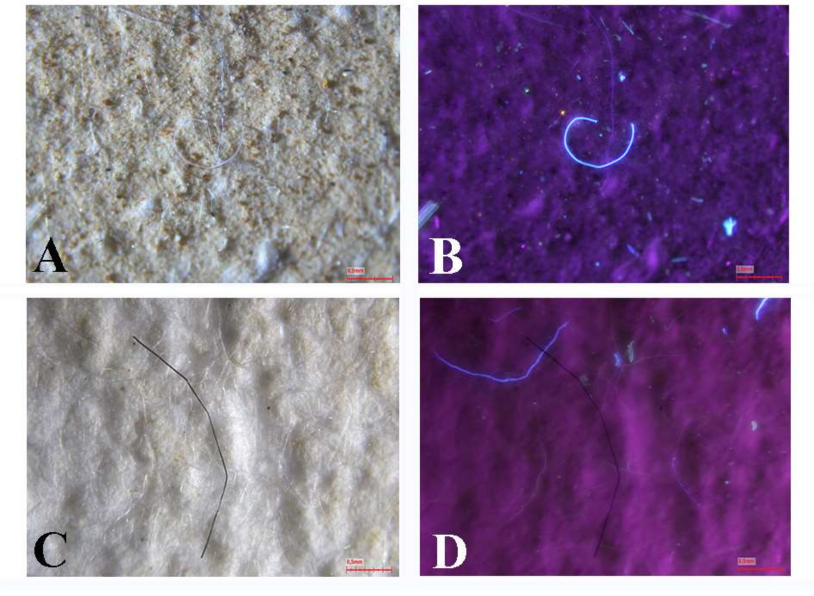

Fig. 19. Microplastics and microfibres from sediments of Herzegovinian caves. Photos under the microscope, without (A, C) and with (B, D) UV light.

A further detailed comparison was made for MF analysis, observing details showed in longitudinal and x-sectional microscopic images of natural, regenerated and synthetic fibres (e.g. Khan et al., 2017) (Fig. 20).

Since there were few examples in the literature about MPs and MFs, several images were taken during the research and published (see Chapters 2, 3, 4, 5, 6, 7).

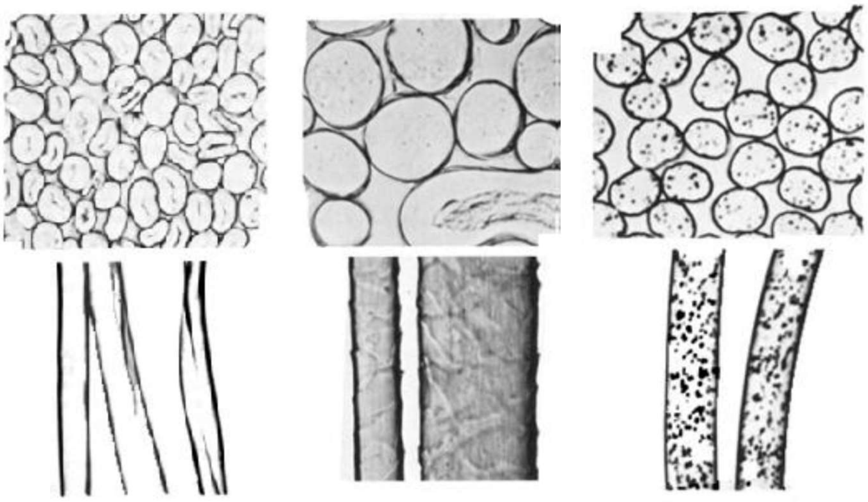

Fig. 2 Cotton, Mercerized

Fig 12. Mohair

Fig. 15 Acrylic, Reg. wet spun, Semi dull

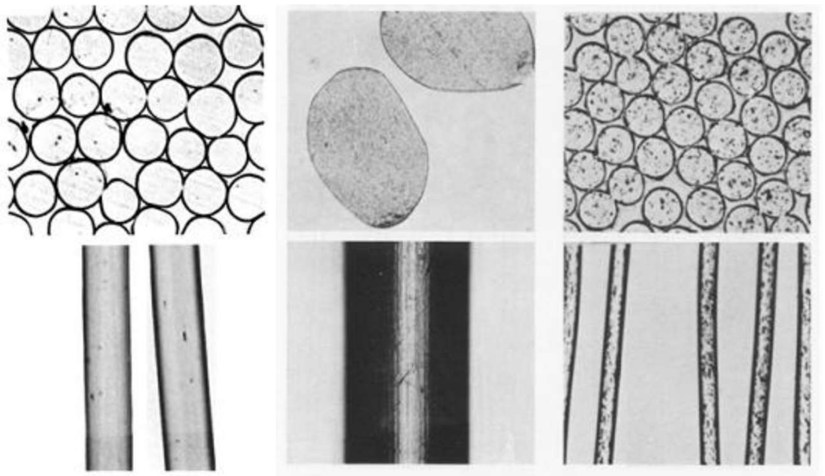

Fig. 17 Nylon, bright

Fig. 20 Polyethylene, high density

Fig. 21 Polyester, Regular melt spun, 3.0 denierper filament, semi dull

Fig. 20. X-sectional and longitudinal view of different common textile fibres (Images from Khan et al. (2017)).

3.6.2 Spectroscopy

In this research, microparticles on filters were analyzed using a micro-Fourier Transform Infrared Spectroscope (μFTIR).

Fourier Transform Infrared spectroscopy is a vibrational spectroscopy technique which provides the chemical composition of analyzed microparticles, generating infrared spectra which are compared with reference libraries to determine their composition (Huang et al., 2023) (Fig. 21).

It is a non-destructive, relatively simple and robust method for identifying MPs, with low susceptibility to interference and broad applicability in environmental studies (Huang et al., 2023). It is widely applied for the quantitative detection and chemical characterization of MPs. Samples must be thoroughly dried prior to analysis, as residual moisture interferes with spectral identification. Despite its broad use, FTIR has notable limitations and results can be influenced by polymer heterogeneity, weathering, or the presence of organic matter. In particular, opaque or black microplastics are difficult to analyze (Elert et al., 2017).

Analysis of natural and regenerated MFs can be more challenging compared to synthetics ones. Natural polymers have lower signal intensities compared to synthetic ones and are more prone to dye interference. Natural and regenerated polymers have almost identical FTIR spectra (Comnea-Stancu et al., 2017), and the presence of dyes, oxidation, and microbial degradation can alter cellulose absorption bands (Li et al., 2010; Remy et al., 2015; Zambrano et al., 2019), therefore, it is extremely challenging distinguish between them.

FTIR can work in three main modes: reflection, transmission and attenuated total reflection (ATR).

The reflection mode is suitable for opaque or thickness materials and is suitable for MP and MF analyses in real samples, especially if filtered on metal filters, such as silver or gold coated ones. However, it can suffers from low signal and high noise, resulting in low accuracy and low matching degree with standard maps (Huang et al., 2023).

The transmission mode provides high-quality spectra but requires thin, transparent samples and extensive pretreatment (Huang et al., 2023).

The ATR mode produces accurate spectra with minimal sample preparation and is effective for irregular or very small particles. It is less susceptible to impurities interferences, however, it is expensive and time consuming, limiting large-scale applications in MP analyses (Huang et al., 2023).

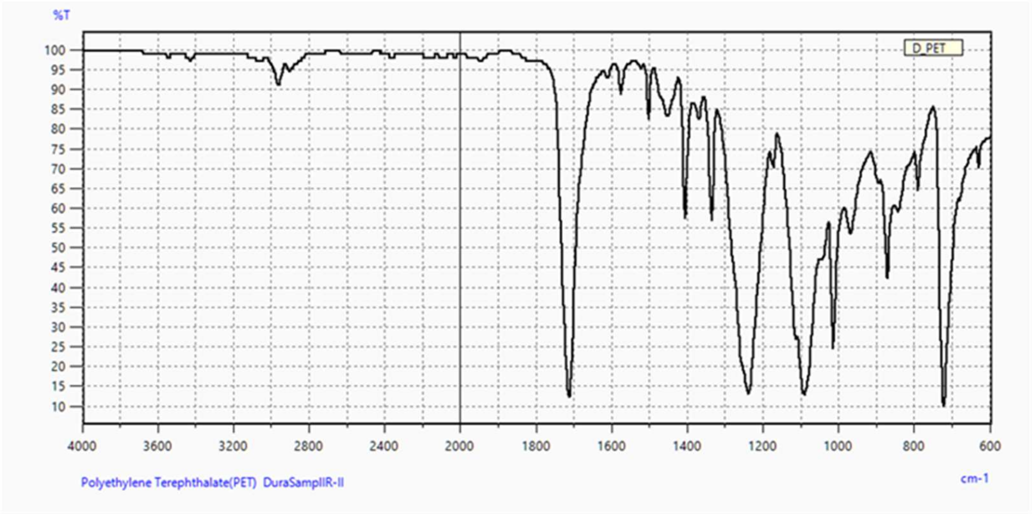

Fig. 21. Spectrum of PET from µFTIR Shimadzu AIM-9000 microscope equipped with a Shimadzu IRTracer-100 spectrophotometer and a Shimadzu ATR with a germanium prism.

Particles on filters were generally verified using the µFTIR Shimadzu AIM-9000 microscope equipped with a Shimadzu IRTracer-100 spectrophotometer and a Shimadzu ATR with a germanium prism (Multimodal Lab, DIATI – Politecnico di Torino). On each filter, different percentages of MPs and other AMPs were analyzed. Particles on glass filters were transferred with a dissecting needle on a silver filter (GVS Life Sciences, Membrane Disk 47 mm), while particles on ANODISC or silver filters were directly analyzed. Particles were analyzed in a spectral range between 4000 and 700 cm^{-1}, with 40 scansions. Atmosphere corrections were applied on the obtained spectra, subsequently compared with the Shimadzu Lab Solution Library ATR Polymer 2, followed by a visual analysis comparison of characteristic bands in the reference spectrum. Spectra were accepted only with a match degree ≥70%.

Due to the malfunction of the above mentioned µFTIR, in the latest research on dripping water in Torri di Slivia cave it was used a µFTIR Nicolet iN10MX, Thermo Fisher Scientific (STIIMA-CNR of Biella) (see Chapter 4C). Particles were analyzed in reflection mode, within the spectral range of 4000 to 650 cm^{-1}, with 24 scans taken per sample. The spectra were analyzed with the Omnic Picta software and verified visually by the operator. Only spectra with a match degree ≥ 70% were accepted.

3.6.3 Image analysis

Based on their color or fluorescence, MPs and MFs could be photographed and automatically quantified by software identification, possibly removing the operator biases, and reducing the processing time, thus enabling faster processing than traditional methods. For those reasons, recently, there has been an increasing interest in image analysis techniques and the AI use, especially for MPs characterization (e.g. Gauci et al., 2019; Guo et al., 2024; Jin et al., 2024; Lorenzo-Navarro et al., 2021; Lorenzo-Navarro et al., 2020; Zhang et al., 2023b; Zhao et al., 2024). Before this research, in 2022 some software were tested for MP counting and analysis.

The Image-J software was used by Mukhanov et al. (2019) to classify MPs into different classes, and the Microplastics Visual Analysis Tool (MP-VAT) was created by Prata et al. (2019) to automatically count fluorescent MPs stained with Nile Red dye under a specific wavelength illumination. However, different factors, such as the staining process, camera conditions and settings, introduce variability in the outcomes, thus requiring improvements that were subsequently implemented in another release of the software (MP-VAT 2.0) (Prata et al., 2020).

Using a MATLAB-based software (Gauci et al., 2019), MP particles extracted from samples originating from different beaches were characterized.

Lorenzo-Navarro et al. (2020) counted and classified MPs particles using as input two images acquired with a high-resolution flat scanner and their System for Microplastics Automatic Counting and Classification (SMACC).

Deep learning applications for MP analysis have been employed to segment synthetic fibres in microscopy images (Wegmayr et al., 2020) and count and classify MPs using pictures taken with cameras or mobile phones (Lorenzo-Navarro et al., 2021).

However, all these programs were often tested on samples created in the laboratory or images taken from plastic fragments cut in controlled environments, not considering the difficulties of analyzing real samples. In images or samples created in a laboratory, MPs can be positioned very far from each other, there are no overlaps of particles, and the contours of the particles are well-defined (e.g. Lorenzo-Navarro et al., 2020). Moreover, sometimes only particles with larger dimensions are counted (e.g. Gauci et al., 2019; Lorenzo-Navarro et al., 2020).

On the contrary, in real samples, particles overlap is frequently observed, and MPs are subject to erosion and biofouling, which often change their shape, color, and contours. Moreover, the number of microplastics in real samples is often higher than those considered in the created images or samples. Finally, laboratory samples have no disturbing factors, while real samples often have particles and sediment powders that can make the background less uniform and clear for the optimal visualization of the particles. One of the last developments consists of a thresholding approach for fluorescence microscope images proposed by Meyer et al. (2017), where machine learning techniques were also employed for the classification of the different typologies of plastics.

Unfortunately, machine learning classification requires supervised training on large datasets, and most of these techniques may not perform well on real-world images if they are too different from training ones, especially when trying to detect and classify tiny and small-resolution objects in a vast uncorrelated background (Liu et al., 2021).

During the research fellow in 2022, together with the colleagues of DISAT, Politecnico di Torino, an automated counting software for MP and MF characterization (MUPL) was developed, using images taken with cameras on filters of real samples selected in different matrices (Giardino et al., 2023) (Fig. 22). The use of automated software limits subjectivity in quantifying MPs and MFs and reduces the processing time compared to traditional methods. The adopted approach relies on traditional image processing techniques where the user tunes process parameters without the need to train the software on microplastic sample images. The software was provided with a very easy-to-use graphical user interface (GUI) which drives the operator along all process workflow and makes it suitable for use by inexpert researchers. The automated counting has been applied to images created with both artificial (created by an image editing software) and real samples of Borgio Verezzi show cave sediments and Po River water collected inside the city of Turin. Detailed information on the development of the software were reported in Giardino et al. (2023).

Apart from the photos used for MUPL validation, this method was employed for MP and MF identification in the sediments of Borgio Verezzi and Toirano caves (Balestra and Bellopede, 2023) (see Chapter 4B).

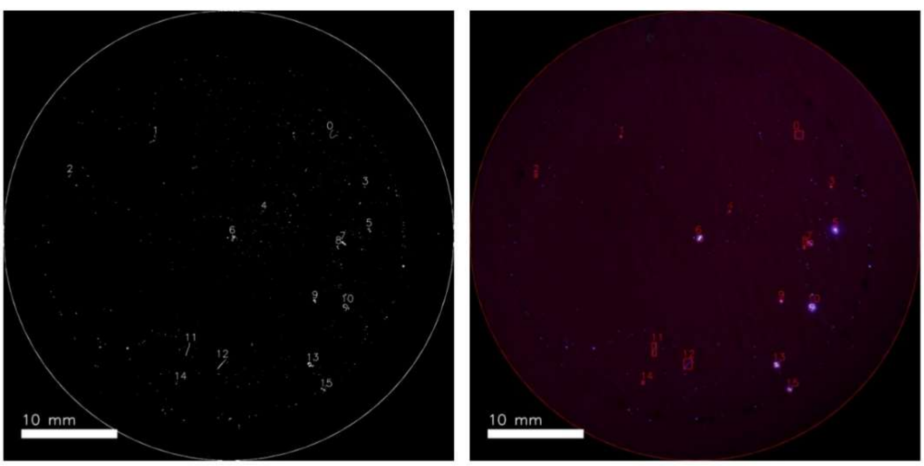

Fig. 22. Example of microparticle automated counting with MUPL software.

Chapter 4

Microplastic pollution in show caves

4A Case Study: The Bossea cave karst system, Piedmont, Italy. Microplastic analysis on sediments, water and fauna: a multidisciplinary approach

Origin of the Chapter
This chapter is adapted from the peer-reviewed articles:

- "Microplastic pollution in show cave sediments: first evidence and detection technique" authored by Balestra and Bellopede (2022b), published in Environmental Pollution, Volume 292, Part A, 1 January 2022, 118261, DOI: **https://doi.org/10.1016/j.envpol.2021.118261.**

  The author of this dissertation was responsible for conceptualisation, methodology, validation, formal analysis, investigation, resources, data curation, writing – original draft, writing – review and editing, visualisation.

- "Preliminary investigations of microplastic pollution in karst systems, from surface watercourses to cave waters" authored by Balestra et al. (2023), published in Journal of Contaminant Hydrology, Volume 252, January 2023, 104117, DOI: **https://doi.org/10.1016/j.jconhyd.2022.104117.**

  The author of this dissertation was responsible for conceptualisation, methodology, validation, formal analysis, investigation, resources, data curation, writing – original draft, writing – review and editing, visualisation.

- "Microplastics in caves: a new threat in the most famous geo-heritage in the world. Analysis and comparison of Italian show caves deposits" authored by Balestra and Bellopede (2023), published in Journal of

Environmental Management, Volume 342, 15 September 2023, 118189, DOI: **https://doi.org/10.1016/j.jenvman.2023.118189.**

The author of this dissertation was responsible for term, conceptualisation, methodology, software, validation, formal analysis, investigation, resources, data curation, writing – original draft, writing – review and editing, visualisation.

- "(Micro-)plastics in saturated and unsaturated groundwater bodies: first evidence of presence in groundwater fauna and habitats" authored bySforzi et al. (2024), published in Sustainability, Volume 16(6), 2024, 2532, DOI: **https://doi.org/10.3390/su16062532.**

  The author of this dissertation was responsible for validation, sample collection, data curation, writing - original draft preparation, writing - review and editing.

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4.1 General introduction

This research aimed to investigate MP and MF pollution in a karst system, analysing sediments, water, and fauna, to understand the extent of the problem with a multidisciplinary point of view, providing new insights into their environmental impacts, and propose correct methods of monitoring and analysis.

Sediments, water and fauna of the Bossea karst system, Piedmont, Italy, were analysed. After the sediment analysis inside the Bossea cave, the water of the system was analysed from surface watercourses to springs, developing an extensive sampling inside the cave, in the speleological and tourist areas. The species *Proasellus franciscoloi*, a stygobiont (i.e. specialist of underground water environments) crustacean living in this system, was analyzed with other aquatic organisms together with CNR-IRET and Università degli Studi di Firenze. Finally, the presence of bisphenols was investigated in the water of the system, to verify possible correlations with the presence of MPs. Analysis on these samples were conducted during the period abroad at Universitat de València.

4.2 The study area

Bossea cave (Grotta di Bossea) (Fig.s 23, 24, 25) is located in a protected area of Frabosa Soprana (CN) municipality, Piedmont, Italy. It has a single entrance at 836 m a.s.l. and develops for about 2800 m in a tectonic contact between Permotriassic meta-volcanics and middle Triassic carbonate rocks and dolostone of the Dolomie di San Pietro dei Monti formation (Antonellini et al., 2019). Bossea cave was opened to the public in 1874, making it the first show cave of Italy; today receives about 18000 tourist/year. The cavity is crossed for about 1.5 km by a subterranean collector (Mora River), with a 50 to 1200 L/s flow rate which directly flows in the Corsaglia River. Different *Ursus spelaeus* bones were found in this cave and are exposed in the central saloon.

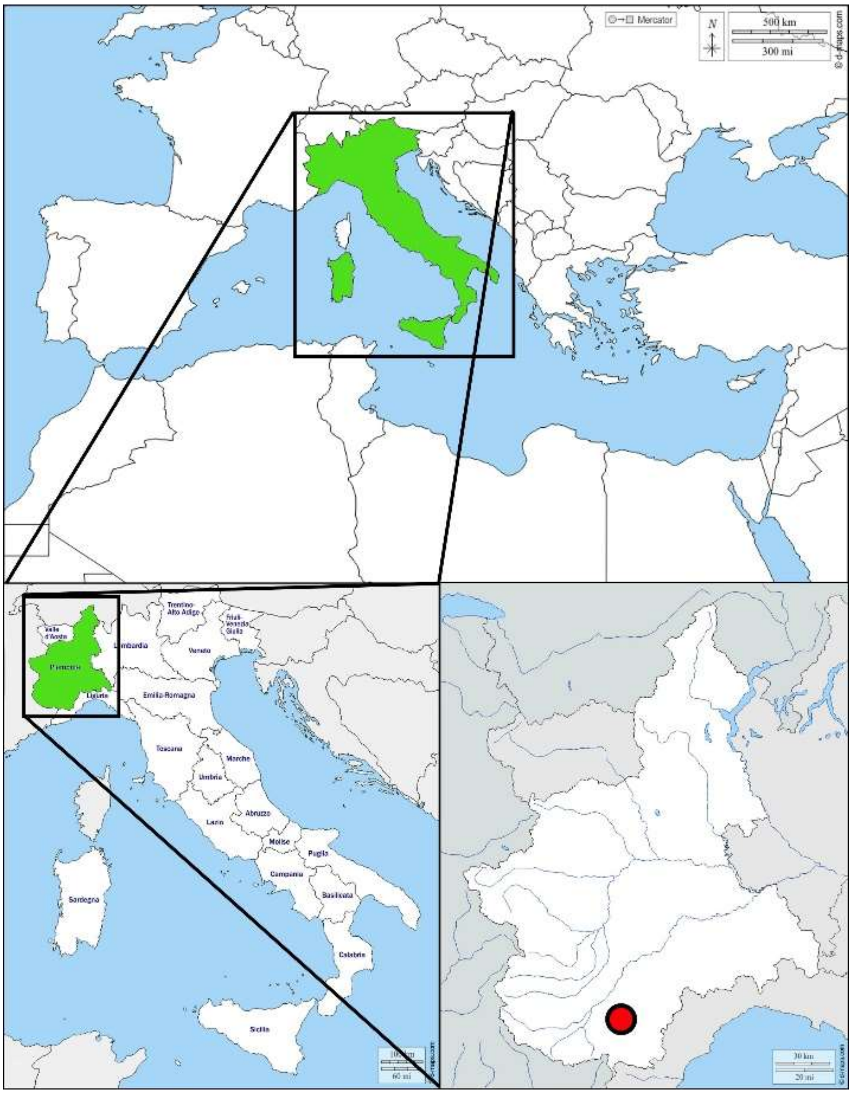

Fig. 23. Location of the study area, SW Piedmont (Italy). (Maps used for the plate and modified, retrieved from: https://d-maps.com/carte.php?num_car=3126&lang=en, https://d-maps.com/carte.php?num_car=4828&lang=it and https://d-maps.com/carte.php?num_car=8256&lang=en). Image from Balestra et al. (2023).

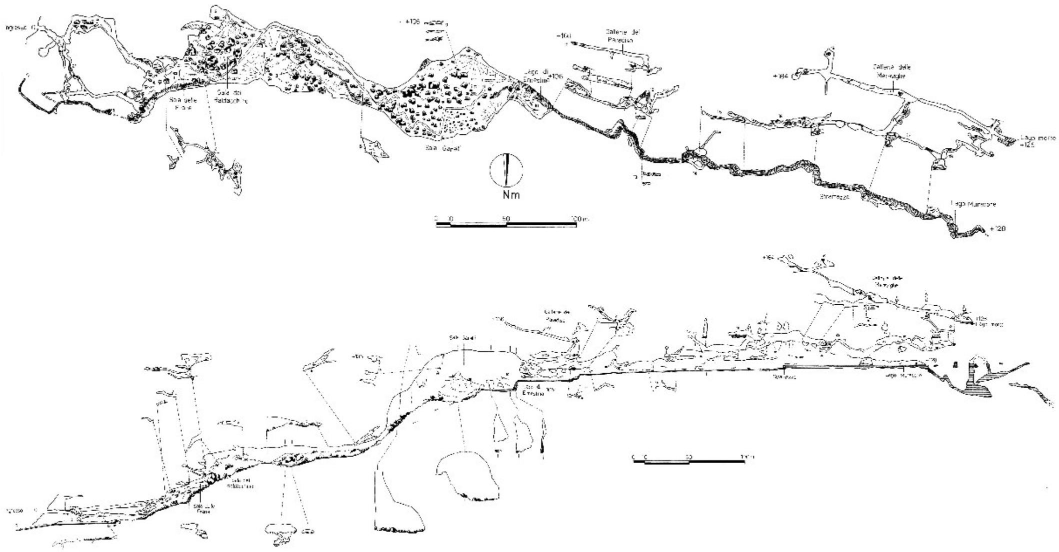

Fig. 24. Bossea cave. Survey by Elia and Calleris (1988).

Fig. 25. Bossea cave waterfall, at the end of the tourist path. Photo: V. Balestra

Several underground karst laboratories are located in Bossea cave to study radon activity (Peano et al., 2011), subterranean biology (Balestra et al., 2022b; Lana and Balestra, 2020), hydrogeology (Balestra et al., 2022a; Fiorucci et al., 2015; Vigna, 2020) and hypogeal meteorology (Balestra et al., 2021), managed by Struttura

Operativa Bossea CAI (Peano and Fisanotti, 1994), Biologia Sotterranea Piemonte – Gruppo di Ricerca, and the DIATI (Politecnico di Torino), working together with ARPA Piemonte and INRIM.

The Bossea karst system feeding area is characterized by a belt of carbonate rocks laterally confined by poorly permeable rocks (quartzites and meta-volcanics) (Fig. 26). Bossea cave represents the final part of the karst system developing in the Maudagna-Corsaglia watershed, between the Prato Nevoso basin and the Corsaglia River (Fig. 26).

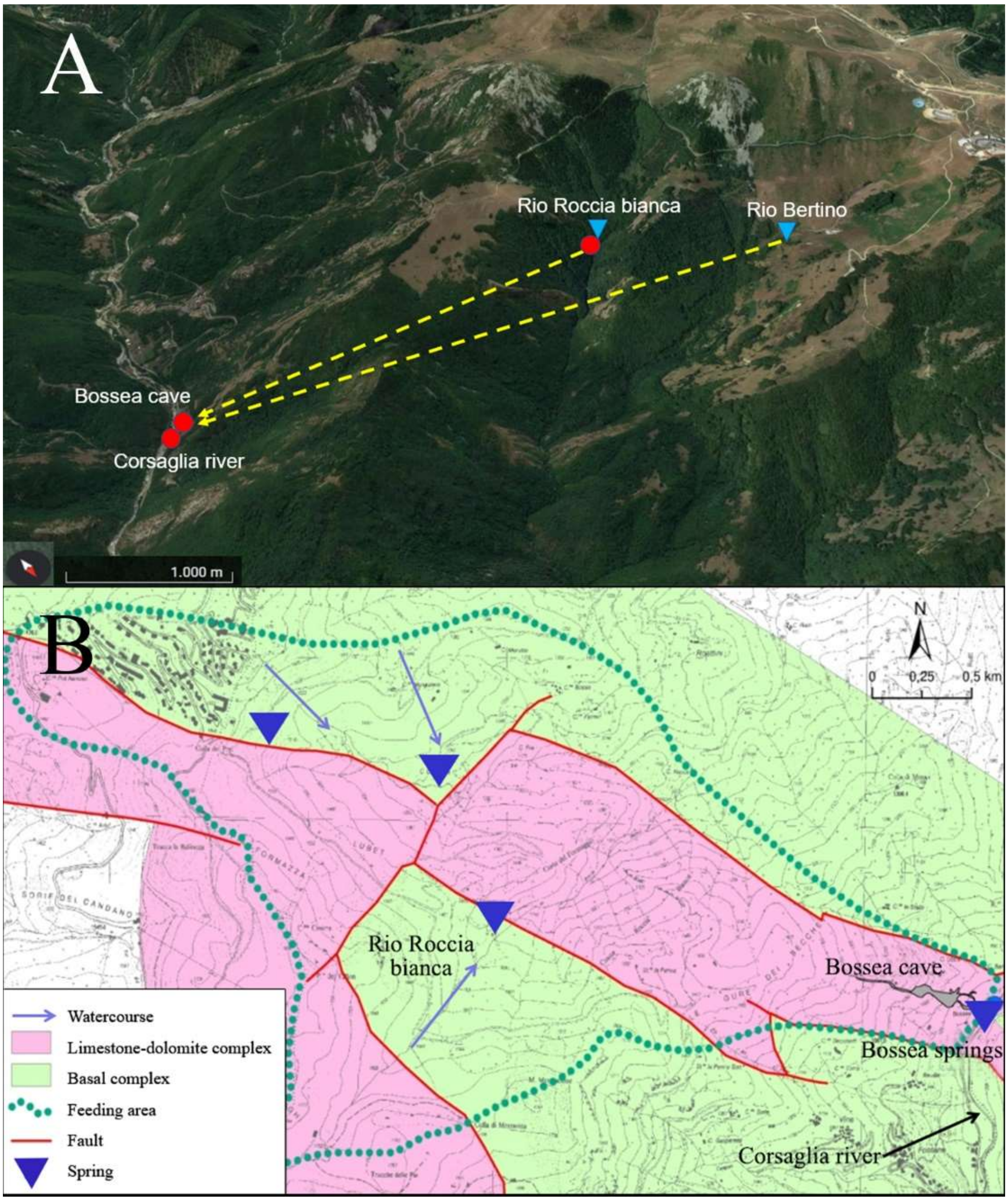

Fig. 26. Bossea karst system. A: Turquois triangles for the main surface water supply (Rio Roccia bianca and Rio Bertino), red circle for sampling areas, yellow arrow for the results of the staining tests carried out previously: the waters of the secondary supply contributing the Bossea karst system recharge (Rio Roccia bianca e Rio Bertino) trough sinkholes in the sub-riverbed, ending up in the collector of the Bossea Cave. Map from https://earth.google.com [accessed: 07/06/2022], modified; B: Bossea karst system feeding area. Map from Vigna (2020), modified. Image from Balestra et al. (2023).

The recharge of this aquifer (Fig. 26B) is mainly linked to primary and/or secondary inputs: the primary inputs (or authigenic) are by rain or snow melt, directly recharging the aquifer, whereas the secondary inputs (or allogenic) of concern are the surface runoff waters continuously recharging the aquifer trough sinkholes in the sub-riverbed (Vigna, 2020). Rio Roccia bianca is the main secondary supply contributing to the Bossea karst system recharge (Fig. 26). A series of tests were previously carried out in the two main absorbent valleys (Rio Roccia bianca and Rio Bertino) (Vigna, 2020), using two different dyes (Tinopal and fluorescein), to examine their arrivals in Bossea Cave. This aquifer has a water circulation along karst conduits and fractures, with an extensive and well-developed saturated zone, and can therefore be described as a system with interconnected drainage, with a medium-high permeability (Banzato et al., 2011).

Bossea Cave offers an ideal situation to study the groundwater circulation in a carbonate rock mass: a main water collector, called Mora River, and several water supplies with different flow rates, related to rock fractures, are present (Fig. 26B). Continuous monitoring data carried out in Bossea Cave by the DIATI - Politecnico di Torino team - revealed that the collector has a flow rate ranging from 50 to 1,200 L/s, developing for about 1.5 km in the cave and directly flowing in the Corsaglia River, with a set of springs (Banzato et al., 2011; Fiorucci et al., 2015; Fiorucci and Vigna, 2015; Peano et al., 2011; Vigna et al., 2017). The discontinuities are poorly or not karstified, characterised by low flow circulation ranging from 0.01 to 2.5 L/s (pools of water or drips), such as Polla delle anatre, one of the most important monitoring points in the Bossea Cave, tracked for over 40 years, with a highly constant flow rate (0.5 to 2.5 L/s) (Balestra et al., 2022a; Vigna et al., 2017). Moreover, secondary inputs continuously recharging the aquifer were previously monitored, proving their arrivals in Bossea Cave and highlighting the surface and underground environment connections. Finally, as the hydrodynamic response to rainfall events is characterised by a rather rapid increase in flow (Banzato et al., 2011), the movement of pollutants from the surface to the underground environments could be rapid, making this type of karst systems more subject to MP pollution.

4A.1 Microplastic pollution in sediments

Origin of the Chapter

This chapter is adapted from the peer-reviewed article "Microplastic pollution in show cave sediments: first evidence and detection technique" authored by Balestra and Bellopede (2022b), published in Environmental Pollution, Volume 292, Part A, 1 January 2022, 118261, DOI: **https://doi.org/10.1016/j.envpol.2021.118261.**
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4.3 Introduction

Over the past decades, the interest in the underground karst environments and its natural wonders has grown remarkably, not only from the scientific viewpoint, but also from an economic perspective. Cavities transformed into show caves may be easily damaged, making it important to follow strict rules before, during and after development to maintain the aesthetic and scientific values of the caves (Cigna and Forti, 2013). Tourist caves are the most attractive natural features of geotourism, allowing more than 50,000 visitors/year in the major show caves of the world (Cigna, 2016; Cigna and Forti, 2013). However, human presence in these caves has led to several environmental issues such as pollution, cave climate change, corrosion of speleothems, lampenflora and variations in cave species abundance and distribution. Visiting the cave, tourists transport dust and lint (organic and synthetic fibres from clothing) into caves, covering and damaging speleothems and walls and providing an artificial food source for different species (Baker and Roberts, 2015; Chelius et al., 2009; Christman, 2019; Jablonsky et al., 1993; Pate, 1999). Lint can damage speleothems indirectly by providing nutrients for acid-producing organisms that can dissolve limestone (Jablonsky et al., 1993) and can be incorporated into the cave formation growth. Some lint fibre analyses gave a synthetic fibre content between 30 and 75% (Christman, 2019; Jablonsky et al., 1993). Moreover, only few works on lint in caves had been done until 2022 (e.g. Chelius et al., 2009; Christman, 2019; Jablonsky et al., 1993).

Assessing MP contamination in caves is crucial for different reasons: they can be ingested by cave animals, endanger the fragile ecosystems of the caves, irreversibly damage speleothems and paleontological or archaeological findings depositing on them and pollute karst aquifers. Therefore, the aims of this study were: i) to summarise a valid non-invasive and non-expensive analytical technique for the separation of MPs from cave sediments and their identification, highlighting the importance of organic matter removal, and ii) to investigate and discuss, for the first time, the abundance, shape and morphological characteristic of MPs in show cave sediments.

4.4 Method

Thanks to this research, the first standardization of the method for detecting MPs in cave sediments was done. For more detailed information about the methodology see Chapter 3.

4.4.1 Field sampling

Five superficial sediment samples (upper 5 cm) were collected near the tourist paths, in different 1x1 m areas of the cave (Fig. 27). One sample was taken in a non-touristic cave zone, visited only by researchers and speleologists, used also to reach the Secondary Lab (Fig. 27). About 300 g of superficial sediment for each sampling area were collected with a metal spoon and placed in glass boxes. For more detailed information about sampling method see Chapter 3.

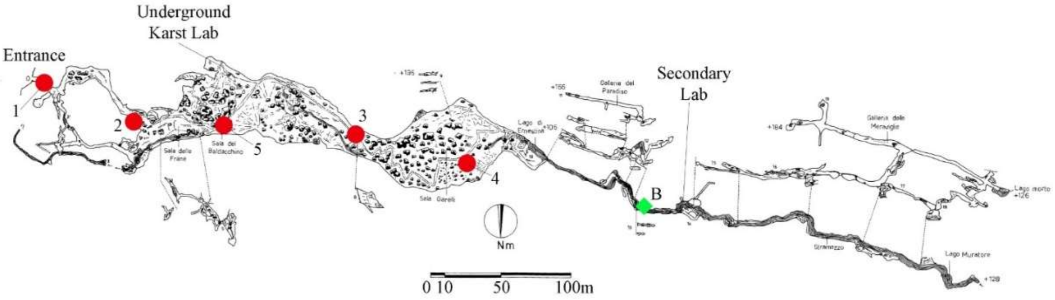

Fig. 27. Bossea Cave sampling areas. Red circles for microplastic sampling in the touristic zone, green turbot for microplastic sampling in the speleological tract to reach the Secondary Lab. Survey by Elia and Calleris (1988), modified. Image from Balestra and Bellopede (2022b).

4.4.2 Microplastic identification

During this research, only microscopy was possible in our laboratory. Therefore, microparticles on filters were observed alongside with and without a UV flashlight under a Leitz ORTHOLUX II POL-MK microscope equipped with a DeltaPix Invenio 12EIII 12 Mpx Camera. MPs were counted and characterized according to strict selection criteria to avoid misidentification (Crawford and Quinn, 2016;

Hidalgo-Ruz et al., 2012; Noren, 2007). A cut off of the particles smaller than 0.1 mm was made as suggested in European Commission (2013) and particles that could not be reliably identified as MPs were not take into consideration.

All counted MPs were described using the standardised size and colour sorting system (SCS) (Crawford and Quinn, 2016). X-sectional and longitudinal microscopic image of natural, regenerated and synthetic fibres were used for helping in MFs identification (Khan et al., 2017).

4.5 Results and discussion

4.5.1 First evidence

Overall, 707 MPs were counted on 18 filters, 15 from the sediments of the touristic area and 3 from sediments collected in the speleological traits of the cave that contain the internal laboratory. MPs were found in all sediment samples, non-touristic traits included. However, the non-touristic sediment sample had about one third of the MP average value found in the samples of the tourist section of the cavity. The MP abundance, their shape and fluorescence are shown in Fig. 28 and Table 2.

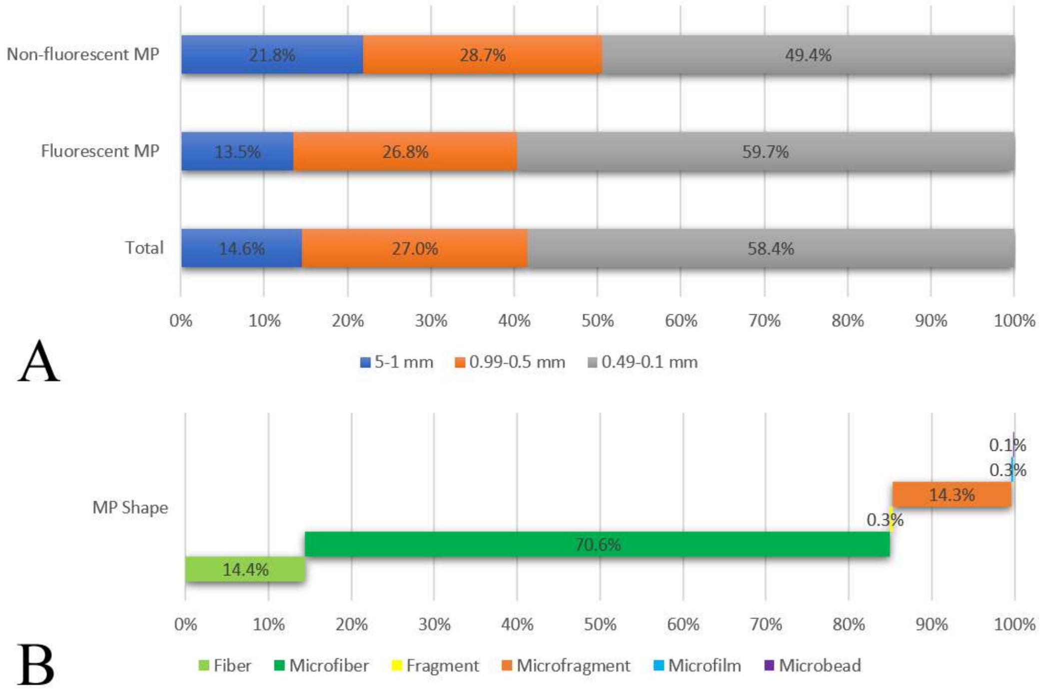

Fig. 28. Microplastic size and shape found in Bossea cave sediments. A: size percentages. B: shape percentages. Image from Balestra and Bellopede (2022b).

Table 2. Abundance, shape and fluorescence of the analyzed microplastics found in Bossea cave sediments.

Filter	MP abundance	Fluorescent MPs	Non- Fluorescent MPs	Fibre	Microfibre	Fragments	Microfragments	Film	Microfilm	Pellet	Microbeads	Foam	Microfoam
1.1	46	43	3	4	41	0	1	0	0	0	0	0	0
1.2	35	30	5	3	32	0	0	0	0	0	0	0	0
1.3	51	46	5	12	39	0	0	0	0	0	0	0	0
2.1	38	30	8	3	34	0	1	0	0	0	0	0	0
2.2	20	14	6	5	14	0	0	0	1	0	0	0	0
2.3	31	30	1	1	30	0	0	0	0	0	0	0	0
3.1	31	29	2	5	26	0	0	0	0	0	0	0	0
3.2	33	23	10	11	19	0	2	0	0	0	1	0	0
3.3	38	36	2	11	27	0	0	0	0	0	0	0	0
4.1	54	48	6	4	41	0	9	0	0	0	0	0	0
4.2	150	143	7	5	90	2	54	0	0	0	0	0	0
4.3	57	51	6	2	27	0	28	0	0	0	0	0	0
5.1	28	24	4	5	23	0	0	0	0	0	0	0	0
5.2	24	18	6	6	17	0	1	0	0	0	0	0	0
5.3	23	20	3	6	17	0	0	0	0	0	0	0	0
B1	13	11	2	6	4	0	2	0	1	0	0	0	0
B2	23	16	7	10	10	0	3	0	0	0	0	0	0
B3	12	8	4	3	8	0	1	0	0	0	0	0	0
TOT	708	620	87	102	499	2	102	0	2	0	1	0	0
%	100	87.7	12.3	14.4	70.5	0.3	14.4	0.0	0.3	0.0	0.1	0.0	0.0

Fibres (Fig. 29A, B, C, D) represented the majority of the MPs present in the cave sediments, respectively 14.4% between 1 and 5 mm and 70.6% between 0.1 and 0.99 mm of length, followed by fragments (14.3% microfragments and 0.3% fragments) (Fig. 29 E, F). Some microbeads (0.1%) and microfilms (0.3%) were also observed, whereas no foam was found in cave sediments.

As Bossea Cave is located in a relatively untouched valley, with only a small mountain village, we suggest that the MPs discovered in the cave are mainly originated from the daily tourist activities in the show cave. Globally, more than 60% of all textiles were produced from synthetic fibres (Henry and Klepp, 2018), and therefore, the shedding of fibres from clothes could be the sources of the MPs discovered in the cave. Fragments were also present in Bossea cave sediments, especially in sampling area 4. Probably, MP fragments were produced during the electric system works, near the sampling area.

Figure 28A shows the different sizes of the collected MPs. According to Crawford and Quinn (2016), MPs are categorised in two different classes: microplastics (1–5 mm) and mini-microplastics (1 mm–1 µm). In this investigation, mini-MPs accounted for 85.4% of the total MPs found in cave sediments, of which 58.4% were shorter than 0.5 mm.

The average MP abundance of each sampling area and their different types are shown in Table 3. The abundance of microplastics along the tourist path varied from 2,500 to 8,700 items/kg. The lowest MP abundance in the cave sediments was found in sampling site 5. The number of MP particles was higher in sampling areas 1 and 4, the monitoring zones that were nearest and furthest to the cave entrance. These data could be related to the air circulation in the cave, which is stronger near the entrance because of air exchange with the outside and the narrowing of the vessel, and near the end of the touristic traits because of the presence of the waterfall and the narrowing of the cave. Otherwise, these data could be related to the time of tourist visits to the cave. Probably, these results are linked to all of these and other factors, which should be studied in more detail to better understand the MP pollution in caves. In the cave sediment sample taken from the non-touristic but speleological area, we found a mean of 1,600 items/kg.

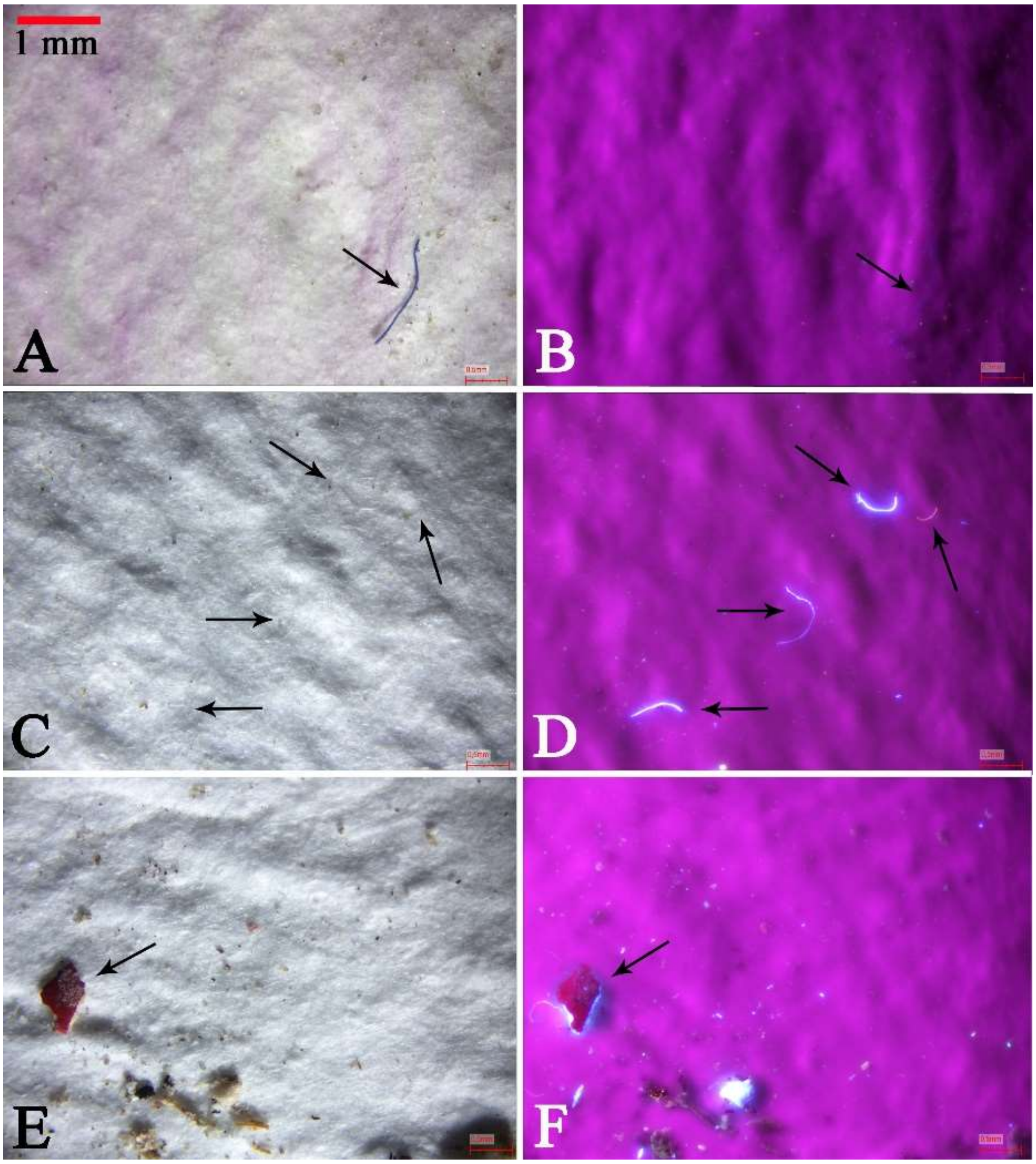

Fig. 29. Microparticles on filters observed with and without a UV flashlight under a microscope. A-B: blue fibre non-fluorescent under UV light. C-D: transparent fibres, fluorescent under UV light. E-F: red fragment visible with and without UV light. Image from Balestra and Bellopede (2022b).

Table 3. Microplastic abundance in cave sediments and their shape [items/10 g of sediment].

Area	MP abundance average	Fibre	Microfibre	Fragment	Microfragment	Film	Microfilm	Pellet	Microbead	Foam	Microfoam
1	44.0	6.3	37.3	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0
2	29.7	3.0	26.0	0.0	0.3	0.0	0.3	0.0	0.0	0.0	0.0
3	34.0	9.0	24.0	0.0	0.7	0.0	0.0	0.0	0.0	0.0	0.0
4	87.0	3.3	52.7	0.7	30.3	0.0	0.0	0.0	0.0	0.0	0.0
5	25.0	5.7	19.0	0.1	3.9	0.0	0.0	0.0	0.0	0.0	0.0
Not touristic	16.0	6.3	7.3	0.0	2.0	0.0	0.3	0.0	0.0	0.0	0.0
Touristic path	43.9	5.5	31.8	0.1	7.1	0.0	0.1	0.0	0.0	0.0	0.0

Comparisons with other cave sediment abundances were not possible during this research, being the first on cave sediments. Moreover, a standardized method for assessing MPs in sediments did not exist. However, despite different measurement methods and treatments greatly affect the results, some considerations about MPs abundance in sediments of different environments could be equally done. The MP abundance values found in the show cave were similar to those found in coastal marine sediments of China (5,020 to 8,720 items/kg) (Qiu et al., 2015) or in the intertidal zone of beaches along Nova Scotia (2,000 to 8,000 items/kg) (Mathalon and Hill, 2014). However, MP values in this work were high compared to those found in the Lagoon of Venice sediments (672 to 2,175 items/kg) (Vianello et al., 2013), in Scapa Flow, Orkney (730 to 2300 items/kg) (Blumenröder et al., 2017), or in marine and freshwater sediments from different parts of the world (30–1,900 items/kg) (e.g. Ballent et al., 2016; Matsuguma et al., 2017; Phuong et al., 2018; Tsang et al., 2017). This fact could be linked to the confined cave environment, which, in many areas, is hardly influenced by air currents or water flows, favoring MP deposition.

The highest MP abundance was fluorescent under UV light (87.7%) (Fig. 29D, F), while 12.3% were not fluorescent (Fig. 29B). Fluorescent MPs can be photographed and automatically quantified by a counting software, limiting subjectivity in the quantification (Prata et al., 2019). Therefore, different counting software packages will be tested in the future to better represent the percentages of MPs in show caves.

Figures 30 and 31 showed the different colours of the collected microplastic fibres. Of the fluorescent fibres (Fig. 30), 84% were transparent, followed by beige (4.6%), blue (3.6%), pink (1.9%) and red (1.5%) ones. Fibres with other colours accounted for less than 1% of the total fluorescent fibres. Non-fluorescent MP fibres (Fig. 31) were mainly blue (46.1%) or black (22.4%), followed by grey, brown and red ones (between 5 and 7%). The colour of microplastics often provides an indication of the chemical pollutants which contaminated them. Different researchers found high levels of pollutants on yellow and black microplastics (e.g. Frias et al., 2010; Karapanagioti et al., 2011). In Bossea cave, about a fifth of non-fluorescent fibres found is black, therefore, further investigations should be carried out in the future. Moreover, the colour of microplastics can be correlated with the consumption by organisms (e.g. Carpenter et al., 1972; Romeo et al., 2015), being confused with natural food. In natural caves there is no light, therefore, the colour or fluorescence of MP could be not of particular interest for cave animal ingestion. However, the colour could be interesting for the external fauna, especially aquatic organisms, which could be subjected to contamination by MPs transported from the inner stream of the cave.

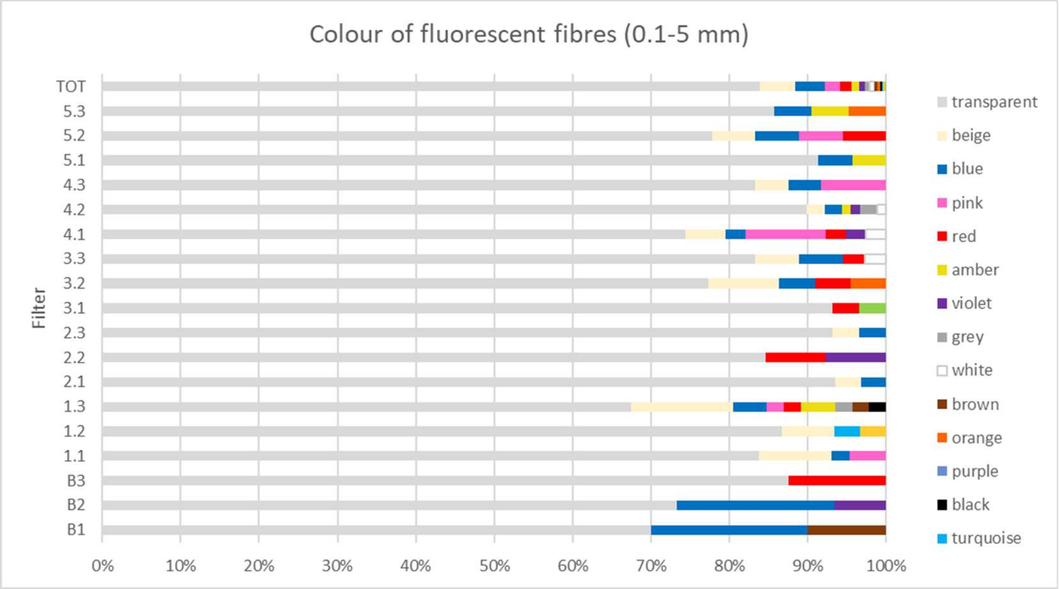

Fig. 30. Colours of fluorescent fibres from sediments sampled in different Bossea Cave areas. Image from Balestra and Bellopede (2022b).

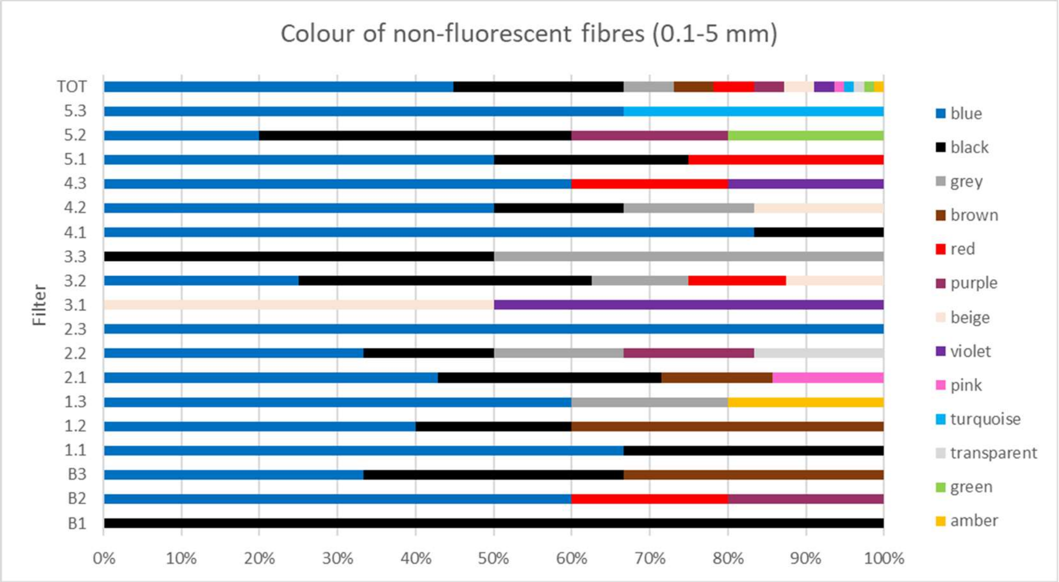

Fig. 31. Colours of non-fluorescent fibres from sediments sampled in different Bossea Cave areas. Image from Balestra and Bellopede (2022b).

The deterioration of the fibres depends on a combination of factors as biodeterioration, temperature, pH and relative humidity of the environment, moisture, reactivity and nature of the material and its progressive natural ageing (Carr, 2017; Szostak-Kotowa, 2004). Biological attack is caused by bacteria and fungi activities, insects and vertebrates (Carr, 2017). The synthetic fibres are generally considered to be resistant to bacteria, mould and mildew activities, however, certain treatments used on textile products, staining with organic material or blending with a more susceptible fibre can promote growth (Szostak-Kotowa, 2004). Moreover, mould and mildew grow increases in higher relative humidity environments (Szostak-Kotowa, 2004). Microbial growth causes loss of strength, elongation and discolouration (Szostak-Kotowa, 2004). Therefore, the high number of transparent fibres found in Bossea cave could be related to biodeterioration, given also the high relative humidity of the cave environment.

Bossea cave is rich in troglobitic and endemic fauna, and MPs can be ingested by cave animals and endanger the fragile ecosystems of the caves. Geologic features are the primary attraction of the show cave, and MPs can irreversibly damage speleothems deposited on them and on the paleontological remains. MPs can directly damage speleothems, being incorporated into the cave formation growth, sometimes colouring them, or indirectly, by providing nutrients for acid-producing organisms that can dissolve limestone (Jablonsky et al., 1993). Moreover, the cavity is crossed by a subterranean river that directly flows in the Corsaglia River, and MPs can pollute karst aquifers. It must be considered that karst aquifers are open systems, even susceptible to contamination by surface pollutants, and therefore, the areas above the caves must also be monitored.

4.6 Conclusion

This study documented the presence of MPs in the examined show cave, filling a gap in the study of microplastic pollution, providing useful references for further research. A valid non-invasive and minimally manipulative technique to separate and detect MPs in cave sediments was tested. This method is eco-friendly, incurs low costs, and the equipment is easily obtainable. From the analysis on cave sediments, fibre-shaped and mini-microplastics dominated the samples, suggesting that synthetic clothes of visitors are the main source of MP pollution in cave. MPs in cave could contaminate potable water, polluting watercourses and nearby environments and irremediably damage speleothems and cave ecosystems. The methodologies that enable the detection of MP contamination are crucial to understand the gravity of the problem and define strategies for cave conservation. Substantial efforts must be made to protect caves resources, implementing new strategies and providing education. Cave conservation should become a priority for the management of the cave resources.

4A.2 Preliminary investigations of microplastic pollution, from surface watercourses to cave waters

Origin of the Chapter
This chapter is adapted from the peer-reviewed article "Preliminary investigations of microplastic pollution in karst systems, from surface watercourses to cave waters" authored by (Balestra et al., 2023), published in Journal of Contaminant Hydrology, Volume 252, January 2023, 104117, DOI: **https://doi.org/10.1016/j.jconhyd.2022.104117**
The author of this dissertation was responsible for conceptualisation, methodology, validation, formal analysis, investigation, resources, data curation, writing – original draft, writing – review and editing, visualisation.

4.7 Introduction

Regarding MP pollution in freshwater resources, research is still in its early stages. During the research period, the potential contamination of groundwater was often only mentioned, and a limited number of studies focused on groundwater MP pollution (e.g. Khant and Kim, 2022; Mintenig et al., 2019; Panno et al., 2019; Samandra et al., 2022; Selvam et al., 2021; Viaroli et al., 2022). Moreover, the invisibility of the subterranean karst system structure makes difficult the study of such systems, even if they preserve a precious asset such as groundwater.

MP contamination of groundwater originates from multiple sources, including wastewater, litter, atmospheric deposition, and polluted soils and surface waters. Atmospheric transport plays a key role in long-distance MP dispersion, influenced by wind patterns and precipitation dynamics (Allen et al., 2019). In the hydrogeological cycle, atmospheric water contribution is the main aquifer recharge. The presence of MPs in precipitation was detected in cities and remote areas (Allen et al., 2019; Liu et al., 2019b), highlighting the omnipresence of this contaminant. Precipitation-induced deposition facilitates the infiltration of MP into soils (Zhou et al., 2021), with smaller particles migrating downward through pores and fractures (Fahrenfeld et al., 2019; McGechan, 2002; Viaroli et al., 2022), or redistributed via bioturbation (Lwanga et al., 2017), accumulating in the hyporheic zone (Frei et al., 2019) and reaching groundwater system (Chia et al., 2021; Wanner, 2021). Hydrogeological settings, aquifer types and seasonal flow regimes significantly influence MP transport and need to be considered when designing sampling strategies and selecting detection methods.

Karst aquifers are groundwater environments usually made up of carbonate rock, constituting about 25% of the global drinking water sources (Panno et al., 2019). The hydrodynamic regime of these aquifers is affected by the geology and the rock cluster fracturing, the karstification degree and the local meteorological conditions (Braun, 1984; Chauve et al., 1990; Hottelet et al., 1993; Moindrot et al., 1988).

Besides being important drinking water resources, karst ecosystems and waters are particular habitats for troglobitic and endemic species (e.g. Balestra et al., 2022b; Barzaghi et al., 2021), which may be vulnerable to pollution (Manenti et al., 2018; Sket, 1999), and karst caves have an exceptional scientific value (Balestra et al., 2021; Cigna and Forti, 2013).

The open nature of karst aquifers makes them vulnerable to contamination by surface pollutants, which can be transported through the rock fractures (White, 1988) or can directly access the karst systems through some caves, especially tourist ones, increasing the pollution risk (Balestra and Bellopede, 2022b; Balestra et al., 2021). Fractured and karst aquifers are subjected to greater pollution risk as MPs are prone to circulate throughout the discontinuities, even if mechanical dispersion may attenuate the contaminant concentration in some zones (Viaroli et al., 2022).

Before this research, only one study on MP pollution was done in waters of karst systems, monitoring springs and wells under low-flow conditions (Panno et al., 2019). Studies on cave waters, as well as MP pollution in surface and underground waters of the same karst area, were lacking. Therefore, investigations were required for understanding the system dynamics and monitoring the state of the environmental health, detecting the possible pollution and its sources. The aims of this study, which was the first of its kind, were therefore i) to preliminarily investigate MP pollution in karst systems, from surface watercourses to cave waters, to provide a reference for further research, and ii) to discuss the abundance, morphological characteristic and types of MPs in the karst system.

4.8 Method

4.8.1 Field sampling

For data collected in flowing water, it is essential to take into account the water flow rate to evaluate the correct MP percentage, which clearly depends on the water flow. Moreover, the season could affect the concentration of MPs in the water. Hence, sampling at different times could be performed to understand the impact of the season. However, these first samples taken punctually in the karst system are important to verify the presence of MPs in surface and subterranean waters; these data are scarce in karst areas and absent in caves. If MPs occur in the system, subsequent analyses can be carried out to verify the impact of the seasons.

Sampling points were chosen to check pollution, starting from the surface water streams that recharge the karst system examined up to the springs, passing through the underground structures that accumulate in the water reserve and, therefore, can retain and/or transport pollutants.

One surface water sample was taken from Rio Roccia bianca, upstream the Bossea cave (Fig. 26), four groundwater samples were collected from different areas of the Bossea cave (Figs. 32, Table 4), and one surface water sample was taken from the Corsaglia River, where the water of the collector emerges (Fig. 26). In the Bossea cave, two samples were collected nearer to the tourist path (Uovo and Sala frane) and two in different non-tourist areas (Sifone and Polla delle anatre) (Fig. 32, Table 4) as reference samples of unpolluted waters because these areas are not frequented by tourists or in close contact with human activities. In the tourist section, the collector water flows from the innermost areas towards the entrance of the cave, where the Sala frane sampling area is located. In this zone, the collector water flows quickly before leaving the cave.

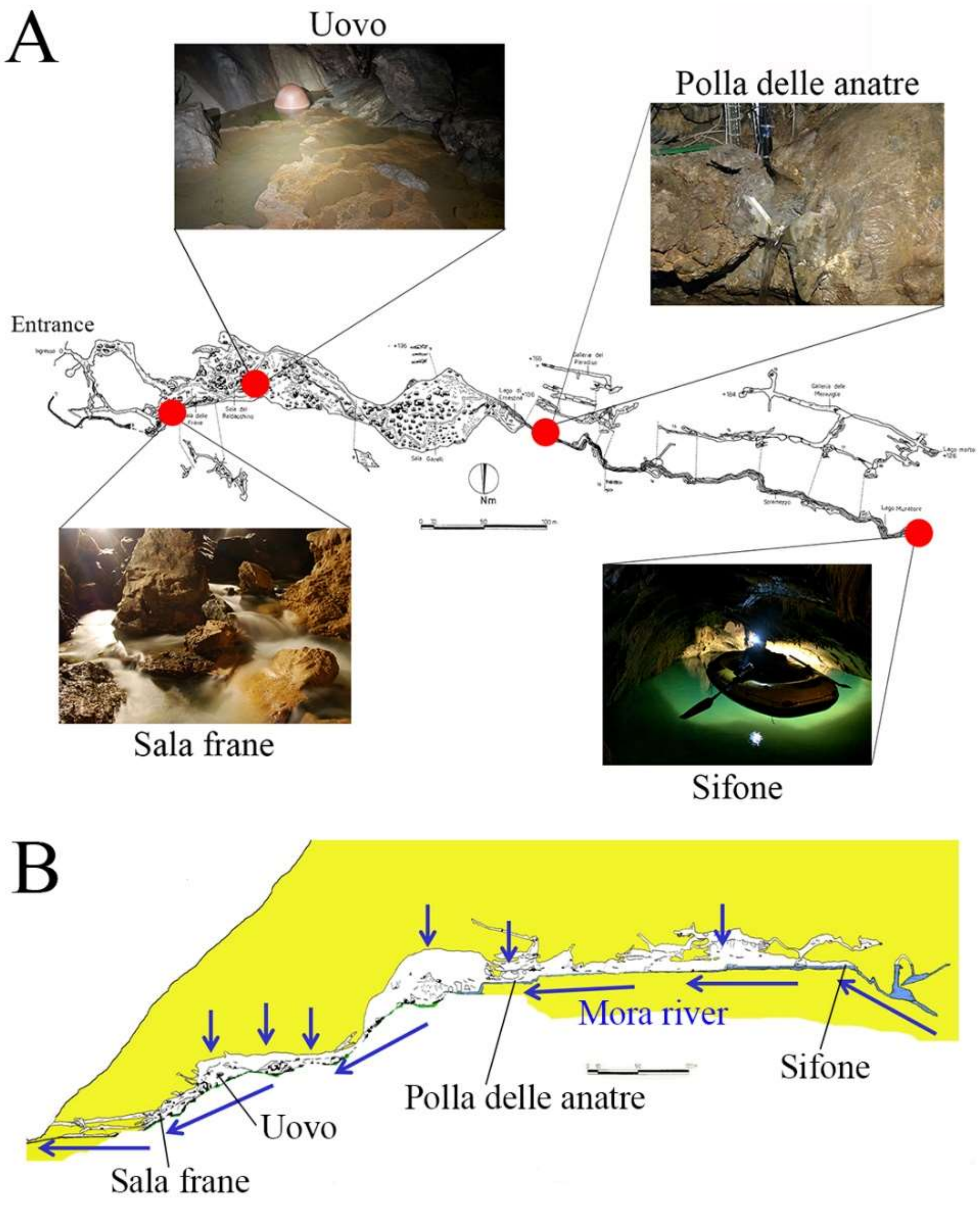

Fig. 32. Bossea cave. A: Bossea Cave sampling areas. Red circles for water sampling. Survey (plan) by Elia and Calleris (1988)), modified. Photos by V. Balestra and B. Vigna; B: Bossea Cave displacement of water with sampling areas. Blue arrows for primary and secondary water intakes. Survey (section) by Elia and Calleris (1988), modified). Image from Balestra et al. (2023).

Groundwater and surface water samples were collected in September 2021 under low-flow conditions. Being a preliminary investigation, water samples were collected into 1-L glass vessels and capped immediately to prevent atmospheric contamination. For more detailed information on sampling method see Chapter 3.

Table 4. Bossea cave sampling area characteristics.

Sampling area	Distance from the entrance	Sampling site type	Water characteristics	Flow rate	Tourist/non-tourist area
Sifone	100 m	Spring	The water comes out from the rock, from below, in a siphoning section, and accumulates in a lake	-	Non-tourist area
Polla delle anatre	150 m	Small pool of water entering in the inner collector	The water flows out of a fracture in the rock and is collected in a small tank with a weir for flow monitoring. The flow has the typical characteristics of an interconnected drainage system	0.5 to 2.5 L/s	Non-tourist area
Uovo	400 m	A small amount of the collector's water conveyed by speleothems and landslides along secondary paths	The water flows slowly between different gours	-	Tourist area
Sala frane	700 m	Collector	The water flows quickly	50 to 1,200 L/s	Tourist area

4.8.2 Laboratory analysis and identification

MP samples were processed according to the previously published method for cave sediments (Balestra and Bellopede, 2022b), adapted for the liquid matrix and improved to determine the particles using the IR spectroscopy. For more detailed information on the used methodology see Chapter 3.

Each water sample was filtered through an 0.8-μm pore size silver filter (GVS Life Sciences, Membrane Disk 47 mm) to allow a better analysis by IR spectroscopy. Dried filters were subjected to OMR through the application of 2 mL of 15% hydrogen peroxide solution, left to react for 30 min at room temperature and dried again for 2 h at 50°C.

MPs on filters were observed alongside with and without an UV flashlight under a Leitz ORTHOLUX II POL-MK microscope equipped with a DeltaPix Invenio 12EIII 12 Mpx Camera. Particles smaller than 0.1 mm were cut off. Particles that could not be identified as MPs were not take into consideration. Randomly, 10% of MPs of each filter were analyzed using a μFTIR, in reflection mode. For more detailed information on microscopic and spectroscopic techniques used, see Chapter 3.

4.9 Results and discussion

MPs were found in all water samples, highlighting MP pollution in the Bossea karst system, from surface watercourses to underground waters. Although for data collected in flowing water, it is essential to take into consideration the water flow rate and the season to evaluate the correct MP abundance over time, this first investigation in the karst area is important because it highlights the presence of MPs in the surface and underground waters, even in areas not directly affected by human activities. Taking into account the continuous monitoring data carried out in the Bossea cave by the DIATI, it is possible to associate the number of MPs to the Bossea collector (70 L/s) and Polla delle anatre (0.5 L/s) flow rate in September 2021.

4.9.1 Microplastic abundance

MPs were found in all water samples, including the two samples collected in the non-touristic areas of the Bossea cave. Microplastic abundance, shape, dimension and fluorescence are shown in Table 5. The concentration of MPs in cave water varied from 12 to 54 items/L, with a mean of 28 items/L. The MP abundance in the surface water of Rio Roccia bianca was 23 items/L, whereas in the Corsaglia River, it was 29 items/L (Table 5).

Comparisons with other show cave water MP abundances were not possible at the beginning of this research, because this study was the first one dealing with cave waters; however, a comparison with other groundwater and surface water samples from different areas of the world was possible (Table 6). The only work on a karst area made before this research, monitoring springs and wells from two karst aquifers in Illinois, USA, documented the presence of a maximum MP concentration of 15.2 items/L (mean of 7.9 items/L) in springs and a maximum concentration of 4.4 items/L (mean of 2.8 items/L) in wells, under low-flow conditions (Panno et al., 2019). Selvam et al. (2021) analyzed 24 groundwater samples from wells and bore wells, collected in the post-monsoon season in coastal south India, pointing out a median concentration of 4.2 items/L, with a maximum concentration of 10.1 items/L. Other values are reported in Mintenig et al. (2019) for NW Germany groundwater, with 5.6 items/L. Much higher values were found in an alluvial unconfined groundwater aquifer in Victoria, Australia, with an average concentration of 38 ± 8 items/L (Samandra et al., 2022). Very low values (1 items/L) were found in chalk and sandstone aquifers of the UK (Johnson et al., 2020). Surface water samples in the same studied aquifers were analyzed by Selvam et al. (2021), showing 7.8 items/L, with a maximum of 19.9 items/L., whereas Mintenig et al. (2019) reported 2.9 items/L and Samandra et al. (2022) 0.4 items/L. The microplastic abundances found in our study are high compared to those found in subterranean and surface waters of Northwest Germany (Mintenig et al., 2019), Illinois, USA (Panno et al., 2019) or South India (Selvam et al., 2021), whereas they were lower than those detected in groundwaters examined in Victoria, Australia (Samandra et al., 2022). However, it should be considered that the size of MPs taken into account, the sampling point, the flow rate, the season and the sources of MPs in the catchment may impact the concentrations of MPs in waters.

Table 5. Abundance [items/L], shape, size and fluorescence of microplastics found in Bossea system waters. Shape and size were defined using the SCS Method (Crawford and Quinn, 2016).

Filter	MP	Fluorescent MPs	Non fluorescent MPs	Fibre	Microfibre	Fragments	Microfragments	Film	Microfilm	Pellet	Microbeads	Foam	Microfoam
Sifone	54	44	10	12	40	0	2	0	0	0	0	0	0
Polla delle anatre	12	12	0	2	8	0	1	0	0	0	1	0	0
Uovo	30	25	5	2	25	0	3	0	0	0	0	0	0
Sala frane	16	13	3	2	14	0	0	0	0	0	0	0	0
Cave total	112	82	30	18	87	0	6	0	0	0	1	0	0
%	100	73.2	26.8	16.1	77.7	0.0	5.4	0.0	0.0	0.0	0.9	0.0	0.0
Rio Roccia bianca	23	18	5	2	20	0	1	0	0	0	0	0	0
Corsaglia river	29	27	2	8	21	0	0	0	0	0	0	0	0
Karst system total	164	127	37	28	128	0	7	0	0	0	1	0	0
%	100	84.8	15.2	17.1	78.0	0.0	4.3	0.0	0.0	0.0	0.6	0.0	0.0

Table 6. Microplastic pollution comparison of groundwater and surface water samples from different areas of the world.

Reference	Country	Aquifer type	Groundwater sampling characteristics	Groundwater MP concentration	Surface water MP concentration
Mintenig et al. (2019)	Germany	-		5.6 items/L	2.9 items/L
Panno et al. (2019)	USA	Karst aquifer	Spring and wells Low-flow conditions	Springs: Max 15.2 items/L Mean of 7.9 items/L Wells: Max 4.4 items/ Mean of 2.8 items/L	-
Johnson et al. (2020)	UK	Chalk and sandstone aquifer	-	1 items/L	-
Selvam et al. (2021)	India	Costal aquifer	Wells and bore wells Post-monsoon season	Max 10.1 items/L. Median 4.2 items/L	Max 19.9 items/L 7.8 items/L
Samandra et al. (2022)	Australia	Alluvial unconfined aquifer		Mean 38±8 items/L Min 16 items/L Max 97 items/L	0.4 items/L

In non-tourist areas of the cave, the MP abundance in waters was similar to that in tourist areas, and a high number of MPs in the karst system water was found in the inner area of the cave, where the water comes out of a syphoning area. However, the Bossea cave sediments collected in tourist areas contained more than twice as many MPs as those collected in non-tourist areas, suggesting that pollution is related to the passage of tourists. The MP presence in cave water, including water coming out of the rock in the non-tourist sections (Sifone and Polla delle anatre), suggested pollution linked to hydrogeologic connections from the surface to the underground aquifers. The high presence of fractures in the rocks of this karst area could favour pollutant transport in the underground environment, as suggested in Panno et al. (2019). It is reasonable to assume that the high degree of karstification in different zones, the presence of a well-organised outflow network and the plenty water flows play a fundamental role in the transport of MPs in this karst system. The contact between water and sediments could increase the MP concentrations in the Bossea karst system water. MP pollution in the soil of this karst area could be related to the intense winter and summer activities in the zones above the karst system, and the area is rich in ski resorts (Prato Nevoso country). The presence of waste in these areas is not unusual, possible sources include plastic litter from anthropogenic activities and synthetic fibres from clothes. In these areas, surface waters that feed Rio Roccia bianca and Rio Bertino streams were collected, the main secondary supplies contributing to the Bossea karst system recharge (Vigna, 2020). A series of tests with dyes, previously carried out in the two main absorbent valleys, indicated that the waters coming from Rio Roccia bianca and Rio Bertino arrive in Bossea Cave in a relatively short time. During lean periods, the Rio Roccia Bianca waters arrive in the Bossea Cave collector after 4 days (speed of about 644 m/day), whereas in flood periods, they arrive after only 1 day (speed of about 2,460 m/day). Moreover, Rio Roccia bianca provides a much higher quantity of water to the Bossea systems than Rio Bertino water. Although currently, there are no studies on the atmospheric transport of pollutants in this area, soil pollution could be related to or aggravated by the atmospheric transport of particles depositing on the ground thanks to precipitation and wind as well as any other contamination between adjacent environments, linked to human activities from the near countries of Fontane and Prato Nevoso. In underground environments, tourists are carriers of alien particles such as lint, organic matter, dust and pollutants (Addesso et al., 2022; Balestra and Bellopede, 2022b; Jablonsky et al., 1993; Liu et al., 2021; Puławska et al., 2021), and therefore, waters in the tourist parts of the cave (Uovo and Sala frane) could be enriched with pollutants, such as MPs in cave sediments (Balestra and Bellopede, 2022b), which could be transported into different parts of the cave and outside, in the Corsaglia River. These data did not show a definitive link to the suggested sources of pollution but offered information about the hydrogeologic connections, showing opportunities for future research on MP dynamics in subterranean waters in karst environments.

4.9.2 Microplastic shape and size

MPs were categorized according to the SCS described in Crawford and Quinn (2016) (Table 5). Fibres represented the majority of the MPs present in cave and surface waters (17.1% between 1 and 5 mm and 78% between 0.1 and 0.99 mm of length), followed by fragments (4.3% micro-fragments) (Table 5 and Fig. 33). Some microbeads (0.6%) were also observed, whereas no foam and films were found in this karst system. As in previous research, fibres and fragments are the main shapes present in the groundwaters, albeit at different concentrations, such as 94% fragments (Samandra et al., 2022) or 100% fibres (Panno et al., 2019). In this work, fibre-shaped fragments dominated the samples. As more than 60% of the world textiles are produced from synthetic fibres (Henry and Klepp, 2018), synthetic clothes are the main source of MP pollution in this karst system. The fragment source could be linked to human activity on the surface or the presence of degraded waste in soil or could have been produced during the electric system works in the cave.

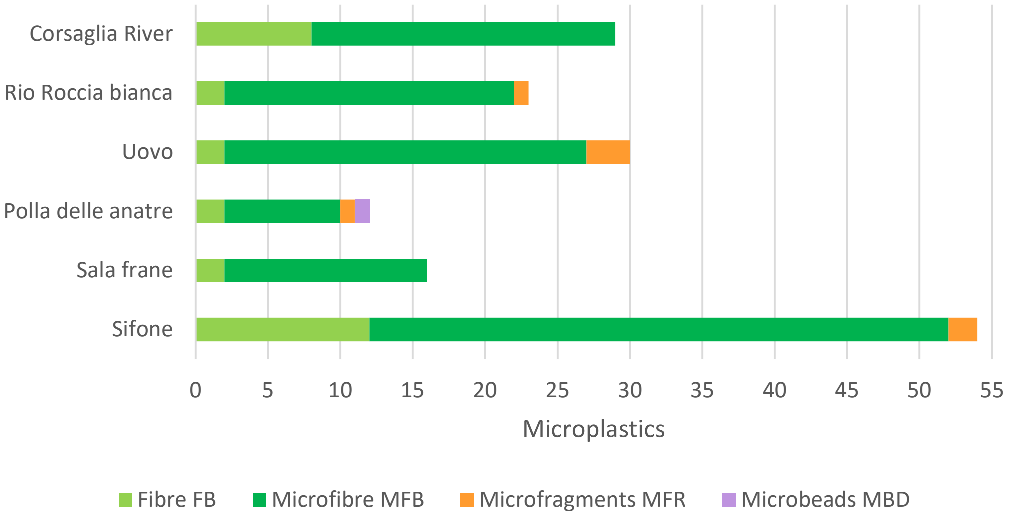

Fig. 33. Microplastic shape and size for the examined karst system waters. Image from Balestra et al. (2023).

According to Crawford and Quinn (2016), MPs were categorized into microplastics (1–5 mm) and mini-microplastics (1 mm–1 µm). Using visual identification under a microscope facilitated the analysis of MP particles from 5 to 0.1 mm. In this investigation, mini-MPs accounted for 82.9% of the total MPs found in the water karst system and for 84% of the MPs found in cave water only (Fig. 33 and Table 5).

The MP shape and size percentages found in Bossea cave waters were similar to those described for cave sediments (Balestra and Bellopede, 2022a, b). However, the means of transport and the source could be different. Microplastic found in the Bossea cave sediments could be mainly related to the passage of tourists along the cave path and the speleologists crossing the non-tourist passage to reach the internal laboratory (Balestra and Bellopede, 2022b). In contrast, the sources of MPs in the cave waters can be linked to cave tourism and pollution of the land above. The sources and transport of MPs in the cave should be studied in more detail to better understand MP pollution in karst areas.

As suggested in Panno et al. (2019), the relative size of MP particles offers some insight into their movement. The MPs found in underground waters in this study were similar to those collected in the surface waters. In this karst area, water migrates from the surface to the underground environments via sinkholes and rapidly enters the fractures and crevices of the underlying aquifers, reaching the cave and finally discharging into the Corsaglia River. Through the soil pores and along irregular fractures and crevices, the larger MPs would be impeded, and only the smaller MP fractions would reach and migrate through the karst aquifers.

4.9.3 Microplastic fluorescence and colour

The highest MP abundance was fluorescent under UV light (84.8%) (Fig. 34, Table 5), with a percentage similar to that found for MPs in cave sediments (87.7%) (Balestra and Bellopede, 2022a, b).

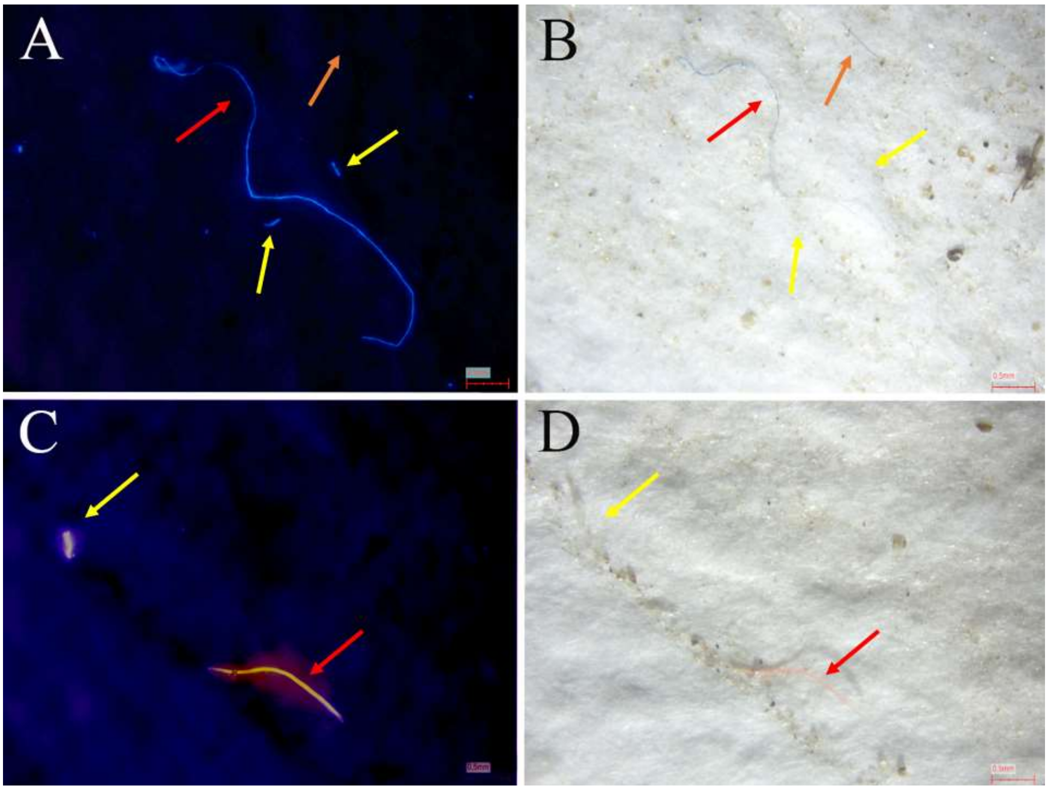

Fig. 34. Microplastic particles on filters observed under a microscope, with and without a UV flashlight. Yellow arrows for transparent microplastics, fluorescent under UV light, orange arrows for coloured microplastic, non-fluorescent under UV light, and red arrows for coloured microplastics, fluorescent under UV light. A–B: microplastic fibres; C–D: microplastic fibre and fragment. Image from Balestra et al. (2023).

Figure 35 showed the different colours of the collected MPs. Of the fluorescent particles (Fig. 35A), 46% were transparent, followed by red (16.5%), blue (10.8%), beige (7.2%) and amber (4.3%) ones. The MPs with other colours accounted for less than 4% of the total fluorescent ones. Non-fluorescent MPs (Fig. 35B) were mainly black (68%) or blue (20%), followed by green, transparent and violet ones (4%). The major part of the fluorescent particles in water are transparent, as reported in Balestra and Bellopede (2022b) for Bossea cave sediments. The other main colours are similar, with the exception of red particles, which are more abundant in water. The non-fluorescent MP particles were mainly black and blue, confirming the data found for Bossea cave sediments (Balestra and Bellopede, 2022b). However, the percentages were different, as well as the colour of the less abundant MPs. These facts could be related to the presence of particles with different densities, more easily transportable in water, with a different degree of biodeterioration or a diverse pollution source. Transparent MPs were also prevalent in groundwaters analyzed in Selvam et al. (2021), followed by white and blue ones, whereas in Panno et al. (2019), 65% of the analyzed MPs were blue and/or clear, whereas the other common colours were red (15%) and grey (13%).

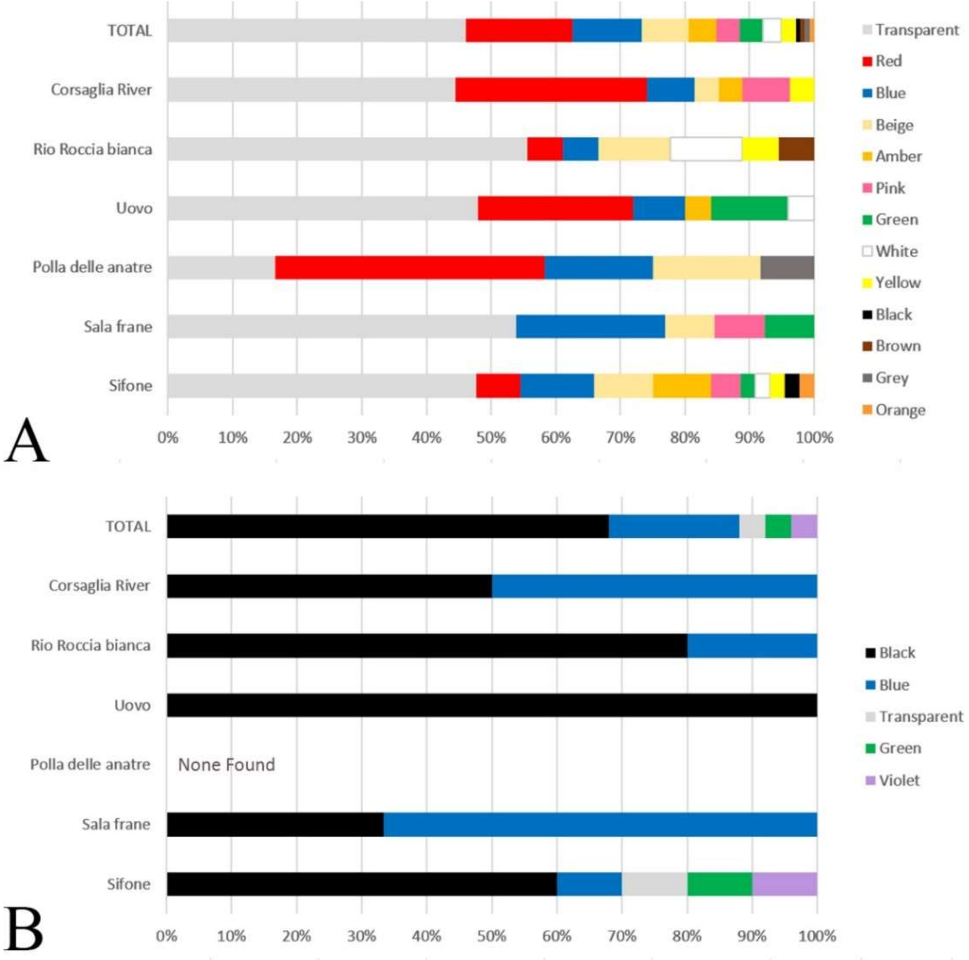

Fig. 35. Percentage of colours of microplastic particles on filters from water sampled in different Bossea karst system areas. Uovo, Polla delle anatre, Sala frane and Sifone were sampled in Bossea Cave. A: Percentages of colours of fluorescent microplastics. B: Percentages of colours of non-fluorescent microplastics. Image from Balestra et al. (2023).

The MP colour could provide an indication of the chemical pollutants; for example, yellow and black MPs could indicate high levels of pollutants (Frias et al., 2010; Karapanagioti et al., 2011). In the Bossea karst system, about 68% of non-fluorescent MPs were black, and future investigations should be carried out to monitor possible environmental contaminations. Moreover, the colour of MPs can be correlated with the consumption by organisms (Carpenter et al., 1972; Romeo et al., 2015; Shaw and Day, 1994), being confused with trophic resources. In natural subterranean systems, there is no light, and therefore, the colour of MPs does not play a particular role in hypogeal animal ingestion. However, the colour and fluorescence of MP particles may be relevant for less cave-adapted and epigeal organisms in watercourses and adjacent habitats.

4.9.4 Microplastic characterization

A set of several methodologies is the optimal choice in MP analysis, therefore, spectroscopy was selected to confirm the results obtained via visual counting under the microscope.

In this study, 10% of MPs in each filter was analyzed by micro-FTIR, taking random MP particles (Fig. 36). Polyethylene (51.4%) and polyvinyl alcohol (31.4%) were the main types of MPs found in the karst system waters, followed by polyester (8.6%), ethylene vinyl alcohol (2.9%), polyvinyl chloride (2.9%) and acrylic adhesive (2.9%). Considering cave groundwaters only, polyethylene (56.5%) and polyvinyl alcohol (30.4%) were the main types of MPs.

Polyethylene, polyester and polyvinyl chloride were also found in other analyzed groundwaters (Mintenig et al., 2019; Panno et al., 2019; Samandra et al., 2022; Selvam et al., 2021). Other different MPs were identified in previous works, such as polyamide (nylon) (Mintenig et al., 2019; Samandra et al., 2022; Selvam et al., 2021), epoxy resin (Mintenig et al., 2019), polypropylene, polystyrene, polycarbonate and polymethylmethacrylate (Samandra et al., 2022). The MP composition is useful to understand the likely sources of pollution. Many of the plastics found are used in the production of textiles, corroborating earlier assumptions on the origin of microplastics found in the waters of the karst system.

Anyway, natural, semi-synthetic, synthetic fibres and different types of MPs contain chemical additives such as dyes, whitening agents and other pollutants (Barker, 1975; Lee et al., 2020; Luo et al., 2019), which can be discharged into the environment and ingested by aquatic organisms (McNeish et al., 2018). Therefore, further studies on the different pollutants associated with MPs and textile fibres are necessary to quantify them and verify the possible environmental impacts.

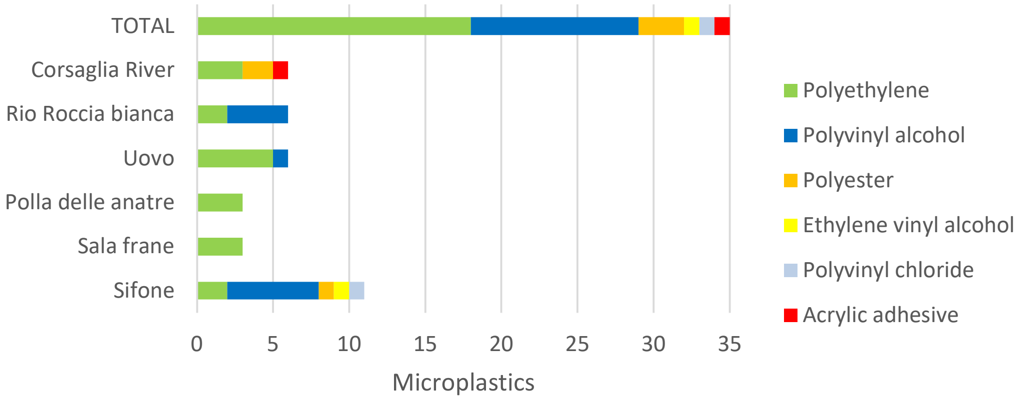

Fig. 36. Identification of 10% of the MP particles of each filter by micro-FTIR spectroscopy. Image from Balestra et al. (2023).

4.10 Conclusion

This preliminary investigation documented the presence of MPs in the examined karst system, from surface watercourses to cave waters. The MP concentration found varied from 12 to 54 items/L. MPs less than 1 mm dominated the samples (82.9%), and fibre was the main shape present (95.1%). The detection of MP contamination is essential to define the issues related to pollution and establish strategies for karst areas and water conservation. This work suggests that karst morphology and associated aquifers could allow the movement of MPs into underground water flow systems, providing useful references for further research. Future studies are needed to understand MP dynamics and transport in the karst system, to determine pollution sources and to understand the potential effects on organisms and ecosystems. As karst aquifers are open systems susceptible to contamination by surface pollutants, the superficial areas should also be monitored. Greater efforts should be made to protect karst areas and underground resources, implementing new strategies to monitor and valuate MP pollution and providing education to all stakeholders to find adequate solutions, following the environmental sustainability principles, especially for the management of water resources.

4A.3 First evidence of microplastic presence in stygobiont fauna

Origin of the Chapter

This chapter is adapted from the peer-reviewed article "(Micro-)plastics in saturated and unsaturated groundwater bodies: first evidence of presence in groundwater fauna and habitats" authored by Sforzi et al. (2024), published in Sustainability, Volume 16(6), 2024, 2532, DOI: **https://doi.org/10.3390/su16062532.**

The author of this dissertation was responsible for validation, sample collection, data curation, writing - original draft preparation, writing - review and editing.

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4.11 Introduction

Given their small size, MPs are highly bioavailable and their large distribution in ecosystems makes them a potentially hazardous substance (Bergami et al., 2020). This is a matter of concern as some organisms may exchange plastics for prey or ingest them with food (Andrade et al., 2019; Expósito et al., 2022). The impact of MP pollution may be related to their ability to act as carriers of other pollutants, such as bisphenol A (BPA), or heavy metals (Cheng et al., 2023; Selvam et al., 2021), and to the additives present in plastics, such as plasticizers, UV stabilizers, which can be released into the environment or transferred through the food chain posing serious health problems (Anbumani and Kakkar, 2018). However, there is also a lack of studies investigating the presence of MPs in groundwater metazoans.

Subterranean ecosystems host stygofauna, which includes groundwater-obligate invertebrate and vertebrate species (Malard et al., 2023). These animals -also named stygobites- have evolved morphological (depigmentation, anophthalmia/microphthalmia, elongation of sensory organs), physiological, and behavioral adaptations to survive and thrive in permanently dark environments characterized by low oxygen concentration and scarce trophic resources (Rétaux and Jeffery, 2023). They often represent phylogenetically ancient species with a point-like distribution and individuals living in small, clustered populations (Cooper et al., 2023).

Groundwater communities are characterized by low abundance and alpha diversity, reduced functional diversity, and high beta diversity (Zagmajster et al., 2023). Therefore, these communities are poorly resilient and can be severely affected by the deterioration of their environmental conditions (Hose et al., 2022). Groundwater animals provide valuable ecosystem services, e.g. many of them feed on viruses and bacteria (Mermillod-Blondin et al., 2023). They are key players in the recycling of organic carbon in subterranean environments and, through their movement and fecal release, contribute to the remixing and spiking of sediments (Deng et al., 2014). This activity contributes to keeping open the interstitial spaces (thereby facilitating water passage, oxygenation of sediments, and dilution of pollutants) and spreading bacteria into environments not yet colonized (Mermillod-Blondin et al., 2023).

Despite existing research highlighting the impacts of MPs on aquatic benthic organisms, such as reduced feeding (de Sá et al., 2015), stunted growth, and induced toxicity and oxidative stress (Jeong et al., 2016), there is a notable lack of evidence regarding their effects in groundwater organisms. Even the fundamental aspect of MP ingestion by groundwater fauna remains unexplored. This knowledge gap is particularly alarming, given the critical role of groundwater fauna in organic carbon recycling within these ecosystems. If these organisms were to replace their natural diet with MPs, they could severely disrupt this essential ecosystem service, undermining the health and stability of groundwater environments.

To fill these gaps, this research aimed to assess the potential MP ingestion by stygobitic invertebrates residing in groundwater bodies, evaluating the occurrence of these pollutants in groundwater invertebrate fauna to better understand the extent of MPs contamination and its implications for the depletion of ecosystem services. Addressing these knowledge gaps is not only crucial for preserving groundwater ecosystems but also aligns with the overarching goals of environmental sustainability and the protection of our planet's vital resources.

This research was carried out by CNR-IRET and Department of Chemistry “Ugo Schiff”, Università degli Studi di Firenze, in collaboration with the author during the PhD research, to investigate the presence of MPs in *Proasellus franciscoloi*, a species of crustacean taken from the system of the Bossea cave, Piedmont, and other aquatic organisms from a cave and two springs of Tuscany, Italy. The collection of animals in Bossea cave was carried out by the author and other members of the association Biologia Sotterranea Piemonte – Gruppo di Ricerca (BSP), a non-profit association for the study and conservation of subterranean habitats and species. This research concerned the analysis of MPs in groundwater and several stygobiont crustaceans from Tuscany and Piedmont groundwater bodies. Only the part concerning the Bossea system fauna analysis will be given below.

4.12 *Proasellus franciscoloi* (Chappuis, 1955)

Proasellus franciscoloi (Fig.s 37, 38) is a groundwater-dwelling isopod belonging to the family Asellidae, endemic to karst aquifers in NW Italy. It is a stygobitic species, exhibiting evident troglomorphic traits such as depigmentation and loss of visual organs. It inhabits interstitial and phreatic zones, playing an essential ecological role as a detritivore within subterranean food webs. Due to its strict dependence on pristine groundwater conditions, *P. franciscoloi* is highly sensitive to physicochemical changes and contamination. The species could be considered a bioindicator of groundwater quality, particularly in vulnerability assessments of karst ecosystems. Despite its ecological relevance, little is known about its population dynamics, reproductive biology, and tolerance thresholds to emerging contaminants. Conservation strategies require further research on its habitat requirements and the impacts of anthropogenic pressures on its survival. Understanding its ecological niche contributes to groundwater biodiversity conservation and sustainable management of karst water resources.

Fig. 37. Proasellus franciscoloi *in Bossea cave. Photo: © V. Balestra*

4.13 Method

4.13.1 Sampling

The specimens of *P. franciscoloi* were collected at "Ramo superiore" within Bossea cave, in the speleological area through which it is possible to reach the Secondary Lab (undersaturated karst habitat). The specimens can easily be found on the wooden beams of the old walkway collapsed in the water, which is used by researchers of BSP as a natural underground lab to study stygofauna directly in their environment.

For more detailed information on sampling, see Chapter 3.

4.13.2 Analysis

Sorting and identification of fauna

Sorting and identification were carried out with a LEICA M80 stereomicroscope at 16× magnification, followed by preservation of deceased individuals in glass vials containing 96% ethanol. A LEICA M205C stereomicroscope with an integrated camera was used to take pictures of each specimen (Fig. 38), and LAS software (Leica Application Suite, version 4.7.1) was then used to measure body size. Conversion formulas were used to convert body size (length and width in mm) to biovolume. To convert biovolume to fresh weight, a specific gravity of 1.1 was assumed.

Fig. 38. Lateral (left) and ventral (right) perspectives of a female specimen of the isopod Proasellus franciscoloi (Chappuis, 1995). This species exhibits typical adaptations of the groundwater-obligated fauna, i.e., elongation of sensory appendages, depigmentation, anophthalmia, and elongated and slender body shape. The ventral side also reveals a large and rich-in-vitellum egg, a recurrent trait of the hypogean fauna. Image from Sforzi et al. (2024).

Contamination control

To reduce the risk of sample contamination, several precautionary measures were adopted. Yellow PP laboratory coats and blue latex gloves were used at every stage of MP processing and all treatments were performed in a clean room under a laminar flow hood. In addition, the laboratory bench was regularly cleaned, and, when possible, the use of plastic equipment was avoided, using only glass or metal instead. Glassware and metal tweezers were washed and rinsed with ultrafiltered MilliQ water and then acetone (Chromasolv, Honeywell, Germany) and let dry under a laminar flow hood covered with aluminum foil, to prevent airborne contamination. Potential sources of contamination were assessed by performing blank controls (Scopetani et al., 2020). Field blanks were performed leaving clean filters uncapped near collection points to evaluate airborne contamination during the entire sampling sessions; once in the laboratory, procedural blanks were performed by subjecting ultrafiltered MilliQ water through the sample processing steps along with environmental samples. Additionally, to evaluate atmospheric deposition, a wet filter was exposed uncapped under the laminar flow hood during each sample processing step. All blanks were analyzed following the same methods as the samples.

Microplastic extraction

The fauna samples were treated according to the methodology outlined in Di Lorenzo et al. (2023b). Briefly, initially, the specimens were rinsed with ultrafiltered MilliQ water to eliminate any external particles, following ISO Standard Method SS-EN ISO 6330. Subsequently, the specimens were carefully loaded into 10 mL glass vials using a steel needle. The vials were filled with 10 mL of 30% H_2O_2 (30% Sigma Aldrich), sealed with aluminum foil, and maintained at a controlled temperature of 60 °C for 72 hours to digest the organic matter. Then, samples were vacuum filtered through a 2 cm ceramic Büchner funnel diameter onto glass fibre filters (CHMLAB GROUP, GF3 grade) and dried. These filters could be directly subjected to spectroscopic analysis, however, inspection through fluorescence microscopy required further processing using the lipophilic dye Nile Red (Prata et al., 2021). A few drops of Nile Red dye solution in ethanol were poured to cover all the filters and were left to dry for 24 h, covered with aluminum foil.

Although this technique does not enable the chemical characterization of the stained polymers, it has proven to be an ideal protocol for the determination of MPs in environmental and biological samples, as it allows the detection of smaller objects when combined with microscopy (e.g., ~1 μm, (Prata et al., 2021)). Particles with dimensions larger than 5 μm fall within the spatial resolution guaranteed by the FTIR microscope coupled to the FPA detector, available for this study for spectroscopic analysis. The integrated use of these two microscopic techniques has allowed to identify and quantify natural (i.e., cellulose) and synthetic fibres and fragments with dimensions ≥ 0.5 μm and chemically characterize those ≥ 5 μm present in the digestive tracts of stygofauna (Di Lorenzo et al., 2023b).

Polymer analysis

The shape, color, and size of the polymeric items on each filter were visually evaluated based on their physical characteristics through a stereomicroscope. The chemical composition of each object ≥ 5 μm in size was analyzed by 2D Imaging Fourier Transform Infrared Spectroscopy (FTIR) using a Cary 620-670 FTIR microscope, equipped with an FPA (Focal Plane Array) 128 x 128 detector (Agilent Technologies) and a 15x Cassegrain objective. The analysis was carried out in reflectance directly on the entire filters, using an open aperture and a spectral resolution of 8 cm-1, acquiring 128 scans for each spectrum in the spectral range 3900-900 cm-1. The background spectra were acquired on a golden surface. Each analysis results in a "single-tile" map with a size of 700 x 700 μm2 (128 x 128 pixels), each pixel having a size of 5.5 x 5.5 μm2 and providing an independent IR spectrum. Using micro-FTIR allows combining the advantages of high spatial resolution microscopy with the chemical composition information achievable with an IR spectrometer. In each false color 2D map, the intensity of the characteristic bands of the investigated polymers was imaged. The chromatic scale of the maps shows the increasing absorbance of the bands as follows: blue < green < yellow < red. Agilent Resolution Pro software, from Agilent Technologies, was used to collect and process all spectra. Fragments and fibres were identified by comparing the obtained spectra with published spectra of plastic and cellulosic polymer standards (see Supplementary Materials, Figures S1-S9). Natural and regenerated MFs were identified and included among the MPs because they can pose a threat to biota (Di Lorenzo et al., 2023b; Santini et al., 2022).

Fluorescence microscopy

Fluorescence analysis was carried out for fauna samples. The rationale behind this approach stems from the direct correlation between the size of MPs ingested by organisms and the size of their mouth openings. This relationship justifies the application of a technique that has a higher spatial resolution (~ 0.5 μm) compared to that achievable by IR spectroscopy alone (~ 5 μm). Since the filters required further processing to make the polymers fluorescent, this analysis was performed subsequently to the spectroscopic analysis to avoid any fluorescent interference during the collection of IR signals. Fluorescent stained filters were imaged through an epifluorescence microscope (Nikon TS2R) equipped with a LED illuminator (CoolLED pe-300 Ultra), for which an excitation light spectrum having a maximum at wavelength $\lambda = 490$ nm was selected to excite fluorescence from the stained MPs.

An excitation filter with a bandpass of 455–495 nm was used to further sharpen the excitation light spectrum, and a dichroic mirror was used to direct the filtered light onto the sample. The emitted fluorescence transmitted through the dichroic mirror and filtered by an emission filter with a bandpass of 515–625 nm was collected on a Hamamatsu ORCA-Flash4.0 V3 camera. Images were acquired with a 20× magnification objective (Nikon Plan Apo; N.A 0,75) using the HCImage software provided by Hamamatsu. For each stygofauna pool, a control blank was prepared, processed, and analyzed in the same way as the sample. The particles found in the blank were subtracted from the number of particles found in the sample. Then shape and dimension (intended as maximum length) of the fluorescent particles ≥ 0.5 μm were obtained by using the routine Analyze Particles of the ImageJ software (Brander et al., 2020) with the following settings: size (0.5 μm-infinity), circularity (0.00-1.00).

Micro-computed tomography

Micro-computed tomography (μ-CT) analysis was performed on P. franciscoloi adult female, approximately 5 mm in length. The samples were analyzed by collecting μ-CT data using Skyscan 1172 high-resolution microCT. This system has a sealed, microfocus tungsten X-ray tube with a 5 μm focal spot size. The X-ray was produced by exposing the anode to an electron beam at a range of 60 kV and 150 μA. Sample was placed on a pedestal between the X-ray tube source and the charge-coupled device detector. The 2D X-ray images were captured with a slice-to-slice rotation angle range of 0.3°. The spatial resolution of the images was kept at 11 μm in terms of pixel size. The 3D image of the object's internal structure was reconstructed using a modified Feldkamp algorithm for cone-beam acquisition geometry, realized in Nrecon v.1.6.3.3 software. The alignment and beam hardening corrections were made before starting the re-construction process. CTVox program was used for 3D visualization, while CT-Analyser (CTan) software was used for the image clean up and measurements.

4.14 Results

Preliminary examination with visible light microscopy suggested the potential presence of MPs in the fecal pellets of certain crustacean specimens collected (Sforzi et al., 2024). Length, width, and biomass value of the animal examined in this study were summarized in Table 7. The percentages of fluorescent MPs classified by shape, as well as the mean and standard deviations of length are reported in Table 8. All fluorescent particles found in blank controls were counted and classified by shape, then accordingly subtracted from the relative samples.

Table 7. Length (L; in mm), width (W; in mm), and biomass in dry weight (DW; in μg) of the individual processed in this study. The total biomass (TOT DW; in μg) and the number of particles per individual (items/ind) and biomass unit (items/μg) are also reported.

Taxonomic pool	Length (mm)	Width (mm)	TOT DW (μg)	Items/ind.	Items/μg (TOT DW)
Proasellus franciscoloi (n=1)	7.095	1.656	1,032	191	0.2

In the pool of *P. franciscoloi*, pellets accounted for 93% of the abundance, while fibres and fragments accounted for 1% and 6%, respectively.

Regarding the non-synthetic materials, in the *P. franciscoloi* pool, polysaccharide gums accounted for the highest percentage (63%), followed by cellulose, with 38%.

Table 8. Classification of the fluorescent particles ≥ 0.5 μm found in the specimen. Percentages of pellets, fibres, and fragments (according to classification in Lusher et al. (2020)) are given: for each type the mean (μ) and the standard deviation (SD) of the lengths (μm) are also reported. For pellets the length was considered as the diameter, for fibres as the major dimension, and for fragments as the distance between the two most distant points.

Taxonomic pool	Pellet			Fibre			Fragment		
	%	μ	SD	%	μ	SD	%	μ	SD
Proasellus franciscoloi (n=1)	93	1	1	1	16	3	6	32	37

About 191 MPs, predominantly pellets, were identified in the gut of the *P. franciscoloi* specimen (adult female, approximately 5 mm in length). Given an average diameter equal to 3 μm of the MP pellets ingested by this species, the total volume of ingested MPs was estimated to be << 1 % of its gut volume (1 mm^3), which was measured using RX imagery (Figure 39).

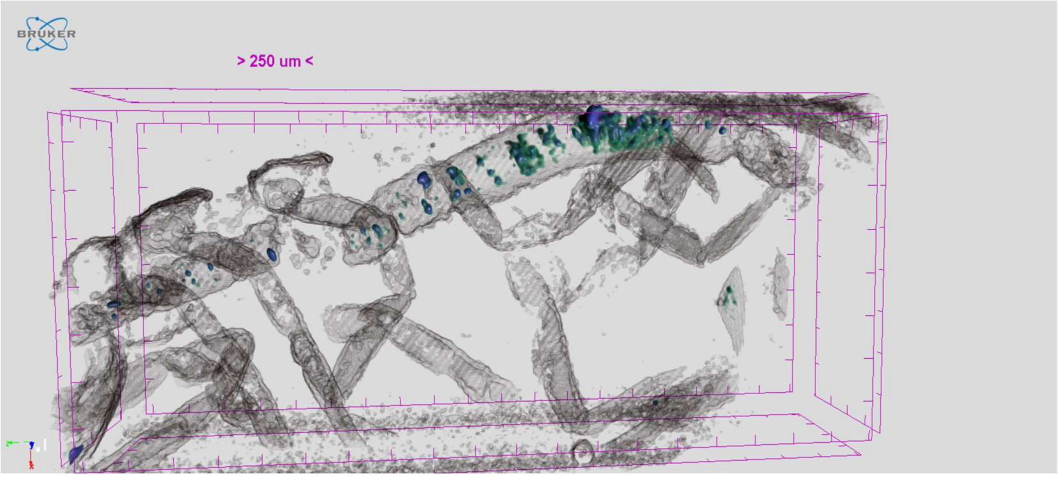

Fig. 39. 3D volume reconstruction from microtomography analysis of the gut of the analyzed P. franciscoloi, *Bossea cave. The pellets are highlighted in green/blue.*

4.15 Discussion

Ingestion of plastic debris by sediment-residing organisms is a well-documented phenomenon as well as the species-specific ingestion pattern (Scherer et al., 2017), however, the discovery of MPs in the gut of the isopod *P. franciscoloi* and groundwater ostracods and copepods marked the first evidence of MPs in groundwater fauna.

The rate of MP ingestion is probably shaped by feeding behaviours and potentially by metabolic rates, which are typically lower in stygobitic species compared to their epigean counterparts (Di Lorenzo et al., 2023a). Accordingly, the number of MPs per µg of dry weight in *P. franciscoloi* was significantly lower than in previous studies on epigean asellids (Pan et al., 2021).

About 191 MPs were identified in the gut of the *P. franciscoloi* specimen, and the total volume of ingested MPs was estimated to be << 1 % of its gut volume (1 mm3. This volume was significantly lower- by an order of magnitude at least- than the 4% gut volume occupancy by MPs in an epigean Asellidae species (gut volume of 1 mm3, as in *P. franciscoloi*) from a small lowland river in north-eastern Belgium (Pan et al., 2021). This discrepancy is likely due to stygobitic species' lower metabolism and reduced feeding rates compared to surface-dwelling relatives (Di Lorenzo et al., 2023a).

All groundwater taxa analysed in Sforzi et al. (2024) ingested mainly MP pellets. The predominance of pellet-shaped ingested MPs in freshwater meiofauna has already been observed in previous studies (Scherer et al., 2017). However, this result is surprising compared to data on MPs pollution that I highlighted in Bossea cave in sediments and waters (Balestra and Bellopede, 2022b; Balestra et al., 2023), in which fibres accounted for the majority of MPs, followed by fragments and other morphologies. However, sediments were collected along the tourist path, out of the water, and the cut-off for MP analysis in sediment and waters was 0.1 mm (Balestra and Bellopede, 2022b; Balestra et al., 2023), while in this study the main focus was on smaller particles (down to 0.5 µm) that can be more easily ingested by groundwater microcrustaceans, based on the studies conducted on their marine and freshwater relative species. For instance, the marine copepod *Acartia tonsa* Dana, 1849, demonstrates selective ingestion of plastic beads specifically within the size range of 13.9 to 59 µm (Wright et al., 2013). In addition, biofouling phenomena increase the density of MP particles in sediments, even of those of small-sized beads, causing them to sink (Liu et al., 2022b). These mechanisms enhance the accessibility of nano- and microplastics to organisms living in sediment, particularly through ingestion, as the size of these particles is comparable to, or even less than, that of sediment grains (e.g. Haegerbaeumer et al., 2019). Moreover, the presence of biofilms on MPs may increase their palatability or detectability to organisms, as observed in some marine crustacean species (Hodgson et al., 2018; Vroom et al., 2017).

Further studies are needed to assess MPs pollution and their potential effects on water chemistry and groundwater obligate species. However, even if the long-term ecological consequences of MP ingestion on groundwater fauna are still unknown, it is reasonable to anticipate that stygobitic species exposed to MPs may experience a spectrum of detrimental impacts (e.g. Zhang et al., 2023a), spanning from disrupted feeding patterns to compromised reproductive health. There is also a crucial need to understand how MPs transfer long food chains and the implications for higher trophic levels. Moreover, groundwater fauna plays critical roles in nutrient cycling, sediment transport, and maintaining the overall health of the ecosystem (Mermillod-Blondin et al., 2023). Therefore, the impairment of these organisms due to MP ingestion could disrupt these vital processes, leading to broader ecological consequences.

Further aspects that would need a deeper investigation are the degradation processes and the ultimate fate of MPs in groundwater environments. Physical, chemical, and biological processes in these ecosystems can affect the degradation of MPs. Once pollution reaches the groundwater, the process is almost irreversible (UNESCO, 2022), and the persistence of pollutants in these environments can be significant, also because the dynamics of groundwater are much lower than those of surface water. Finally, groundwaters often interact with surface waters (Saccò et al., 2024). The impact of MPs in groundwater could, therefore, extend to adjacent aquatic systems, spreading the ecological consequences beyond their point of origin.

4.16 Conclusion

The increasing pollution of groundwaters by MPs raises great concerns for the conservation of sensitive environments and species. Besides impairing groundwater quality, which is often used for drinking purposes, it is expected to cause detrimental effects on species and communities living in this habitat.

Regarding the impact of MPs on groundwater fauna of Bossea system, it was found that examined *Proasellus franciscoloi* ingested mainly MP pellets, while my previous studies on sediment and water samples of the Bossea cave mostly contained polymer fibres. Further studies are advisable to assess the role of MPs as vectors of organic contaminants through ingestion by underground fauna, bioaccumulation and biomagnification in karst systems.

Finally, this study highlighted the importance of selecting an appropriate dimensional cut-off in investigating microplastic in groundwater ecosystems. Selecting an excessively large cut-off risks underestimating the actual ingestion of these particles by groundwater species, especially if their size preferences are not comprehensively addressed.

4A.4 Bisfenol pollution in Bossea karst system water

4.18 Introduction

Emerging organic contaminants (EOCs) are substances potentially harmful to the environment, such as pesticides, pharmaceuticals, microplastics (MPs) and bisphenols (García et al., 2020; Reberski et al., 2022). Many EOCs can accumulate in soil and water, including aquifers, especially through leaching, which involves their movement into deeper soil layers and eventually into groundwater sources (Narevski et al., 2021; Oppeltová et al., 2024; Pérez-Lucas et al., 2018; Reberski et al., 2022; Yamamoto and Yasuhara, 1999). The accumulation of EOCs in aquifers poses serious environmental and health risks, as these water sources are used for drinking water worldwide (Reberski et al., 2022). The transportation of EOCs to aquifers is determined by aquifer characteristics and subsurface conditions, such as hydrogeological factors, groundwater residence time, and environmental conditions (Oppeltová et al., 2024). Chemical, biological and physical processes may control EOCs migration into aquifers too (Stoppiello et al., 2020).

Bisphenol A (BPA) is one the most highly produced chemicals worldwide used in the production of synthetic polymers, such as polycarbonate plastics and epoxy resins, and thermal paper (Hoekstra and Simoneau, 2013; Huang et al., 2012; Michałowicz, 2014). As a result, it is ubiquitously present in a wide range of consumer products, including paper products, electronic devices, toys, and water pipes, but also in medical equipment and dental products (Huang et al., 2012; Michałowicz, 2014). Thermal paper is produced in large quantities, due to its widespread use in register receipts, books, fax papers, and labels. Following recycling, it is also employed in the manufacture of newspapers, tickets, brochures, mailing envelopes, kitchen rolls, toilet paper, and food cartons (Michałowicz, 2014 and references therein). Furthermore, BPA is extensively utilized in the production of polyacrylates, and polyesters, and is incorporated into food contact materials, such as packaging, beverage bottles and lacquered coatings for metal cans, causing chronic human exposure through ingestion of contaminated food and drinks (Arnold et al., 2013; Cooper et al., 2011; Makris et al., 2013; Michałowicz, 2014; Niu et al., 2012). These materials, upon degradation, can represent significant sources of BPA in the environment too (Michałowicz, 2014). Therefore, among bisphenols, BPA is currently the most investigated.

BPA exhibits a wide range of toxicological effects due to its endocrine-disrupting potential, oxidative stress induction, mutagenic potential, as well as hypomethylation activity (Keri et al., 2007; Richter et al., 2007). Experimental evidence has demonstrated that BPA disrupts the physiological regulation of several key hormones, including sex hormones, and is associated with hepatotoxic, immunotoxic, mutagenic, and carcinogenic effects in animals, with implications for human health (Clayton et al., 2011; Keri et al., 2007; Richter et al., 2007). Recent studies also suggested a correlation between BPA exposure and increased risk of obesity, type 2 diabetes, and cardiovascular disease (Shankar et al., 2012; Teppala et al., 2012)

In contrast to its pronounced toxicity in higher organisms, BPA exhibits comparatively lower toxicity toward plants and microorganisms, such as bacteria, fungi and algae, which, in some cases, are even capable of degrade these pollutants (e.g. Shin et al., 2007). These biodegradation processes are exploited in bioremediation strategies aimed to mitigate BPA contamination in different environmental matrices (Saiyood et al., 2013).

The presence of BPA in the environment is exclusively linked to human activities. However, at the moment, identifying the primary source of population and ecosystems exposure to BPA is difficult. Several pathways that contribute to environmental and food contamination have been identified: the major sources include the production, treatment and processing of BPA, as well as the degradation of polymers (epoxy resins and polycarbonates), such as MPs, leading to the release of BPA into ecosystems and the food chain (Michałowicz, 2014 and references therein).

With regard to environmental issues, BPA was investigated especially in atmosphere and water. In atmosphere, BPA is emitted mainly as a result of industrial activity, with concentrations ranging from small amounts (5-15 pg/m3), to more than 1900 pg/m3 (Michałowicz, 2014 and references therein). In surface waters, BPA is generally detected at low concentrations, however, other investigations have demonstrated substantial contamination, especially near urban centers, reaching example value > 90 µg/dm^3 (Michałowicz, 2014; Stachel et al., 2003). In urban effluents, main sources are linked especially with atmospheric fallout, sewer overflows, and runoff (Cladière et al., 2013). Significantly higher concentrations of BPA were detected even in industrial wastewater and in groundwater (Michałowicz, 2014 and references therein).

Groundwaters are fundamental resources and important ecosystems. Unfortunately, groundwaters are under increasing pressure from contamination due to anthropogenic activities (Reberski et al., 2022 and references therein). Generally, groundwaters are less vulnerable to contamination by EOCs than surface waters, however, once in groundwaters, these substances are persistent and difficult to treat (Reberski et al., 2022; White et al., 2019). BPA is one of the most relevant detected compounds in European groundwaters, with a detection frequency of 40%, and a reported maximum concentration reaching 100 µg/L (Lapworth et al., 2015; Loos et al., 2010).

Research on BPA pollution in fresh waters highlighted their presence in surface waters and groundwaters worldwide, however, in karst systems they are less studied, despite karst groundwaters are widely exploited for drinking use (Reberski et al., 2022; Wilkinson et al., 2022). Karst aquifers are particularly susceptible to pollution, due to direct infiltration pathways, such as stream sinks, shafts, and caves (Ford and Williams, 2007; Goldscheider and Hötzl, 1999), which facilitate the rapid transport of pollutants, even reaching rapidly long distances (Worthington and Ford, 2009). Little studies on EOCs were done in karst, but only from a small number of countries, especially from Europe and USA, with few or no studies in many other parts of the world (Reberski et al., 2022 and references therein). However, they provide key insights into patterns of EOCs in karst aquifers, useful for a wide range of applications and conservation measures.

Currently, to the authors' knowledge, there is only one study which analyse BPA pollution in waters of boreholes, streams, wells and caves of the same karst area and possible surces (Oppeltová et al., 2024). Studies on bisphenol pollution in caves, as well as surface and underground waters of the same karst system are lacking. Karst groundwater resources are highly vulnerable to pollution. Given their essential ecological functions and their role as a source of drinking water, it is crucial to implement environmental investigations to monitor environmental health and identify possible sources of pollution.

In this study, a preliminary screening of Bisphenol A (BPA), AP (BPAP), and C (BPC) was conducted in the karst system of Bossea, Piedmont, Italy, analyzing surface watercourses, a subterranean river, cave groundwaters and waters from a conduit overflow, providing a reference for further research. If found, the characteristics of the system and the area will be analysed, as well as the surface anthropogenic activities to hypothesize the possible sources of pollution.

This karst system was previously analysed to understand even MP pollution in sediments and waters, a possible source of BPA pollution, highlighting MP pollution from surface watercourses to cave and springs (Balestra and Bellopede, 2022b; Balestra et al., 2023) (see Chapters 4). Being MPs a possible source of BPA pollution, in parallel to bisphenols, MP pollution was analyzed again, but under high flow conditions, during a rainy day after a long sunny period. This further data enriches the information relating to this karst system regarding MP pollution in waters, previously investigated in the lean period (Balestra et al., 2023). Moreover, other AMPs, such as MFs, were investigated too.

This work wants to be the first evaluation of bisphenol (especially BPA) pollution in the karst systems of Bossea, with the objective of stimulating further researchers in this field to support the development of appropriate conservation measures for these vulnerable environments.

4.19 Method

4.19.1 Sampling

Sampling points were set in surface watercourses, subterranean rives, cave groundwaters, and springs (overflow conduit from the cave) of the same karst system (Fig. 40). Sampling points and sample characteristics are listed in Table 9. A total of 13 samples for bisphenols were collected in parallel to other 13 samples for MP and other AMP analysis. Three sampling points were established in surface watercourses, eight inside Bossea cave, and two in the overflow conduit.

Water bulk samples (Hidalgo-Ruz et al., 2012) were collected using pre-cleaned (filtered ethanol and Milli-Q water) glass jars. For each sampling area, a quantity between 200 and 550 mL of water was collected. Samples were collected on 18-09-2024 during a very rainy day, after a sunny period, with about 5°C near Rio Roccia Bianca watercourse.

For more detailed information on sampling method and contamination control, see Chapter 3.

Unfortunately, sample RIO 2 was damaged during shipment by post to Spain and G6 for MP and MF analysis was broken in the lab.

Table 9. Water sample characteristics in the Bossea karst system.

Sample ID	**Sample name**	**Sampling site type**	**Water characteristics**	**ml**
G1	Cascatella	Cave, speleological area	Small waterflow of the subterranean river	240
G2	Polla delle Anatre	Cave, speleological area	Small water coming entering in the inner collector. The water flows out of a fracture in the rock and is collected in a small tank with a weir for flow monitoring. The flow has the typical characteristics of an interconnected drainage system	250
G3	Ernestina	Cave, tourist area	Lake created by the subterranean river in which the water flow slows down, near the tourist path	250
G4	Milano	Cave, tourist area	Small water coming. The water flows out of a fracture in the rock, into the wall, creating a pool. Really distant from the tourist path	250
G5	Orso	Cave, tourist area	Lake created by the subterranean river in which the water flow slows down, in the biggest hall of the cave	280
G6	Uovo	Cave, tourist area	Pool created by the subterranean river in which the water flows very slow between different gours, near the tourist path	250
G7	Baldacchino	Cave, tourist area	Small water coming. The water flows out of a fracture in the ceiling. Distant from the tourist path	260
G8	Batteri	Cave, tourist area	Very small water coming from the ceiling. The water flows out of a fracture in the rock, creating stalattites. Quite distant from the tourist path	260
S1A	Troppopieno A	Overflow - Spring	Overflow of the subterranean river. The water comes from a conduit in the wall, inside a small room near the accomodation facility of the cave, above the Corsaglia river and the main spring of the subterranea river.	500
S1B	Troppopieno B	Overflow - Spring	Overflow of the subterranean river. The water comes from a conduit in the wall, inside a small room near the accomodation facility of the cave, above the Corsaglia river and the main spring of the subterranea river.	220
RIO1-DX	Rio Roccia Bianca DX	Surface watercourse	Surface watercourse upstream the Bossea cave. Main secondary supply contributing to the Bossea karst system recharge. Secondary right flow	500
RIO1-SX	Rio Roccia Bianca SX	Surface watercourse	Surface watercourse upstream the Bossea cave. Main secondary supply contributing to the Bossea karst system recharge. Secondary left flow	500
RIO2	Rio Roccia Bianca (RIO1-DX + RIO1-SX)	Surface watercourse	Surface watercourse upstream the Bossea cave. Main secondary supply contributing to the Bossea karst system recharge. Main watercourse	540

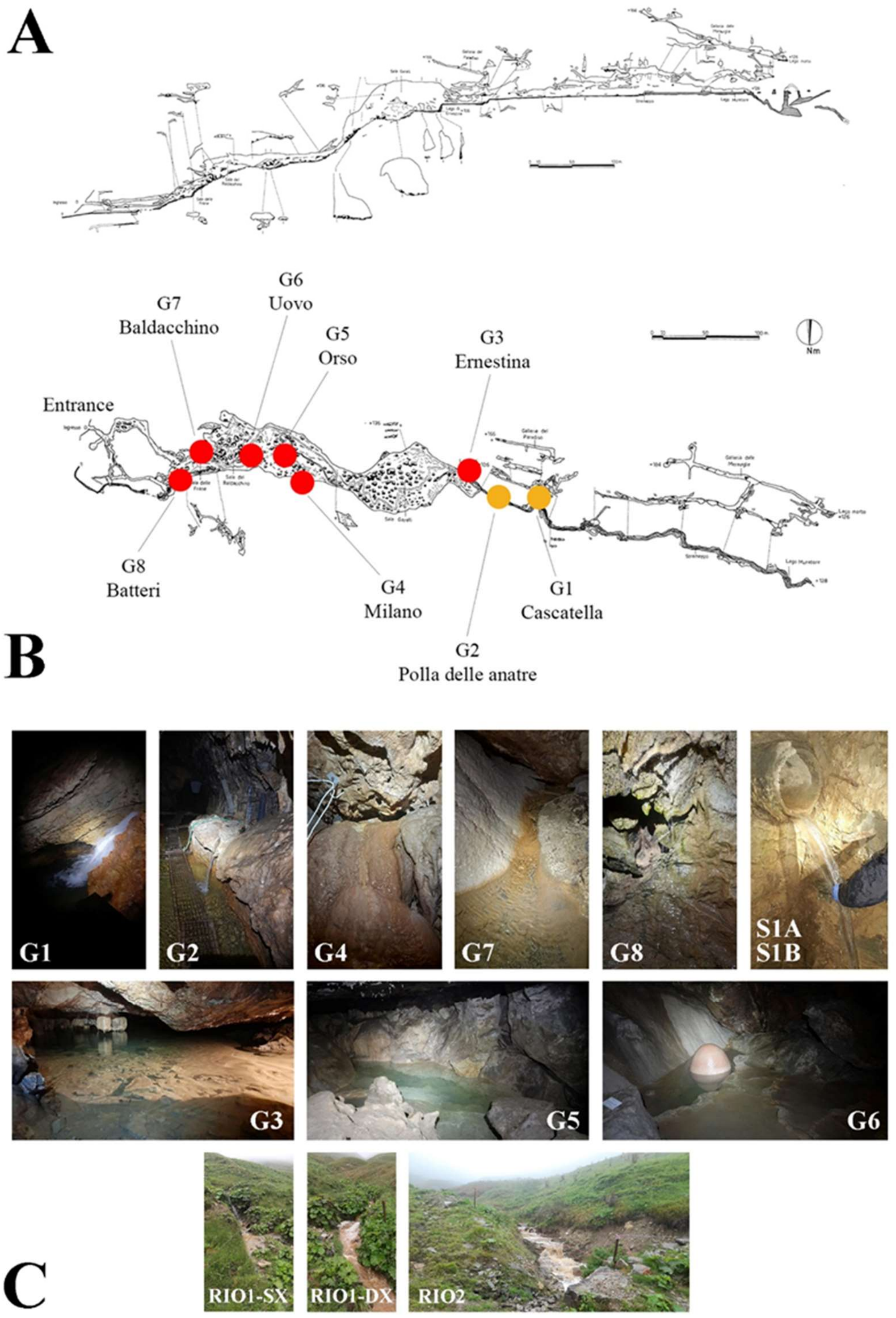

Fig. 40. Sampling points. A, B: Bossea cave surveys. Red circles for water sampling in the tourist area, orange circles for water sampling in the speleological area (Surveys – section (A) and plan (B) - from Elia and Calleris (1988), modified); C: photos of all sampling points (photo S1A-S1B by E. Lana, all the other photos by V. Balestra).

4.19.2 Bisphenols analysis

Bisphenols analyses were conducted in the Department of Analytical Chemistry of the University of Valencia. The methodology is being published, consequently, only a summary will be reported below.

A solid-phase extraction procedure was carried out using a 3D-printed device functionalized with a bisphenol A aptamer. The required sample volume was brought into contact with the device, and the loading step was performed under stirring at 100 rpm for 30 min. Afterwards, the device was washed with water and then placed in contact with a 1:1 (v/v) H_2O:ACN solution to elute the bisphenols. The resulting eluate was subsequently analyzed by HPLC using a C18 column (5 µm, 100 Å, 15 cm). Chromatographic separation was achieved under isocratic conditions with a mobile phase of 40:60 (v/v) H_2O:ACN containing 0.1% formic acid, at a flow rate of 1.0 mL/min. The column temperature was maintained at 30 °C, and the injection volume was 40 µL.

4.19.3 Microplastic and microfibre analysis

Water samples were pre-treated by adding a 30% hydrogen peroxide (H_2O_2) solution at a volume equal to 50% of the total sample volume, covered with an aluminum foil, and left to react for seven days under natural conditions. Each water sample was then filtered through a 1.2-µm pore size silver filter (GVS Life Sciences, Membrane Disk 47 mm).

Particles on filters were observed under a Leitz ORTHOLUX II POL-MK microscope equipped with a DeltaPix Invenio 12EIII 12 Mpx Camera, with and without a UV light. It was established to analyse MPs from 5 to 0.05 mm. Particles not clearly identify as MPs and MFs were not taken into consideration.

Particles were verified using a micro-Fourier Transform Infrared Spectroscope (µFTIR) Shimadzu AIM-9000 microscope equipped with a Shimadzu IRTracer-100 spectrophotometer and a Shimadzu ATR with a germanium prism. All suspected MPs were analyzed. Spectra were accepted only with a match degree ≥70%.

For more detailed information on method and contamination control, see Chapter 3.

4.19.4 Correlations

The Spearman correlation will be used to assess whether the presence of BPA could be directly associated with that of MPs.

4.20 Results

The results confirmed that the Bossea karst system is polluted by BPA (Table 10), with high values in the main part of the sampling points, especially inside the cave. BPAP and BPC were not detected in the analyzed water samples.

Table 10. Detected bisphenols in the Bossea karst system. LOD: ca. 0.9 µg/L

Sample	**[BPA] (µg/L)**	**[BPC] (µg /L)**	**[BPAP] (µg/L)**
G1 - Cascatella	29 ± 3	< LOD	< LOD
G2 – Polla delle Anatre	< LOD	< LOD	< LOD
G3 - Ernestina	< LOD	< LOD	< LOD
G4 - Milano	28.2 ± 1.5	< LOD	< LOD
G5 - Orso	< LOD	< LOD	< LOD
G6 - Uovo	21.9 ± 0.5	< LOD	< LOD
G7 - Baldacchino	23.3 ± 1.4	< LOD	< LOD
G8 - Batteri	14.8 ± 1.4	< LOD	< LOD
S1A	< LOD	< LOD	< LOD
S1B	10.0 ± 1.2	< LOD	< LOD
RIO1 - SX	15.3 ± 1.8	< LOD	< LOD
RIO1 - DX	16.2 ± 2.3	< LOD	< LOD
RIO2	-	-	-

Analyses on AMPs highlighted pollution in the entire system (Table 11), with totally different values between the sampling points. However, MPs were not found in all sampling points.

Table 11. Anthropogenic microparticles found in Bossea karst system.

Filter	**Natural and Regenerated [items/L]**	**Synthetic [items/L]**	**N.D. [items/L]**	**TOT [items/L]**
G1 - Cascatella	91.7	0.0	4.2	95.8
G2 - Polla delle Anatre	136.0	0.0	0.0	136.0
G3 - Ernestina	12.0	4.0	8.0	24.0
G4 - Milano	88.0	0.0	0.0	88.0
G5 - Orso	0.0	0.0	0.0	0.0
G6 - Uovo	-	-	-	-
G7 - Baldacchino	123.1	0.0	3.8	126.9
G8 - Batteri	0.0	7.7	0.0	7.7
S1A	48.0	6.0	2.0	56.0
S1B	0.0	0.0	4.5	4.5
RIO1 - SX	104.0	4.0	12.0	120.0
RIO1 - DX	2.0	2.0	4.0	8.0
RIO2	59.3	0.0	13.0	72.2
Mean	55.3	2.0	4.3	61.6
%	90.7	3.6	5.8	
Cave				
Mean	64.4	1.7	2.3	68.3
%	94.2	2.4	3.3	
Springs				
Mean	24.0	3.0	3.3	30.3
%	79.3	9.9	10.8	
Surface watercourses				
Mean	55.1	2.0	9.7	66.7
%	82.5	3.0	14.5	

No statistical correlation exists between the values of MPs and BPA found in the samples (rs= -0.275, p=0.413), as well as with the type of MPs found (Table 12).

Table 12. Characteristic of microplastics found in the water sample of the Bossea karst system.

	Shape	Size	Colour	Fluorescence	Type
G3 - Ernestina	fragment	0.05	blue	blue	EVOH
G8 - Batteri	fragment	0.05	black	no	copolimer
G8 - Batteri	fragment	0.08	grey	blue	copolimer
S1A	fragment	0.05	blue	no	acrylic
S1A	fragment	0.16	blue	no	copolimer
S1A	fibre	0.16	black	no	PVC
RIO1 - SX	fibre	0.74	yellow	yellow	PET
RIO1 - SX	fibre	0.26	transparent	blue	PAM
RIO1 - DX	fibre	0.66	black	no	PET

4.21 Discussion

BPA was found in major part of the examined water samples, highlighting intense pollution in karst surface and subterranean aquatic karst environments. Comparisons with other karst areas are challenging since methodologies and analyses are different and can affect the results, and the sampling point and environmental conditions may vary concentrations over time. Moreover, there is a lack of studies regarding karst systems. However, some assumptions can be made. Data found using the words “bisphenol”, “BPA”, “karst”, “cave” and “groundwater” in the search engines of scientific online databases were reported in Table 13 for groundwaters and Table 14 for karst systems. All data found regarded BPA concentrations; data about other bisphenols were not found.

Table 13. Comparison of data about BPA in worldwide groundwaters.

Country	Maximum concentration (µg/L)	References
Zambia	0.001	Sorensen et al. (2015)
China	0.027	Du et al. (2016) Dong et al. (2018)
China	0.036	Li et al. (2015) Dong et al. (2018)
New Zeland	0.280	Moreau et al. (2019)
USA	0.430	Bexfield et al. (2019)
Czech Republic	0.655	Oppeltová et al. (2024)
Austria	0.930	Hohenblum et al. (2004)
Germany	1.136	Osenbrück et al. (2007)
French	1.360	Lapworth et al. (2015)
Spain	1.500	Latorre et al. (2003)
Europe	2.299	Loos et al. (2010)
Tennessee, USA	2.300	DRINKING WATER UNIT (2018)
USA	2.550	Barnes et al. (2008)
UK	20	Stuart et al. (2011)
England	100	Lapworth et al. (2015)
Japan	740	Michałowicz (2014) Kawagoshi et al. (2003)

Table 14. Comparison of data about BPA in karst system waters from different areas of the world.

Country	Type	Maximum concentration (µg/L)	References

Zambia	Wells, karstified dolostone	0.001	Sorensen et al. (2015)
USA	Wells, carbonate aquifers	0.430	Bexfield et al. (2019)
French	Chalk groundwater	1.360	Lapworth et al. (2015)
England	Chalk groundwater	100	Lapworth et al. (2015)
Czech Republic	Groundwater (borehole, 100 m depth)	0.655	Oppeltová et al. (2024)
China	Karst groundwater, pore water	0.027	Du et al. (2016) Dong et al. (2018)
China	Groundwater, Loam	0.036	Li et al. (2015) Dong et al. (2018)
Czech Republic	Thermal karst waters (cave, 60 m depth)	0.242	Oppeltová et al. (2024)
Czech Republic	Surface karst waters (borehole, 8 m depth)	0.144	Oppeltová et al. (2024)

BPA concentrations found in the Bossea karst system waters are higher than most of groundwater and karst system waters investigated so far (Tables 11 and 12), with the exception of groundwaters analyzed in UK (Lapworth et al., 2015; Stuart et al., 2011), and Japan, where groundwaters were contaminated with leachate derived from refuse dump (Kawagoshi et al., 2003).

BPA pollution in the environment was investigated especially in atmosphere and water (Michałowicz, 2014). In surface waters, BPA was generally detected at low concentrations, however, other investigations have demonstrated substantial contamination, especially near urban centers (Michałowicz, 2014). In urban effluents, main sources are linked especially with atmospheric fallout, sewer overflows, and runoff (Cladière et al., 2013). Significantly higher concentrations of BPA were detected even in industrial wastewater and in groundwater (Michałowicz, 2014 and references therein).

No statistically significant correlation between MPs and BPA was found in this research, as well as with the type of MPs found. This may be linked to the fact that the samples were taken in parallel, or that bisphenols do not derive from the MPs degradation at the examined sampling points, but rather upstream. Therefore, other sources have to be found for this system.

In this area, possible sources of environmental pollution are linked especially to the atmospheric transport, because the Bossea system in located in a sparsely populated mountain area. In atmosphere, BPA is emitted mainly as a result of industrial activity, with variable concentrations, polluting waters and lands one deposited (Michałowicz, 2014). At about 15 km from Bossea, a paper mill is present, and at least three plastic industries at about 20 km. Another one, at less than 20 km from Bossea was closed in 2022.

BPA is widely used in the paper industry, primarily associated with thermal paper production. In thermal papers, such as receipts, tickets, and labels, BPA acts as a color developer within the heat-sensitive coating. These materials represent the main source of BPA release from the paper sector. During recycling, thermal paper residues often enter mixed paper waste streams, leading to BPA contamination in recycled paper products such as tissues, paper towels, and packaging. Consequently, BPA can be detected in everyday paper-based materials not directly manufactured with the compound. Industrial wastewater and sludge generated during paper processing also serve as secondary environmental sources, enabling BPA dispersal into soil and aquatic systems. Additionally, minor contributions may derive from epoxy resins, inks, and coatings used in paper finishing. However, Bossea system is upstream of the paper mill, therefore, pollution may be linked to airborne emissions of BPA from paper production and recycling facilities. Moreover, the paper mill near Bossea system does not produce materials that should contain BPA, however, it uses recycled paper for its products, which may contain them.

BPA is one the most highly produced chemicals worldwide used in the production of polycarbonates and epoxy resins (Hoekstra and Simoneau, 2013; Huang et al., 2012; Michałowicz, 2014). Furthermore, BPA is extensively utilized in the production of polyacrylates, and polyesters, and is incorporated into packaging, beverage bottles and lacquered coatings for metal cans (Arnold et al., 2013; Cooper et al., 2011; Makris et al., 2013; Michałowicz, 2014; Niu et al., 2012). The plastic industries near Bossea system produce composite process materials and technical films, which could contain BPA.

Nearby snow systems in the ski slopes cannot be ruled out either, as they may have internal coatings with epoxy resins on tanks or pipes, gaskets, sealants or industrial paints with BPA-based epoxy components. However, these quantities should be very low and localized.

Once pollution reaches groundwater, the persistence of pollutants in these environments can be significant, because the dynamics of groundwater are much lower than those of surface water. Therefore, further studies are needed to assess BPA pollution in this area, their possible sources, and their potential effects on water chemistry and species, especially groundwater ones.

Regarding MPs pollution, although it was not possible to sample in the same places as the previous time (Balestra et al., 2023), due to an important heavy rain event, it was possible to observe that interesting differences in MP abundances and characteristics were present (Table 15).

Table 15. Differences between microplastic found and previous research.

Site	Environment	Matrix	MP abundance	Fluorescent MPs	Non fluorescent MPs	Fibre	Fagment	Foam	Film	Pellet/sphere	1-5mm	x<1 mm
Lean period (Balestra et al., 2023)												
Bossea cave	cave	waters	28	73.2	26.8	93.8	5.4	0	0	0.8	18	82
Rio Roccia Bianca	surface water	waters	23	78.3	21.7	95.7	4.3	0	0	0	8.7	91.3
Corsaglia river	spring	waters	29	93.1	6.9	100	0	0	0	0	27.6	72.4
Heavy rainy event (2024)												
Bossea cave	cave	waters	1.7	66.7	33.3	0.0	100.0	0.0	0.0	0.0	0.0	100.0
Rio Roccia Bianca	surface water	waters	3.0	66.7	33.3	100.0	0.0	0.0	0.0	0.0	0.0	100.0
Overflow	spring	waters	2.0	0.0	100.0	33.3	66.7	0.0	0.0	0.0	0.0	100.0

MP amount during the rainy event was really low respect to that found in the lean period (Balestra et al., 2023). This may be linked to the fact that heavy rains may have moved the material, quickly carrying it away. Further studies are needed on MPs to verify their mobilization during rainy or flood events.

During the lean period, MPs resulted especially fibres with big and small dimensions, while during the rainy event, fibres and fragments were found, and all with dimension < 1mm.

PE, PVA, PES, EVOH, PVC and acrylic adhesive were found in the lean period, as similarly in the rainy period, where PET, EVOH, PVC, PAM, acrylic and copolymers were analyzed. Many of these plastics are commonly used in the production of textiles, and, being also fibres, could be related to clothes production, as supposed in Balestra et al. (2023). However, the origin of the fragments could be multiple, both in the cave and outside, so further investigations are needed.

4.22 Conclusion

The results confirmed that the analyzed karst system is polluted by BPA with high values in the main part of the sampling points, especially inside the cave. BPAP and BPC were not detected in the analyzed water samples. However, the presence of BPA cannot be related to the presence of MPs at the moment, therefore, other sources of contamination and transport need to be investigated.

The increasing pollution of groundwaters raises great concerns for the conservation of sensitive environments and species, as well as for the water management. Besides impairing groundwater quality, which is often used for drinking purposes, it is expected to cause detrimental effects on species and communities living in this habitat. Further studies are advisable to assess BPA pollution in karst systems, their sources and the possible link with MP transport and contamination.

4B Case Study: Borgio Verezzi and Toirano caves, Liguria, Italy. Microplastic pollution in sediments: comparisons between methods and caves

Origin of the Chapter

This chapter is adapted from the peer-reviewed article “Microplastics in caves: a new threat in the most famous geo-heritage in the world. Analysis and comparison of Italian show caves deposits” authored by Balestra and Bellopede (2023), published in Journal of Environmental Management, Volume 342, 15 September 2023, 118189, DOI: **https://doi.org/10.1016/j.jenvman.2023.118189.**
The author of this dissertation was responsible for term, conceptualisation, methodology, software, validation, formal analysis, investigation, resources, data curation, writing – original draft, writing – review and editing, visualisation.

Permissions

4.23 Introduction

The potential MP contamination for subterranean environments in 2022-2023 was often only mentioned or limited to groundwater resources (e.g. An et al., 2022; Khant and Kim, 2022; Mintenig et al., 2019; Samandra et al., 2022; Viaroli et al., 2022). However, subterranean environments are open systems, therefore, susceptible to contamination by surface pollutants.

Karst areas are characterized by carbonate rocks, representing the major Earth’s cave systems. Caves are the most important geological heritage worldwide (Cigna and Forti, 2013; Piano et al., 2022), rich in speleothems and minerals (Hill and Forti, 1997), extremely peculiar habitats hosting organisms with interesting ecological adaptations (Culver and Pipan, 2019; Mammola, 2019), and custodians of important drinking water reserves (Moldovan et al., 2020). Indeed, over the past decades, the interest in subterranean karst environments has grown remarkably, from a scientific and economic viewpoint, emphasizing the importance of conservation and sustainable management actions (Chiarini et al., 2022; Cigna, 2013; Cigna, 2016). Thank to their relatively stable environmental conditions, caves can be considered “conservative environments” (Chiarini et al., 2022), able to preserve information for a long time such as paleontological and archaeological remains. However, these characteristics make caves vulnerable environments too, easily damaged by climate variations and pollution, causing an irreparable loss of scientific information and natural habitats (Chiarini et al., 2022; Gillieson, 2011). The open nature of karst systems causes water and air masses exchanged between the external environment and the internal one (Badino, 2010), making them vulnerable also to contamination by surface pollutants, which can be transported through the rock fractures and caves (Balestra et al., 2023; Ruggieri et al., 2017; White, 1988).

When cavities are transformed in show caves, an additional impact is produced (Calaforra et al., 2003; Cigna and Forti, 2013), making it important to follow strict management rules to safeguard their values (Cigna and Burri, 2000; Watson et al., 1997). The installation of lighting systems, the construction of path infrastructures and the passage of people can increase the energy balance of the cave, modify the cave atmosphere and microclimate (Lang et al., 2015a; Lang et al., 2015b), cause the soiling and the corrosion of speleothem surfaces, and introduce alien materials, such as lint, dust, pollutants, spores and other organic materials (Balestra and Bellopede, 2022b; Chelius et al., 2009; Christman, 2019), creating favourable environments for lampenflora growth and bacteria activity (Burgoyne et al., 2021; Havlena et al., 2021; Mulec, 2012; Piano et al., 2021).

Lint is usually defined as an accumulation of fluffy fibres that collect on fabric, however, in caves this term is used to indicate especially natural, artificial and synthetic fibres of clothes, together with dust, skin, hair and other organic materials, brought inside the cave by humans and transported by air or water, accumulating on speleothems and deposits. The impacts of lint in caves is poorly studied (Burger and Pate, 2001; Jablonsky et al., 1993), however, some observations have been done: lint can damage speleothems indirectly by providing nutrients for acid-producing organisms which can dissolve limestone (Jablonsky et al., 1993), and directly, being incorporated into speleothems during their growth. Before the beginning of my research, previously analysis of cave lint gave a synthetic fibre content between 30 and 75% (Christman, 2019; Jablonsky et al., 1993).

In caves, MPs can pollute karst water, be consumed by organisms, endanger the underground ecosystem and irreversibly damage speleothems and paleontological or archaeological remains. In addition, the economic impact of the possible speleothems and subterranean habitats damage is not to underestimate: show caves draw over 70 million people every year in more than 1,200 caves worldwide, amounting up to 800 million Euros in entrance fees alone, employing about 25,000 people directly and 100 times more considering connected tourist activities (Chiarini et al., 2022).

During the research fellow period in 2022, MP pollution investigations in the sediments of Bossea cave, Piedmont, Italy, started, developing the first method for MP analysis in show cave sediments (Balestra and Bellopede, 2022b). This work was later carried out during this doctorate, also working in the caves of Toirano and Borgio Verezzi, Liguria, Italy.

The aims of this study were: i) to improve the separation method for microplastic pollution detection in cave deposits, ii) to investigate, for the first time, the presence, abundance, and characteristics of MPs in the sediments of the Liguria Region show caves, Italy, and iii) to discuss data on MP pollution in deposits between Italian show caves with extremely different peculiarities from a climatic, environmental, touristic and economic point of view. Specifically, I wanted to investigate the following questions: a) are microplastics present in all show caves? b) is the MP amount always greater along the tourist route than the speleological areas? c) the MP amount increases with the decrease in the size considered?

4.24 The study area

Sediment samples were collected in Borgio Verezzi and Toirano show caves, two Ligurian karst caves of the Northwest of Italy, following the first monitoring in the sediments of Bossea show cave, Piedmont, Italy (Balestra and Bellopede, 2022b) (Fig. 41), within the national project PRIN "SHOWCAVE", a multidisciplinary research project to study, classify and mitigate the environmental impact in tourist caves (Balestra et al., 2021; Isaia et al., 2021). A summary of the main features of the three examined show caves is shown in Table 16.

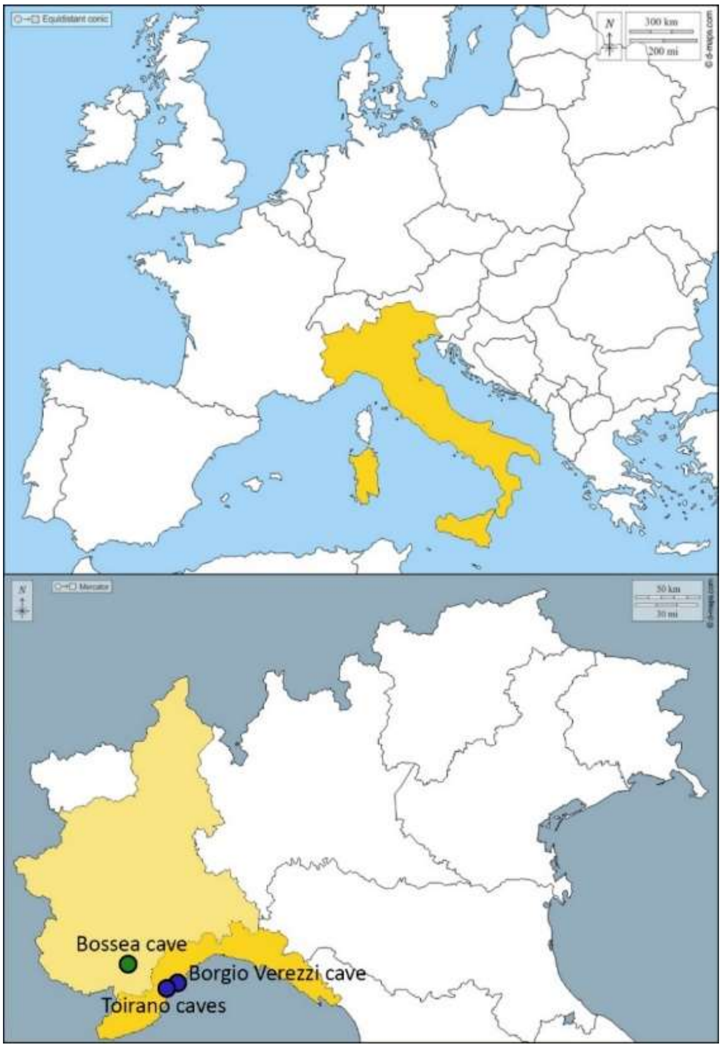

Fig. 41. Location of the examined show caves. Borgio Verezzi and Toirano caves are located in Liguria and Bossea cave in Piedmont, NW Italy. (Maps used for the plate and modified, retrieved from: https://d-maps.com/carte.php?num_car=2254&lang=it and https://d-maps.com/carte.php?num_car=24424&lang=it). Image from Balestra and Bellopede (2023).

Table 16. Main features of the examined Italian show caves: Borgio Verezzi, Toirano and Bossea caves.

	Toirano caves	Borgio Verezzi cave	Bossea cave
Italian Region	Liguria	Liguria	Piemonte
Country	Toirano (SV)	Borgio (SV)	Frabosa Soprana (CN)
Surface cover of the karst system	Woods, rocks	Woods, houses, roads	Woods, pastures, rocks, ski slopes
Altitude	186-201 m a.s.l.	32-36 m a.s.l.	836 m a.s.l.
Number of entrances	2	4	1
Air circulation	Multi-entrance cave	Multi-entrance cave	Single entrance cave
Average temperature	15°C	16°C	9°C
Linear development	1778 m	1000 m	2800 m
Total length of the tourist path	970 m	800 m	3000 m
N. tourist/year (2021-2022)	40000	33000	18000
Palaeontological and archaeological finds	Along the tourist path (Bàsura cave)	In a protected non-touristic area (near the Palaeontological entrace)	In non-touristic areas and in cases along the tourist path (Bear saloon)
Water near the tourist path	Lakes and dams	Lakes	Lakes, subterranean river, waterfall and dams
Date of sample collection	2021	2021	2020 (Balestra and Bellopede, 2022b)

4.24.1 Toirano caves

Toirano caves (Fig. 42) are located in the Toirano municipality, Liguria, Italy, and develop in the Dolomie di San Pietro dei Monti formation. They are characterized by two different caves: the Bàsura cave (Grotta della Bàsura, 186 m a.s.l., 890 long) and the Lower S. Lucia cave (Grotta di Santa Lucia inferiore, 201 m a.s.l., 778 long), connected by an artificial 110 m tunnel. The caves are thus considered a unique multi-entrance cave. Recently, an hypogenic speleogenenis origin of these caves has been supposed (Columbu et al., 2021). Bàsura cave was explored in 1950 and became touristic in 1953. In 1960, Lower S. Lucia Cave was discovered and the artificial tunnel achieved in 1967 allowed the expansion of the tourist path, organizing a one-way route of about 970 m through the two cavities. Today, the cavity receives about 40,000 tourist/year. Bàsura cave is famous worldwide for its finds: it preserves human footprints of the *Homo sapiens* groups from the upper Paleolithic (Romano et al., 2019), animal footprints (Avanzini et al., 2018), and countless bones of the extinct *Ursus spelaeus* of Pleistocene (Giacobini and D'Errico, 1985; Rellini et al., 2021; Zunino et al., 2022). Peculiar speleothems and evocative minerals characterised these caves (Martini, 2008).

Fig. 42. Toirano caves. Photo: V. Balestra

4.24.2 Borgio Verezzi cave

Borgio Verezzi cave (Grotta di Valdemino) (Fig. 43) is located in the municipality of Borgio (SV), Liguria, Italy. It was discovered in 1933 by three children who entered the cave through what is now called “the entrance of children”, at 34 m a.s.l. Other three entrances are present in this cave: the tourist entrance, 32 m a.s.l, the palaeontological entrance, 36 m a.s.l., and the British entry, 36 m a.s.l. Thanks to these multiple entrances Borgio Verezzi cave has a significant air circulation (Balestra et al., 2021).The cavity was opened to the public in 1970 and today receives more than 33000 tourist/year. The cave develops for about 1000 m in the Dolomie di San Pietro dei Monti formation, with an 800 m touristic path. Borgio Verezzi Cave genesis seem to be linked to the fresh and salt water mixing (Balestra et al., 2021). The cave is made up of a large collapse hall where a series of freshwater lakes are present, and it is rich in coloured speleothems. Different paleontological finds datable between 500,000 and 750,000 years ago were found in the cavity, witnessing the alternation between glacial and hot periods (Breda, 2015 and references therein).

Fig. 43. Borgio Verezzi cave. Photo: V. Balestra

4.24.3 Bossea cave

See Paragraph 4.2.

4.25 Method

4.25.1 Field sampling and data collection

In all examined show caves, six sampling areas of 1x1 m were defined: five near the tourist path (named with the name of the cave and a number) and one in a non-touristic cave zone (named with the name of the cave and the letter W (white)) (Fig. 44). For each sampling area, a minimum of 150 g of superficial sediments (upper 5 cm) were collected. Sediments samples were collected in 2020 in Bossea cave (Balestra and Bellopede, 2022b) and in 2021 in Borgio Verezzi and Toirano caves.

For more detailed information on sampling see Chapter 3.

Considering caves as conservative environments (Chiarini et al., 2022), although the number of tourists and the environmental conditions may vary during the year, the sediments along the tourist path are never moved; as a result, there is an accumulation of pollutants over time. The concentration of plastics in the cave sediments in the sampling areas could vary in case of exceptional flood events, which would lead the stream to invade the tourist path and/or siphon from the entrance, moving the sediments, or bringing inside the caves new water containing outside pollutants. Otherwise, in the case of water and air pollution monitoring, seasonal variation could occur, therefore, monitoring over time could be crucial.

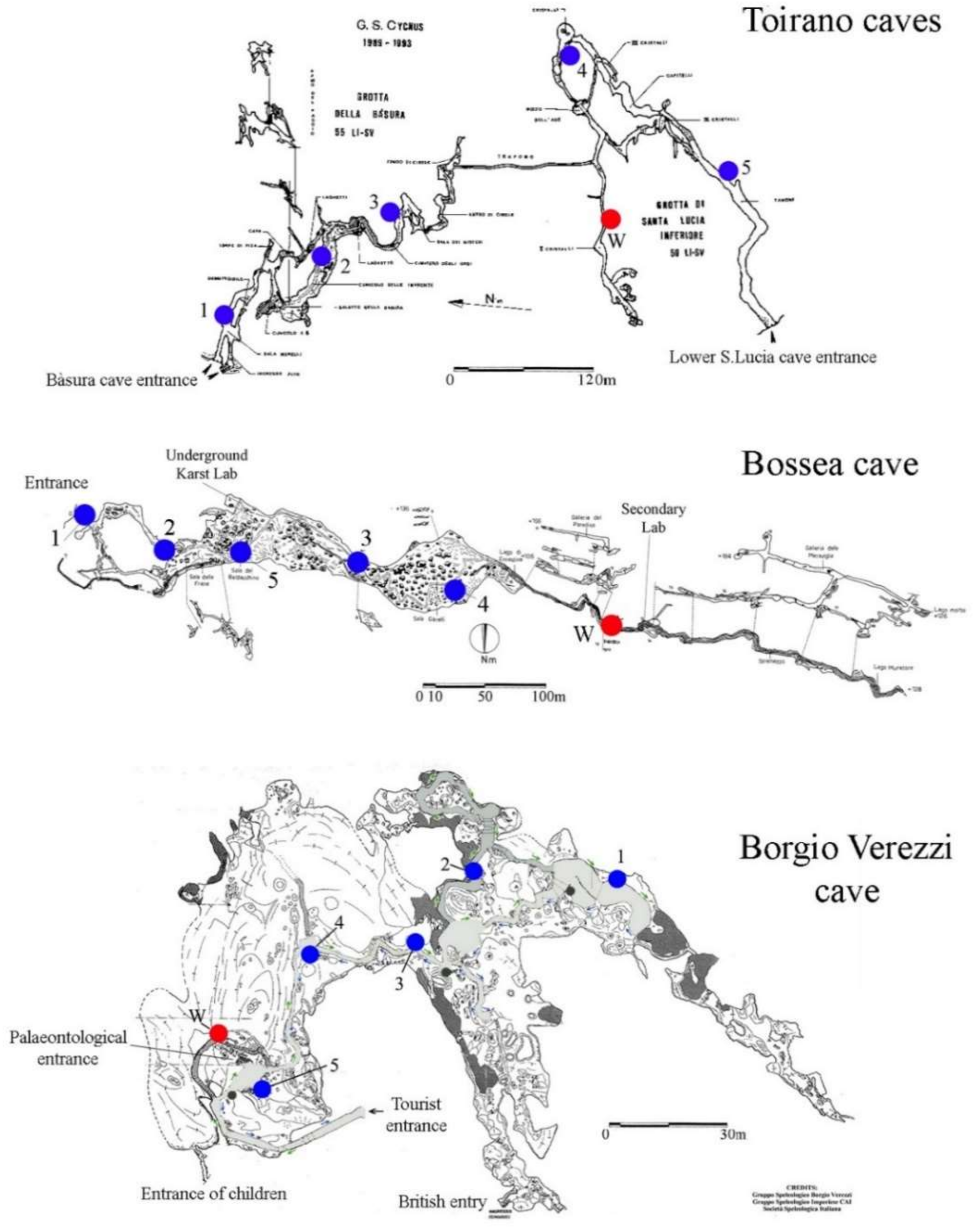

Fig. 44. Sampling areas in the three investigated show caves. Blue circles for sampling points in touristic areas, red circles for sampling points in the speleological ones. Survey of Toirano caves by Gruppo Speleologico Cycnus (from http://www.openspeleo.org/openspeleo/), modified. Survey of Bossea cave by Elia and Calleris (1988), modified. Survey of Borgio Verezzi cave by Gruppo Speleologico Borgio Verezzi, Gruppo Speleologico Imperiese CAI and Società Speleologica Italiana (from http://www.openspeleo.org/openspeleo/), modified. Image from Balestra and Bellopede (2023).

4.25.2 Laboratory analysis

Sediment samples of Bossea cave were previously analyzed using the method described in Balestra and Bellopede (2022b); samples collected in Borgio Verezzi and Toirano caves were analyzed according to this method, improved and tested for these sediments, rich in organic matter, as better explained in Chapter 3.

Blank controls on milliQ water, H_2O_2 and NaCl solutions were done to determine possible contamination during laboratory analysis.

For more detailed information on the method, see Chapter 3.

4.25.3 Microplastic identification and characterisation

Particles on filters of Bossea cave were observed with and without a UV flashlight (Alonefire SV10 365 nm UV flashlight 5W) under a Leitz ORTHOLUX II POL-MK microscope equipped with a DeltaPix Invenio 12EIII 12 Mpx Camera, with 2.5x, 4x, 10x or higher magnifications (Balestra and Bellopede, 2022b). MPs were analysed up to 0.1 mm. Particles not clearly identifiable as MPs were not take into consideration. Detected MPs were characterised according to the Standardised size and colour sorting system (SCS) (Crawford and Quinn, 2016). For more detailed information see Chapter 3.

Microplastics on filters of Borgio Verezzi and Toirano caves were counted and characterised by shape and size using the automated software MUPL (Giardino et al., 2023) through the creation of high-definition photographs under UV light. Taking into account the limitations of image analysis and the size of the pixels, MPs on filters were counted from 5 to 0.4 mm, and categorized in fibres, fragments and particles, according to the MUPL shape factor. Starting setting parameters were calibrated using MP abundance found on photos of six filters for each cave, counted using ImageJ and verified under microscope. These photos were chosen taken into account the characteristics of filters (background, number of fluorescent particles, kind of fluorescence) to best represent the entire sample. The parameters were set to have a total mean error <10% and an error on each filter <15%. MUPL band pass filter was used only on filters with a not-uniform background. Being MUPL investigations limited to fluorescent particles between 5.0-0.4, one third of Borgio Verezzi and Toirano caves filters were observed under microscope too, with and without UV light, to count and characterize smaller MPs (0.39-0.1 mm) and non-fluorescent one (5-0.1 mm).

Finally, to obtain a confirmation of the MPs identification and their chemical composition, randomly 12% of the total MPs found in the Toirano and Borgio Verezzi caves was verified using the µFTIR. Particles on glass filters were manually transferred on a silver surface (filter GVS Life Sciences, Membrane Disk 47 mm, 0.8 µm pore size) before identification. Spectra were accepted only with a match degree ≥80%, as suggested in Fossi et al. (2017).

For more detailed information, see Chapter 3.

4.26 Results and discussion

4.26.1 Notes on improved steps for microplastic separation from cave deposits

The concentration of hydrogen peroxide solution used for OMR in previous research in different environments varies from 15 to 30% (e.g. Mathalon and Hill, 2014; Prata et al., 2019; Zhang et al., 2019). According to Nuelle et al. (2014), 30%

hydrogen peroxide solution could damage MP particles and dissolve smaller ones, reducing also MP fluorescence intensity under the microscope. However, for samples rich in organic material 15% H_2O_2 could be not enough.

Analysis of sediments from the examined show caves provided several considerations for future research. Post-treatment with 30% H_2O_2 on filters is not recommended, as it damages filter surfaces - causing bubbles and uneven textures - which hinder microscopic characterization and automated image analysis. Although some of the organic matter present in cave sediments is macroscopically visible, a substantial fraction of micro-components remain, resulting in poor filter quality if left untreated or inadequately treated. As a result, pretreatment with 30% H_2O_2 (preferred over 15%) is more effective. Application of a 1:1 volume of 30% hydrogen peroxide solution directly to the sediments, prior to density separation with NaCl, yielded cleaner and more uniform samples. The filters obtained with this pretreatment showed smooth and minimally contaminated backgrounds, optimal for both microscopic identification and automated image analysis.

As it is possible to observe in Tables 17 and 18, the photos of Borgio Verezzi cave were quite uniform respect to Toirano caves ones, allowing the calibration of the MUPL parameters in shorter time, and consequently the MP characterization too. In addition, filters with a cleaner background were better also for visual identification under microscope.

Table 17. Setting parameters used with the software MUPL for Toirano caves microplastic counting on filters.

Filter (10g)	Abundance MPs	Abundance MPs MUPL	% Error	Band pass filter	Background removal filter	Canny lower threshold	Canny upper threshold	Close filter size	Close filter size iterations
Toirano 1-1		19		0.3-5.5	70	120	150	5	1
Toirano 1-2		6			10	170	210	3	1
Toirano 1-3	15	16	6.7	0.3-5.5	70	120	130	5	1
Toirano 2-1		9			30	70	200	5	3
Toirano 2-2	14	13	7.1		40	95	210	5	1
Toirano 2-3		11			30	70	200	5	3
Toirano 3-1	7	8	14.3	0.3-5.5	50	50	120	3	3
Toirano 3-2		15		0.3-5.5	50	50	120	3	3
Toirano 3-3		25		0.3-5.5	50	50	120	3	3
Toirano 4-1		6			10	170	230	3	1
Toirano 4-2		4			10	170	230	3	1
Toirano 4-3	4	4	0.0		15	110	120	3	3
Toirano 5-1		8			10	150	210	3	1
Toirano 5-2		10			10	80	230	5	2
Toirano 5-3		5			10	180	210	3	1
Toirano W1		12			40	90	110	3	1
Toirano W2	3	3	0.0		10	90	110	3	1
Toirano W3	15	16	6.7		40	90	110	3	1
Mean	9.7	10.6	5.8						

Table 18. Setting parameters used with the software MUPL for Borgio Verezzi cave microplastic counting on filters.

Filter (20g)	Abundance MPs	Abundance MPs MUPL	% Error	Band pass filter	Background removal filter	Canny lower threshold	Canny upper threshold	Close filter size	Close filter size iterations
Borgio 1-1		25			15	100	155	5	1
Borgio 1-2	10	10	0.0		15	100	155	5	1
Borgio 1-3		22			20	100	155	3	1
Borgio 2-1	25	23	8.0		15	100	155	5	1
Borgio 2-2		22			15	100	155	5	1
Borgio 2-3		18			15	100	155	3	1
Borgio 3-1		27			15	100	155	3	1
Borgio 3-2		17			15	100	155	3	1
Borgio 3-3		9			15	100	155	5	1
Borgio 4-1		17			15	80	155	5	1
Borgio 4-2		78		0.3-5.5	20	50	105	3	3
Borgio 4-3	23	24	4.3		10	100	155	5	1
Borgio 5-1		11			15	100	155	5	1
Borgio 5-2		7			15	100	155	5	1
Borgio 5-3		21			15	100	155	5	1
Borgio W1	13	13	0.0		15	100	155	5	1
Borgio W2	17	16	5.9		30	100	120	5	1
Borgio W3	10	11	10.0		15	100	155	5	1
Mean	16.3	20.6	4.7						

4.26.2 Microplastic abundance and size

The use of MUPL software (Giardino et al., 2023) allowed us to detect fluorescent MP particles between 5.0 to 0.4 mm, their size and shape. According to Crawford and Quinn (2016), MPs are categorised in microplastics (5-1 mm) and mini-microplastics (<1 mm). Microplastics were found in all cave sediment samples of Toirano and Borgio Verezzi show caves, non-touristic areas included (Fig. 45, 46).

In Toirano caves an average of 1060.0 items/kg was found in the touristic areas and of 1033.3 items/kg in the not-touristic ones (Fig. 45A, 46A). Figures 45C and 46C show the size average percentage of the collected MPs from 5 to 0.4 mm: MPs accounted for 25.3% and mini-MPs for 74.7%. One mesoplastic from 10 to 5 mm was found in sampling areas 1, 5 and in the non-touristic one.

In Borgio Verezzi cave an average of 1103.3 items/kg was found in the touristic areas and of 666.7 items/kg in the not-touristic ones (Fig. 45D, 46A). Figures 45F and 46C show the size average percentage of the collected fluorescent MPs from 5 to 0.4 mm: MPs accounted for 27.5% and mini-MPs for 72.5%. Two mesoplastics from 10 to 5 mm were found in sampling areas 3 and 4, and one in the non-touristic area.

For a data comparison about MP pollution in sediments of the Italian show cave, Bossea cave data were reworked to provide information on MPs from 5 to 0.4 mm. Figure 46 presents a comparison between the average MP abundance values within the 5-0.4 mm range, their size and shape across the three examined show caves. Bossea cave data were determined with visual counting under microscope, Borgio Verezzi and Toirano caves with MUPL automated software. In all caves MP average abundance in the touristic areas was greater than in not-touristic ones and MPs were always more than 1000 items/kg (Fig. 46A). However, the ratio of the MP amounts found in the three caves was much different. Borgio Verezzi and Toirano caves MP amounts in the touristic areas were similar (1103.3 and 1060.0 items/kg), however, MP abundance in Borgio Verezzi non-touristic areas was about half of the touristic ones (667.7 items/kg). Moreover, MP abundance in Toirano caves was similar to that found in its touristic zones (1033.3 items/kg) (Fig. 46A). Microplastic amount in the touristic areas of Bossea cave was about the double as many as those detected in Borgio Verezzi and Toirano caves (1906.7 MPs/kg), despite the number of tourists/year was about half. However, MP abundance in Bossea cave speleological areas was little more than a third of that found in the touristic zones (733.3 items/kg) (Fig. 46A).

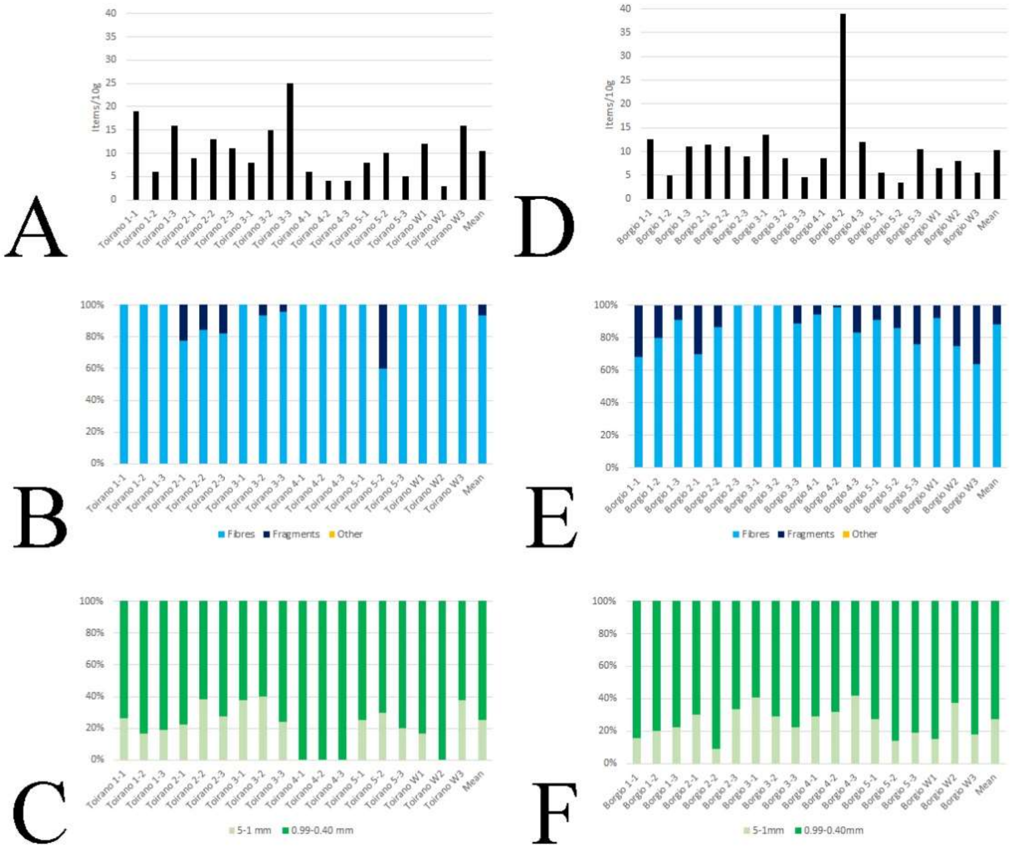

Fig. 45. Toirano and Borgio Verezzi caves samples abundance, shape and size percentages detected by MUPL software (fluorescent particles from 5 to 0.4 mm). Toirano caves samples abundance (A), shape (B) and size (C) percentages. Borgio Verezzi cave samples abundance (D), shape (E) and size (F) percentages. Image from Balestra and Bellopede (2023).

Figure 46C shows the size average percentage of the collected MPs in the three examined caves, from 5 to 0.4 mm. Despite the different OMR steps and the different methodologies used for the MP detection in the three caves, MPs<1mm accounted for more than 70% in sediments of all caves (Fig. 46C), highlighting MP amount increase with decreasing in considered size.

In the first study carried out in Bossea cave, MPs have been detected by means of visual counts under the microscope, which allowed to observe particles between 5 and 0.1 mm, highlighting the presence of an average of 4390 items/kg dry sediments along the tourist path and 1600 items/kg in the speleological section of the cave; about 60% of MPs found in Bossea cave sediments were shorter than 0.5 mm (Balestra and Bellopede, 2022b). MUPL automated software is a valid and very quick method to count and characterize larger MPs, however, particles below a certain size are impossible to be identified with image analysis and only fluorescent MPs are counted. Visual identification under microscope could be a useful method to identify not-fluorescent MPs and detect smaller particles. Therefore, one third of Toirano and Borgio Verezzi caves filters were observed under microscope too, with and without UV light, to count and characterize MPs up to 0.1 mm and compare data. Microplastic abundance, size and fluorescence in Bossea, Borgio Verezzi and Toirano caves obtained with visual counting under microscope are reported in Fig. 46D, E, F.

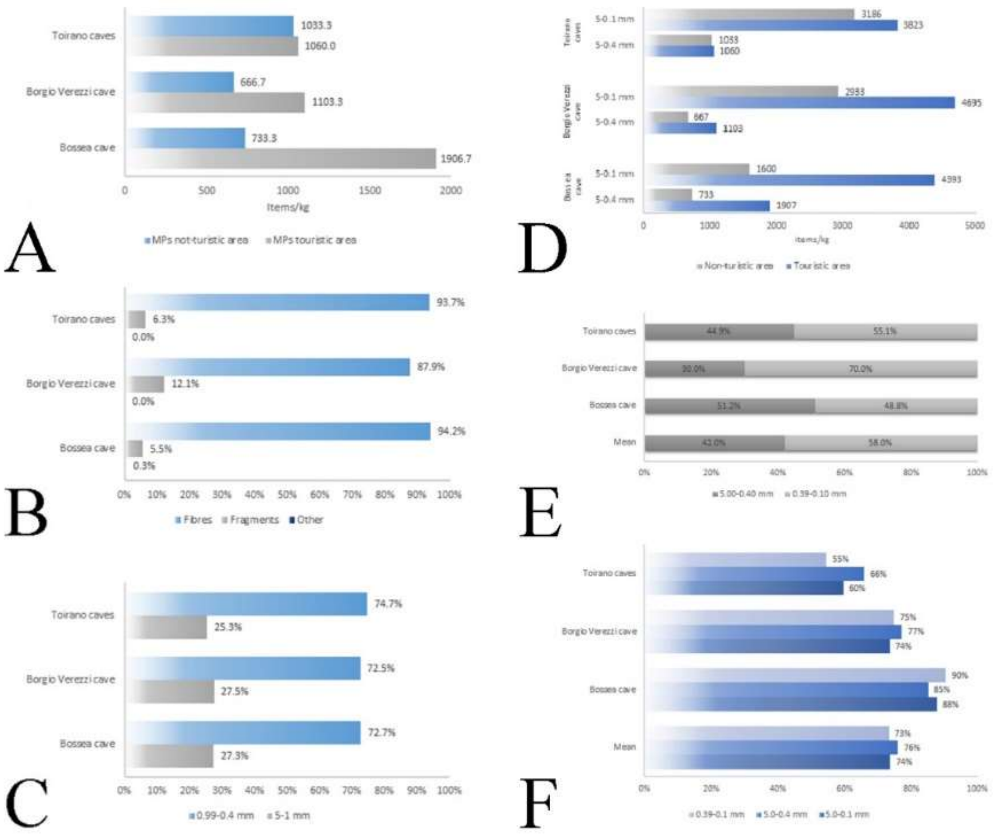

Fig. 46. Comparison between MP abundance values and their morphological characteristics average percentages of the three examined show caves. A: MP abundance (5-0.4 mm fluorescent particles only); B: MP shape (5-0.4 mm fluorescent particles only); C: MP size (5-0.4 mm fluorescent particles only); D: MP abundance considering different size range; E: MP size (visual counting under microscope for 5-0.1 mm particles); F: Fluorescent MP percentages considering different size ranges (visual counting under microscope for 5-0.1 mm particles). Image from Balestra and Bellopede (2023).

Microplastic abundance between 5-0.4 mm and 5-0.1 mm in the three examined show caves are shown in Figure 46D, highlighting the MP amount increase with decreasing in considered size, as shown in figure 46C. Moreover, the difference in the MPs amount between the tourist and non-tourist areas of the examined caves were visible both from the results obtained with MUPL analysis (5-0.4 mm) and visual identification under microscope (5-0.1 mm), despite the particle quantities was significantly different (Fig. 46D). In Toirano caves an average of 3823 items/kg dry sediments along the tourist path and of 3186 items/kg in the non-touristic area was detected. In Borgio Verezzi cave an average of 4695 items/kg dry sediments in the tourist area and of 2933 items/kg in the speleological one was detected. Borgio Verezzi and Bossea caves exceeded 4000 items/kg in the touristic area, however, MPs were considerably less in the non-touristic one (2933 items/kg in Borgio Verezzi cave and 1600 items/kg in Bossea cave) (Fig. 46D). Instead, in Toirano caves MP abundance exceed 3000 items/kg in both touristic and non-touristic areas.

The high MP amount in the examined show cave sediments could be linked to the conservative cave environment. Microplastic amounts in the touristic areas of the caves could be related to the number of visitors, to the time of tourist visits and to the extended stay time in some characteristic zones of the caves. However, it is not possible to exclude MP contamination from the external environments, due to the open nature of the karst systems. Climate variations and human activities in the recharge area of the karst systems or near the cave entrances could contaminate underground environments, such as tyre pollution transported by air flow inside the cave or pollutants carried by groundwaters and percolation waters (Balestra et al., 2023).

As Toirano caves are located in a relatively wooded valley, MPs discovered in the cave sediments were probably originated from the tourist activities in the show cave, bringing into the cave especially synthetic fibres of clothes and garbage. However, it is not possible to exclude MP contamination from the external environments. The non-touristic area of the Toirano caves is rich in beautiful speleothems and mineral formations and it is easy to access, therefore, it is often visited by speleologists and researchers which can carry particles in this zone. Moreover, in the Toirano caves washings of the tourist path are carried out with pumped water, which can move the deposited material, accumulate it in certain areas or remove it from others.

Microplastics discovered in the sediments of Borgio Verezzi cave could be mainly originated from the daily tourist activities, bringing into the cave synthetic clothes, lint, dust and garbage. Different concerts and events have been made into the cave in summer seasons and Christmas holidays, reaching also a hundred of person in a single night. Moreover, the surface area above this cave is covered by woods, olive trees, rocks, houses and roads built in the past. The paleontological entrance is located in a private farmland, the British entry near the municipal road, the tourist entrance and the entrance of children near houses and restaurants. Therefore, it is possible that the MPs found in this cave could came from the surface activities. During particular rainy and flood events, spills of surface streams were recorded, flowing directly into the cave through the main entrances, which brought into the cavity different materials such as organic matter and plastic, including in the non-touristic area. Moreover, in the past, the non-touristic area was crossed by the tourist route, therefore, the material transported by tourists could still be present. Finally, the air currents present in the cave often head towards this area, so they could be a source of transport of micro particles.

The high number of MPs found in Bossea cave could be linked to the fact that it has been open for much longer than the other examined show caves. However, high values could be related also to the air flows and the external exchanges, very different from those in Borgio Verezzi and Toirano caves, because of the presence of a single entrance in Bossea cave and of the large size of the internal halls of the cavity. The examined non-touristic area of the Bossea cave is less easily accessible than those of the other two caves, however, it is travelled by speleologists and researchers, therefore, there may be transport and storage of particles. In addition, the area is crossed by an underground collector and different secondary inputs which could transport MPs in this area. However, the water flow could also bring downstream the accumulated material during flood events, cleaning or polluting the area.

At the moment of this research, comparisons with other show caves were possible only with few cavities of the world. In Valentić et al. (2022) MPs pollution in sediments and water of two karst region of Slovenia was investigated. In the Postojna region the authors collected samples in the Postojna cave system and in the Planina cave system, finding MPs only in Postojna cave water. In the Škocjan region, about 60000 items/m^3 were found in Škocjan caves system sediments and about 6667 items/m^3 in Kačna cave sediments, finding MPs also in the water samples; instead, any MP was found in Jama 1 v Kanjaducah. These values are very low compared to those found in the examined Italian show caves, especially considering 38 million tourists visited Postojna cave to date (Šebela, 2019). However, the used methods were different and rimstone dams in the Postojna cave system are regularly cleaned with water by the management company (Mulec, 2014; Valentić et al., 2022), therefore, the quantity of MP in the Postojna–Planina cave system could be much greater than the values found in this study, as highlighted by the authors. It is however very interesting to observe that also in Slovenian karst systems the highest concentrations of MPs were found in the tourist parts of both examined cave systems.

Figure 46E highlights that an average of 58% of MP particles analysed under microscope had a dimension between 0.39 and 0.1 mm, emphasizing MP abundance increase with the decrease in the size considered and underlining the importance of combining several methods for monitoring MP pollution in environments. MUPL automated software allowed to identify and characterise MPs from 5 to 0.4 mm in a short time and with an error less than 6%, instead, visual identification under microscope on part of filters, allowed to detect a larger number of MP particles of small dimensions. The combination of several methods is useful to improve and validate the results, however, each method has its limits: in this case, the used methodologies do not allow the identification of particles smaller than 0.1 mm. Therefore, the presence of smaller MPs or nanoplastics was not monitored and the plastic amount present in the cave sediments could be reasonably much greater than the quantities found so far.

4.26.3 Microplastic shape

In Toirano caves fibre shape dominated all cave sediments samples (93.7%), while 6.3% of particles were fragments (Fig. 45B, 46B). In Borgio Verezzi cave fibre shape dominated all cave sediment samples (87.9%), while 12.1% of particles were fragments (Fig. 45E, 46B). Other shapes were not detected by the MUPL software in both caves.

Comparing the data of the three examined caves (Fig. 46B), fibres represented the majority of the MPs present in the sediments of all caves (87.9-94.2%), followed by fragments (5.5.-12.1%); other shapes were present in not relevant quantity (0.3%) or absent. Also in this case, the values found for the sediments of the three caves were very similar, despite the use of different methodologies for the MP detection and characterization. Up to 60% of world textiles production are synthetic (Barrows et al., 2018; Boucher and Friot, 2017), suggesting that synthetic clothes could justify the high quantity of MP fibres found in cave sediments. Microplastic fragments could have been produced during the electric systems works, during the activities to make the cave tourist or from waste deterioration.

4.26.4 Microplastic fluorescence

Figure 46F underlines the importance of visual identification under microscope, in order to not lose the non-fluorescent particles (about 25%) during MP detection. The fluorescent particle abundance percentages varied for each cave, however, they are similar regardless of the size taken into the account for each cave (Fig. 46F): about 60% in Toirano caves, 74% in Borgio Verezzi cave, and 88% in Bossea cave, with a percentage mean value of about 74%.

4.26.5 Characterization of microplastic by µFTIR-ATR

In Borgio Verezzi cave sediments were found PA (polyamide), PE (polyethylene), PET (polyethylene terephthalate), PVAc (polyvinyl acetate), PVFM (polyvinyl formal), PAM (polyacrylamide), EVOH (ethylene vinyl alcohol) and copolymer (Fig. 47).

Sediment samples of Toirano caves contained PET, PAM, PP (polypropylene), EVA (ethylene vinyl acetate) and copolymer (Fig. 47).

The most common identified particles are polyesters (PET) and polyolefins, such as PE and PP. Most of the analyzed particles were identified by the FTIR library as plastic materials, however, it was established to validate only spectra with match ≥ 80% as suggested by Fossi et al. (2017). Spectra of PP and PET are shown in Fig.s 48 and 49.

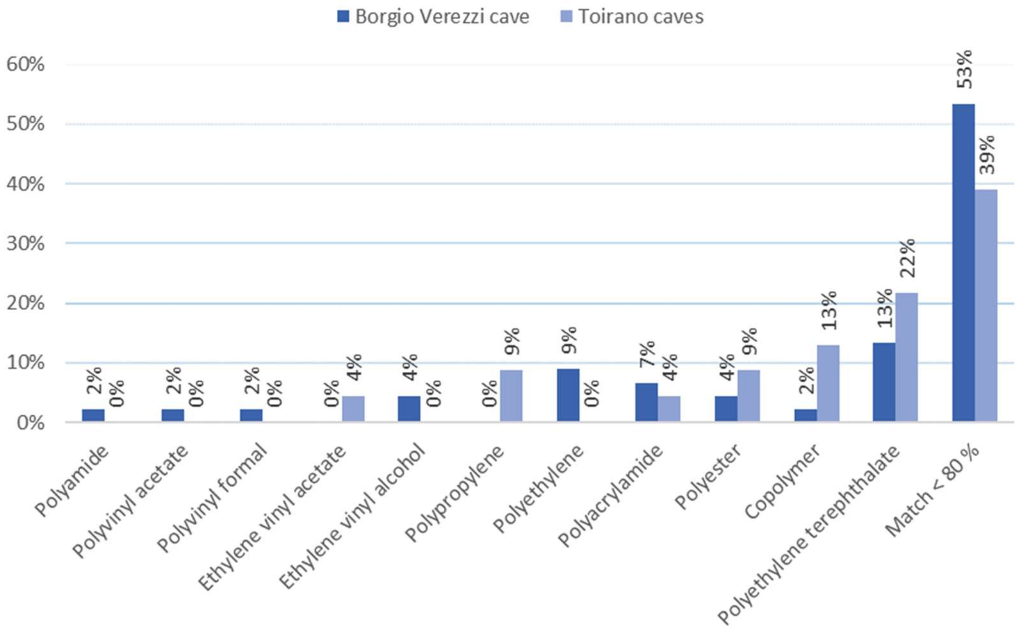

Fig. 47. Plastic typology found on 12% of the total MPs of Borgio Verezzi and Toirano caves. Image from Balestra and Bellopede (2023).

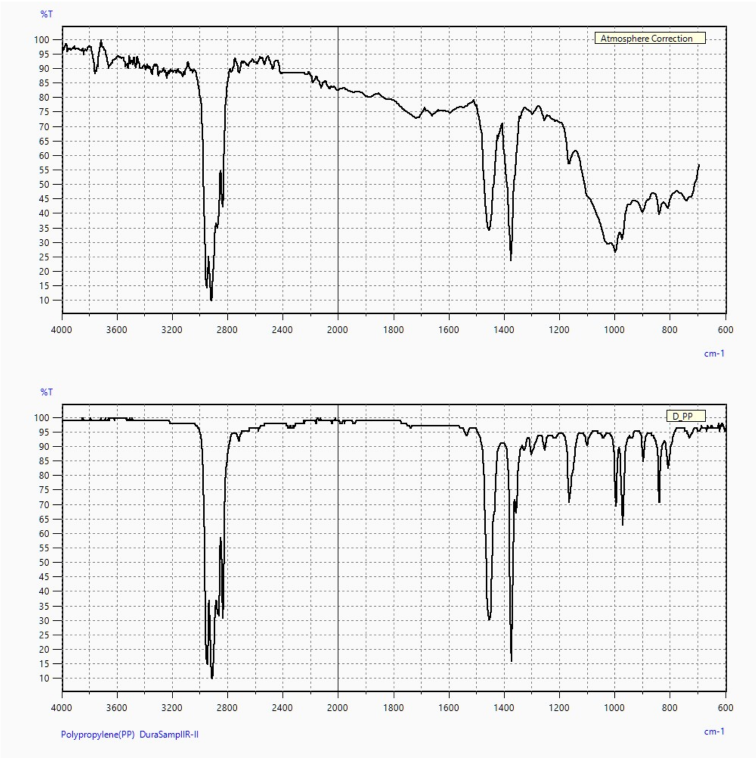

Fig. 48. Spectrum of PP found in the sediments of Toirano caves. µFTIR Shimadzu AIM-9000 microscope equipped with a Shimadzu IRTracer-100 spectrophotometer and a Shimadzu ATR with a germanium prism. Match 86.9%.

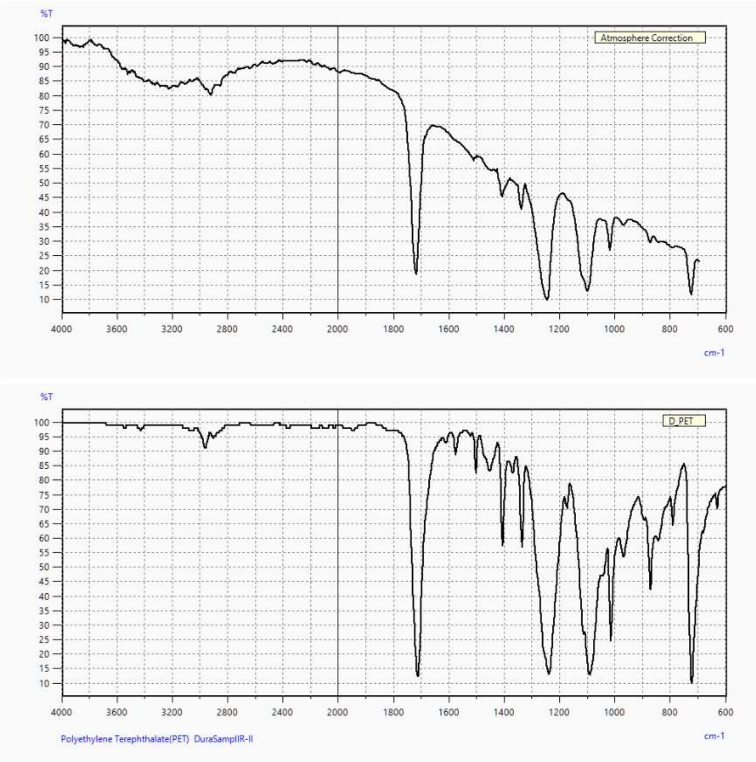

Fig. 49. Spectrum of PET found in the sediments of Borgio Verezzi cave. µFTIR Shimadzu AIM-9000 microscope equipped with a Shimadzu IRTracer-100 spectrophotometer and a Shimadzu ATR with a germanium prism. Match 85.5%.

MP identification by spectroscopy is useful to understand the possible origin of pollution: many of the plastics found in Borgio Verezzi and Toirano caves are used in the production of textiles, supporting the assumptions on the primary origin of microplastics in cave sediments due to the tourist clothes.

Comparisons with other show caves during the research period, in 2022-2023, were possible only with Slovenian cavities. PE, PET and PP were found in sediments of Slovenian caves of the Škocjan region, together with PU (polyurethane) microparticles (Valentić et al., 2022). Being the karst systems open environments, it is important to take into account also the polymers found in the water of the karst systems: PE, PET, PP, PA, PS (polystyrene) have been found frequently, together with PVA (polyvinyl alcohol), polyester, EVOH, PVC (polyvinyl chloride), acrylic adhesive, PU, polyacrylamide, EVA, EPDM (ethylene propylene diene rubber), PMMA (polymethyl methacrylate), PBT (polybutylene terephthalate), PTFE (Polytetrafluoroethylene), copolyester, TPV (thermoplastic vulcanizates) and EBA (ethylene butyl acrylate) (An et al., 2022; Balestra et al., 2023; Romano et al., 2023; Valentić et al., 2022).

4.27 Conclusions

This study documented the presence of MPs in sediments of all examined show caves, filling a gap in the study of MP pollution. Improving organic matter removal technique to separate MPs in cave sediments helped to get better filter for particles identification and characterization. Visual identification under microscope and MUPL automated software was used to obtain as much information as possible and compare data, underlining the importance of combining different methods. MUPL automated software is a valid and very quick method to count and characterize larger MPs, instead, visual counting under microscope is useful to identify non-fluorescent MPs and detect smaller particles, which significantly increase as the size decreases. MPs were present in touristic and non-touristic areas of all caves, with higher amount along the tourist paths. Fibre-shape microplastics less than 1 mm, and polyesters and polyolefins dominated the samples, advising that synthetic clothes are the main source of MP pollution in show caves. Other possible sources of MP pollution in show caves could be linked to tourism activities and surface pollution, providing useful references for further research.

The importance of pollutant monitoring in underground and surface karst environments was emphasizing for conservation purposes, especially regarding important natural resources such as drinking water. MP monitoring is crucial in karst environments to establish the current degree of pollution and define strategies for the protection and management of these geological heritages and their resources, even providing education and implementing new strategies for sustainable development.

4C. Microplastic and microfibre pollution in Torri di Slivia cave dripping water

Origin of the Chapter
This chapter is adapted from the peer-reviewed article "Falling from the ceiling: first evidence of microplastic pollution in cave dripping waters" authored by Balestra et al. (Under review), under review in Journal of Environmental Management.
The author of this dissertation was responsible for term, conceptualisation, methodology, validation, formal analysis, investigation, resources, data curation, writing – original draft, writing – review and editing, visualisation.

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4.28 Introduction

Given their potential impact on ecosystems, the simultaneous analysis of both MPs and other anthropogenic microparticles (AMPs), especially MFs, is gaining importance in environmental research. Although studies on these micropollutants, particularly MPs, have expanded recently, they focused especially on marine environments and biota (e.g. Cutroneo et al., 2020; Leistenschneider et al., 2021; Ugwu et al., 2021). Terrestrial environments remain poorly studied, such as karst areas, despite their high pollution risk.

In karst systems, the presence of MPs and other AMPs is linked to direct and indirect human activities, and is strongly influenced by aquifers hydrodynamics, geological features, karstification degree, local meteorological conditions, and air circulation. In caves, direct sources of contamination include tourism in show caves and the presence of speleologists and researchers in the speleological ones, or litter. Indirectly, pollution in underground environments can originate from litter, landfill, wastewater discharge, agriculture, transports, industrial production, surface water and soil pollution, and athmospheric deposition on the surface (e.g. Allen et al., 2019; Liu et al., 2019b; Oppeltová et al., 2024; Zhou et al., 2021). Once in the soil, these materials can be reduced in dimension and transported especially though leaching in soil pores and rock fractures, which involves their movement into deeper soil layers, accumulating in subterranean environments and waters (Chia et al., 2021; Fahrenfeld et al., 2019; Frei et al., 2019; Lwanga et al., 2017; McGechan, 2002; Pérez-Lucas et al., 2018; Viaroli et al., 2022; Wanner, 2021). Biological, chemical, and physical processes can control pollutants migration into underground environments and waters too (Stoppiello et al., 2020).

To date, only a limited number of studies have investigated MP and MF pollution in caves, focusing mainly on sediments and water samples, in unexplored, speleological and show caves (Balestra and Bellopede, 2025; Piccardo and Bevilacqua, 2024 and references therein). However, scientific literature on this topic is mostly focused on show caves, as they offer easier access forresearchers and present relatively minor logistical difficulties for fieldwork. Show caves can play an important role in pilot studies and advancing knowledge on underground pollution, testing new methods and analyzing preliminary data to verify the health of karst system. Few research has taken into account the entire karst system, from surface to underground environments (e.g. Balestra et al., 2023), and, currently, little is known about contamination sources and potential transport routes in caves. Dripping water in caves can be a significant source of pollution, as water infiltrates through land that may already be contaminated. Into the soil, an accumulation of micropollutants can occur, increasing the possible contamination of water. However, to date, percolation water has never been specifically analyzed.

Evaluating MPs and other AMPs in caves can provide important insights for the management and conservation of karst systems, especially for water, highlighting how indirect pollution can contaminate subterranean environments. In this study, dripping waters were investigated for the first time. Monitoring was conducted in Torri di Slivia karst cave, Friuli-Venezia-Giulia, Italy. The aims of this study are: i) to examine, for the first time, the presence of MPs and other AMPs, especially MFs, in cave dripping waters; if detected, ii) to assess their abundance and characteristics, probable sources, potential risks and ecological impacts that such pollutants may pose to karst environments. This study represent the first preliminary assessment of MP and other AMP pollution in cave dripping waters, with the aim of promoting effective conservation strategies for karst areas, which integrate both surface and subterranean components, especially for water resource protection.

4.29 The Study area

The Torri di Slivia cave, called also Pejca v Lazcu in Slovenian (22-39VG), is located in the Duino-Aurisina municipality, Friulia-Venezia-Giulia Region, in the Italian sector of the Classical Karst Region, at about 109 m a.s.l (Fig.s 50, 51). The cave has two entrances, an artificial one for tourists and a big natural pit of about 30 m (Fig. 61). It develops for about 553 m in Mesozoic (Cretaceous) carbonate rock, (Calcareniti del Molassa formation), primarily massive and fractured limestones, with a total drop of 101.5 m.

The Classical Karst Region (Kras) is a limestone plateau of approximately 900 km^2 spanning NE Italy (Friuli Venezia Giulia region) and SW Slovenia. It extend for about 15-20 km in width and about 40 km in length in the SW-NW direction (Visintin and Cucchi, 2010). The region gives its name to global karst terminology, as the first karst phenomena studies originated here (Gunn, 2004). The plateau is primarily composed of Cretaceous to Eocene carbonate rocks (Comeno/Komen Unit) (Cucchi et al., 1987; Placer, 1981), and is heavily karstified, exhibiting a large number of surface morphologies and over 3,500 known caves in the Italian sector (Zini et al., 2010). Due to their high quality and abundance, the waters of the

Classical Karst have historically supported the regional economic and social development (Zini et al., 2010). However, extensive human intervention has significantly and irreversibly altered this area and the system hydrology (Fornasir, 1929; Gemiti, 2004; Marocco and Melis, 2009). Urbanization, commercial and industrial activities have heightened the vulnerability of these resources in recent decades (Zini et al., 2010).

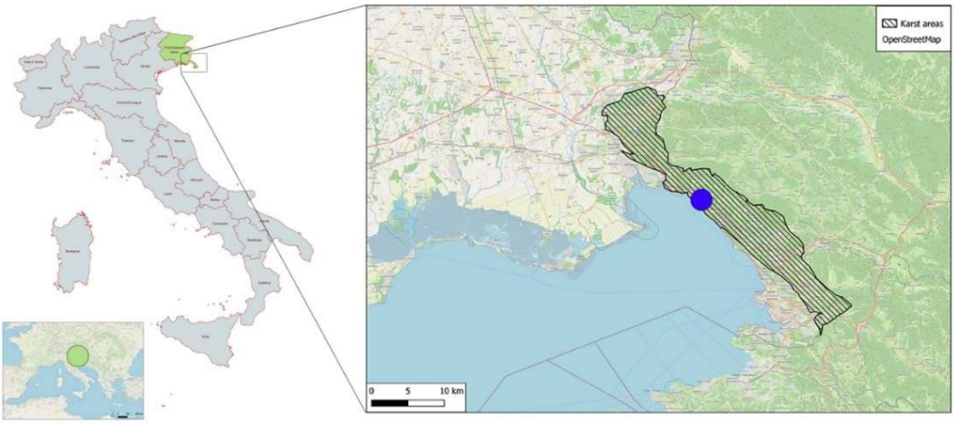

Fig. 50. Study area in the Italian sector of Classical Karst, Friuli-Venezia-Giulia Region. Karst area in black hatch, Torri di Slivia cave in blue circle (Italy map created with mapchart.net, Europe and detail of Italian Classical Karst created with QGIS Desktop 3.12.1 with GRASS 7.8.2 using OpenStreetMap map, modified - openstreetmap.org/copyright).

Different zones of this area are now under environmental protection, however, karst systems are open and close to urban zones, industrial areas, railways and highways, posing ongoing threats to habitats, species and potable waters. Torri di Slivia cave opens near the right carriageway of the Carso highway (Trieste – Monfalcone direction), crossed by the railway of the Trieste – Monfalcone line. Consequently, this cavity results a perfect natural underground laboratory for the study of MP and other AMP pollution in dripping water.

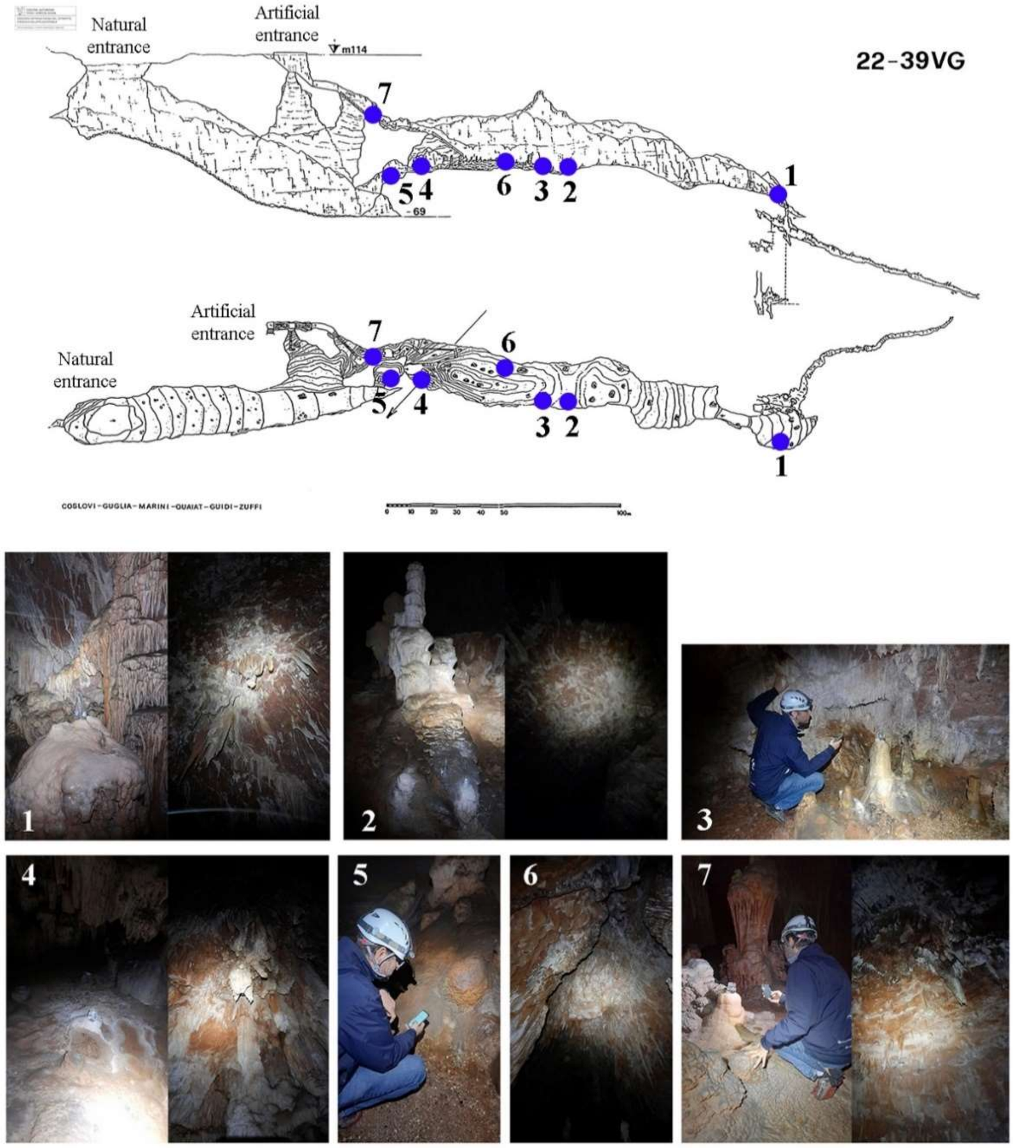

Fig. 51. Torri di Slivia cave surveys with sampling points (blue circles) (Cave surveys by CGEB - Commissione Grotte Eugenio Boegan from https://catastogrotte.regione.fvg.it/scheda/22-Grotta_delle_Torri_di_Slivia; photos by V. Balestra).

4.30 Method

4.30.1 Field sampling

Seven sampling points at different distance from the entrance were defined in Torri di Slivia cave (Table 19, Fig. 51), based on the environmental characteristics of the caves and on the surface morphologies. Samples were named with "Slivia" and the number of the sampling point (from 1 to 7). Monitoring was conducted at the end of March 2025, after an important rainy event.

Table 19. Characteristics of the sampling points and collected samples.

Sampling point	ml	Drops/minute	Characteristics of the sampling point
Slivia 1	102	30	End of the cave, in the oldest and not-touristic part of the cavity. Water was collected under a wall covered by red and white stalactites. Dripping water creates a large stalagmites.
Slivia 2	64	14	At the end of the tourist route, under a white stalagmite. The most distant point from the ceiling. Slow dripping.
Slivia 3	136	31	At the end of the tourist step with the anti-slip mats. Water was collected from a fast dripping from the low wall.
Slivia 4	140	106	Fast dripping from stalactites on the ceiling. The dripping water creates different rimstone dams.
Slivia 5	16	4	At the end of the tourist steps, in a pit with a gavel bottom. Slow dripping near a calcite flow.
Slivia 6	129	94	Under the roost of the bat colony, near big dams.
Slivia 7	132	28	At the end of the first tourist step. Waters was collected from a fast dripping from the ceiling, creating a stalagmites.

The limited availability of dripping water further complicates analysis and monitoring efforts. Despite cave ecological importance and the necessity to understand sources and transport mechanism of microplastics in underground environments and groundwaters, these environments are still little studied. Therefore, any sampling and subsequent analysis are key to improving our understanding of ecosystem health and the indirect impacts of anthropogenic activities. In particular, show caves can play a key role in pilot studies and the testing of new methods. In accordance with the precautionary principle and considering the environmental conditions and the limited availability of dripping water in the cave studied, the volume of the collected sample was limited to approximately 150 ml.

Water bulk samples (Hidalgo-Ruz et al., 2012) were collected using nitrile gloves, dropping water inside glass jars pre-cleaned with bi-filtered absolute ethanol and MilliQ water. Glass jars were left on site for about 20 minutes and then immediately closed with the cap. Blank control was performed using one empty glass jars, placed near the sampling point, for the same time. The jars were coated with anti-impact material to transport them safely in a backpack. Samples were refrigerated at 6 ◦C until analysis in the laboratory.

4.30.2 Laboratory analysis

Chemicals and MIlliQ water were filtered twice through a 1.2 µm glass fibre filter (Enrico Bruno srl) before use, avoiding contamination. Blank control on air was performed. The blank correction method applied in this study follows Shruti and Kutralam-Muniasamy (2023 and references therein)) and references therein, whereby the contribution of pollutants identified in the blanks is subtracted from the results of the analysed samples.

Water samples were analyzed following the previous procedures outlined for water samples in caves and karst systems (Balestra et al., 2024a; Balestra et al., 2024b). The collected water samples were subjected to organic matter removal

(OMR) using an amount of 30% H_2O_2 solution equal to half the sample. Samples were shaken (50 revs/min) for 2 hours at 40°C in a Shaker Water Bath SBS30, Bibby Stuart Scientific. Waters were then filtered through a 1.2 µm pore size silver filter (GVS Life Sciences, Membrane Disk 47 mm).

For more detailed information about contamination control and the method, see Chapter 3.

4.30.3 Microparticle identification and characterization

A combination of microscopic and spectroscopic techniques was employed for a more comprehensive study of MPs and other AMPs.

Microparticles retained on filters were initially examined under a Leitz ORTHOLUX II POL-MK microscope equipped with a 12 Mpx DeltaPix Invenio 12EIII Camera. For MF analysis, detailed morphological comparison was carried out using longitudinal and x-sectional images of natural, regenerated and synthetic fibres (e.g. Khan et al., 2017). Each filter was examined under visible light and a UV 365 nm flashlight. Microparticles between 0.05 and 5 mm were analyzed. Microparticles that could not be confidently identified as MPs or other AMPs were excluded from the analysis.

Spectroscopic analysis on microparticles found on the filters were performed with the micro-Fourier Transform Infrared Spectroscope (µFTIR) Nicolet iN10MX, Thermo Fisher Scientific, in reflection mode, within the spectral range of 4000 to 650 cm^{-1}, with 24 scans taken per sample. The spectra were analysed with the Omnic Picta software and verified visually by the operator. Only spectra with a match degree ≥ 70% were accepted.

For more detailed information about microscopic and spectroscopic techniques used, see Chapter 3.

4.31 Results

4.31.1 Abundance and composition

Based on the values obtained from the blank samples, potential airbone contamination linked to sampling in cave turned out to be about 21 items/sample, and 13 items/sample for laboratory activities, all not synthetic.

MPs were found in three sampling areas, distributed in all the cave: Slivia 2, 4, 6 (Tables 20, 21 and Fig. 52). The value was estimated from 0 to 23 MPs/L of dripping water, with a mean and standard deviation of 6.6±8.7 MPs/L (Table 20). These sampling points were located in the stretch of cave between the railway and the highway E70 (Fig. 52). In particular, Slivia 4 sampling point was exactly under the highway E70 border, and Slivia 2 near the railway (Fig. 52).

Taking into account also natural and regenerated materials of anthropogenic origin, four monitored sampling points resulted polluted: Slivia 2, 4, 6 and 7 (Tables 20, 21 Fig. 52). The total quantities of AMPs found in the sampling points were estimated from 0 to 143.9 items/L, with a mean and standard deviation of 33.8±50.6 items/L (Table 20). Slivia 7 sampling point resulted exactly under the highway E70 (Fig. 52).

Considering the amounts found for each sampling point, Slivia 7 was the most polluted, however, Slivia 6 is the only point with both MPs and natural and regenerated MFs of anthropogenic origin and the one with the major number of found MPs (n=3). Slivia 1, 3 and 5 were not polluted.

Table 20. Anthropogenic microparticle (AMP) abundances for each cave dripping water sample in Torri di Slivia cave, and estimates for one liter of water. AMPs were divided into synthetic (microplastics) and not synthetic (natural and regenerated). Reported values were corrected by subtracting the values found in the blanks

Sampling point	ml	Not synthetic	Synthetic	TOT	Not synthetic /L	Synthetic/L	TOT/L
Slivia 1	102	0	0	0	0.0	0.0	0.0
Slivia 2	64	0	1	1	0.0	15.6	15.6
Slivia 3	136	0	0	0	0.0	0.0	0.0
Slivia 4	140	0	1	1	0.0	7.1	7.1
Slivia 5	16	0	0	0	0.0	0.0	0.0
Slivia 6	129	6	3	9	46.5	23.3	69.8
Slivia 7	132	19	0	19	143.9	0.0	143.9
TOT		25	5	30			
MEAN		3.6	0.7	4.3	27.2	6.6	33.8
St. dev.		6.6	1.0	6.7	50.3	8.7	50.6

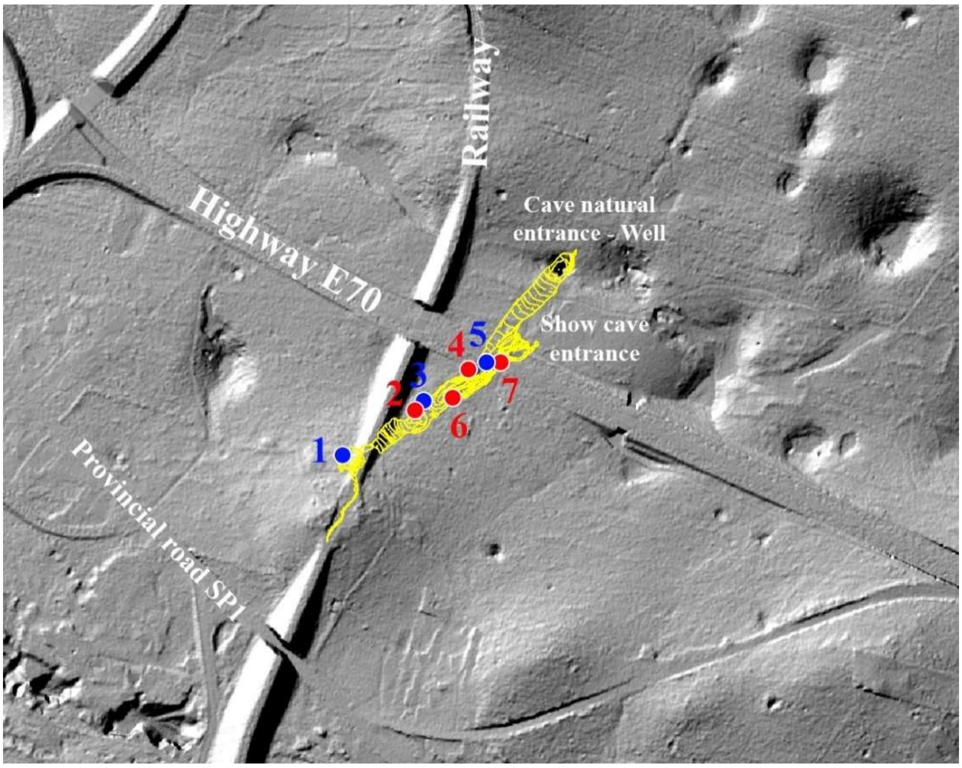

Fig. 52. Sampling points with microplastic pollution respect to surface human activities. Torri di Slivia cave survey in yellow, sampling points with microplastics and/or other anthropogenic materials in red circles, sampling points without pollution in blue circles (Regione Autonoma Friuli Venezia Giulia Lidar survey 2017-2020, modified).

Microscopic and spectroscopic analysis showed that 80.5% of AMPs were natural and regenerated materials of anthropogenic origin, and 19.5% synthetics (Fig. 53). Of the not synthetics, all materials were cellulosic, except for one woolen MF found in Slivia 7. µFTIR analysis with good spectra (>70%) confirmed the presence of polyethylene terephthalate (PET) in Slivia 4 (n=1) and 6 (n=3), and polyurethane (PU) in Slivia 2 (n=1) (Fig. 53).

Table 21. Microplastic characteristics in the Torri di Slivia cave dripping water.

Sampling point	Shape	Size [mm]	Colour	Fluorescence	Type
Slivia 2	Fragment	0.48	Beige	Yellow	PU
Slivia 4	Fragment	0.15	Transparent	Blue	PET
Slivia 6	Fragment	0.17	Beige	Green	PET
Slivia 6	Fibre	0.08	Green	Green	PET
Slivia 6	Fragment	0.11	Beige	No	PET

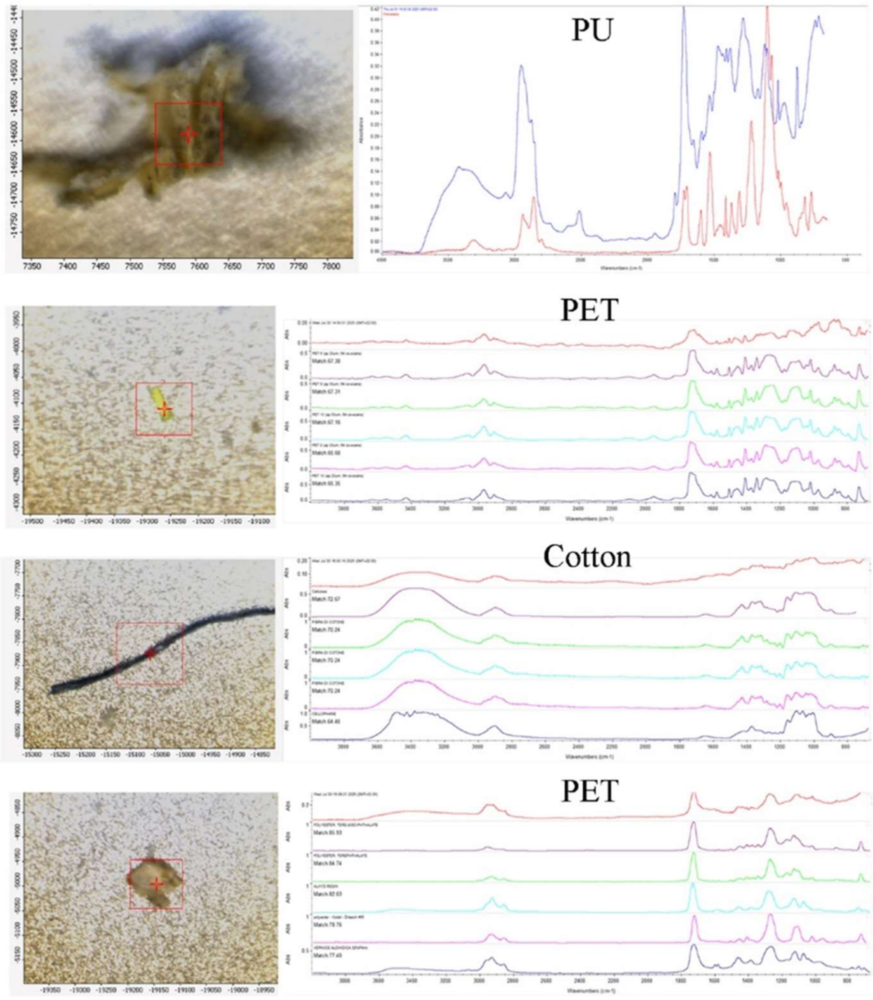

Fig. 53. µFTIR spectra of some microparticles of anthropogenic origin found in the dripping water of Torri di Slivia cave, Italy.

4.31.2 Shape and size

Fibre-shape dominated all samples (81.6%), followed by fragments (17.1%) and film (1.3%) (Table 22, Fig.s 54A, B). All MPs were fragments (n=4), except for one PET fibre. Of the other AMPs, the 94% were natural and regenerated MFs.

Big AMPs (5-1 mm) accounted for only 10.7%, while AMPs < 1 mm were the most abundant (89.3%) (Tab. 22, Fig.s 54C, D). Most microparticles had size between 0.49 and 0.10 mm (51.5%) (Table 22, Fig.s 54C, D).

Table 22. Anthropogenic microparticle characteristics found in the dripping water of the Torri di Slivia cave.

				Fluorescence		Fluorescence colour				Size				Shape		
Sampling point	ml	Items	Items/L	Fluorescent	Not fluorescent	Blu	Red	Green	Other	5-1 mm	0.99-0.50 mm	0.49-0.10 mm	0.09-0.05 mm	Fibre	Fragment	Film
Slivia 1	102	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Slivia 2	64	1	15.6	1.0	0.0	0.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0
Slivia 3	136	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Slivia 4	140	1	7.1	1.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0
Slivia 5	16	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Slivia 6	129	9	69.8	7.0	2.0	5.0	0.0	2.0	0.0	1.3	1.3	5.1	1.3	7.0	2.0	0.0
Slivia 7	132	19	143.9	18.2	0.8	17.5	0.8	0.0	0.0	1.9	4.6	8.4	4.2	17.5	1.1	0.4
TOT		30.0		27.3	2.7	23.5	0.8	2.0	1.0	3.2	5.9	15.4	5.5	24.5	5.1	0.4
Mean		4.3	33.8	3.9	0.4	3.4	0.1	0.3	0.1	0.5	0.8	2.2	0.8	3.5	0.7	0.1
st dev		6.7	50.6	6.3	0.7	6.0	0.3	0.7	0.3	0.7	1.6	3.0	1.5	6.2	0.7	0.1

4.31.3 Fluorescence and colour

The 90.9% of the analyzed AMPs were fluorescent under UV light, of which the major part had blue fluorescence (78.4%) (Table 19, Fig.s 54 E, F).

Of the fluorescent AMPs found 90.8% were transparent, followed by beige (11.5%), green (3.7%) and blue (3.4%) ones; particles with other colours had percentages between 2 and 1.4% (Fig. 54G). Non-fluorescent AMPs were mainly beige (36.6%), black (31.7%), and grey (19.8%); particles with other colours had percentages of 5.9 % (Fig. 54H).

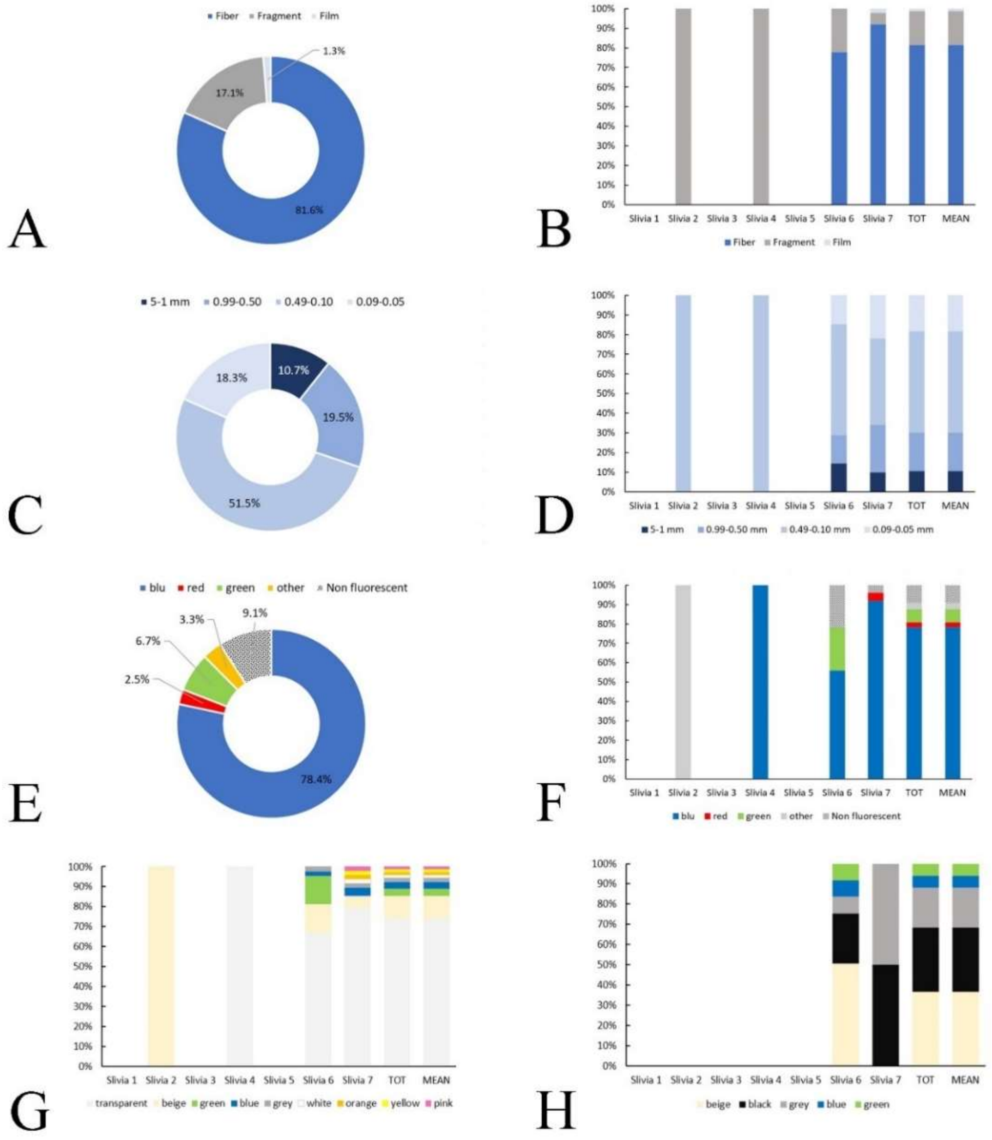

Fig. 54. AMP characteristics in the dripping water of Torri di Slivia cave. A, B: shape; C, D: size; E, F: fluorescence; G, H: colour.

4.32 Discussion

Research on AMPs is hindering by contamination risks, methodological challenges, and sampling and analysis issues.

MPs were found in three out of seven sampling points (Slivia 2, 4 and 6) (Table 17, Fig. 61). Taking into the account also natural and regenerated microparticles of anthropogenic origin, even Slivia 6 and 7 resulted polluted (Table 17, Fig. 51). Sampling points Slivia 1, 3 and 5 had no particles (Table 17, Fig. 51).

To the author knowledge, this is the first research about MP and other AMP pollution in cave dripping waters. Therefore, comparisons with other studies are not possible at the moment. Comparative analysis on MPs and other AMP data, especially MFs, remains challenging due to methodological discrepancies and varying analytical protocols. Differences in particle size criteria, sampling locations, and environmental conditions further contribute to temporal variability in reported concentrations. Nevertheless, some assumptions can still be drawn regarding the presence of MPs and other AMPs in caves.

However, it must be take into the account that different research used diverse methods, the size range of detected microparticles are not the same, some studies include anthropogenic MFs in the abundances, others separate MPs from MFs, and examined caves have different environmental and climatological conditions, and have diverse possible anthropogenic sources.

Pollution in the karst cave dripping waters can comes from the surface anthropogenic activities, rubbish buried, atmospheric depositions, and water infiltrations through fractures and soil. Torri di Slivia cave is crossed on the surface by the highway E70 and the railway (Fig. 52), and is located near the Provincial road SP1, therefore, the presence of MPs and other AMPs may be linked to traffic, road markings, road wear, tire degradation, as well as dust and litter (Andersson-Sköld et al., 2020; Burghardt and Pashkevich, 2023). Moreover, unfortunately, sinkholes near the cave were used as landfills times ago and several buckets of paint had also been poured into the area, especially near the tourist cave entrance. Slivia 2, 4, 6 and 7 sampling points result located in the stretch of cave between the railway and the highway E70 (Fig. 52). In particular, Slivia 2 is near the railway, Slivia 4 is exactly located under the highway border, and Slivia 7, the most polluted, under the highway (Fig. 52). Therefore, pollution in dripping water could be linked to percolation from this road drains. However, Slivia 6, the most polluted by MPs and the only sampling point with both MPs and MFs, is distant from both. Dripping water from this sampling point comes from a very high area of the ceiling, which is characterized by the presence of a colony of bats with thousands of individuals, which could unintentionally carry MPs and MFs in the upper part of the cave, contaminating also dripping waters.

Moreover, even Slivia 1, 3 and 5 are near the highway and the railway, however, they were not polluted (Fig. 52). Consequently, subsequent studies will be done to better understand the possible sources of pollution and water routes from the surface to the cave.

Microscopic and spectroscopic analysis showed that 80.5% of AMPs were natural and regenerated materials of anthropogenic origin (19.5% synthetics), and that fibre-shape dominated all samples (81.6%), followed by fragments (17.1%). Inside the Cliff cave, US, 31% of particles was cellulosic and 29% plastic (Hasenmueller et al., 2023). In natural environments, different research reported an higher abundance of non-synthetic MFs compared to synthetic ones, with cellulosic fibres, particularly cotton, being the most prevalent (Athey and Erdle, 2022 and references therein). In the Italian sector of the Classical Karst, the monitoring of MF pollution in waters of springs and caves highlighted that 64.4% was not synthetic and only 13.4% was MPs (Balestra et al., 2024a). The high presence of fibres in Torri di Slivia cave suggests that pollution in this area could be linked mainly to textiles deterioration. MFs could be transported in the area by atmospheric deposition or could be linked to rubbish buried and geo-textiles deterioration from the highway and the railway built near the cave. Fragments may have originated from the breakdown of waste in the environment or from nearby human activities. These data could confirm the presence and accumulation of MPs and MFs in karst environment over time. In a karst system, pollution may spread more slowly compared to other environments, particularly when transported by water, due to the specific type of karst present in the area. As a result, pollutants may accumulate in rock fractures within the system, reaching the cavity at a later time, for example through dripping waters.

Analysis on MPs in the Torri di Slivia cave showed the presence of PET and PU. All MPs resulted fragments (n=4) except one fibre of PET. PET is one of the most used polymers in the world, with main applications in the packaging, textile and technical-industrial sectors. As packaging, it is mainly used for food and beverages, especially for bottles and containers, due to its light weight and durability. It is also widely used in the production of synthetic fibres, as well as in technical applications such as thermoformed films and trays. PU is a very versatile material with numerous applications in different sectors. Its main applications include thermal insulation in construction, the production of mattress and padding foams, components for the automotive industry, footwear soles, coatings, adhesives and sealants. Moreover, PU is one of the plastics often contained in the paints for automobile or metals. PET and PU were previously found also in the waters of Bossea cave, Trebiciano cave, Mariano well, Postojna cave system, Kačna cave and Škocjan Caves system, as well as in the sediments of some of these caves (Balestra et al., 2024b; Balestra et al., 2023; Valentić et al., 2022).

AMPs < 1 mm (up to 0.05 mm) were the most abundant (89.3%), of which the major part had size between 0.49 and 0.10 mm (51.5%). However, only particles between 5 and 0.05 mm were analyzed in this research, therefore, micropollutants with smaller dimensions could be present and in larger quantities.

As reported in recent research carried out in karst systems and caves (Balestra and Bellopede, 2022b, 2023, 2025; Balestra et al., 2024a; Balestra et al., 2024b; Balestra et al., 2023), the majority of the AMPs found in Torri di Slivia cave were fluorescent under UV light (90.9%). These data indicate that UV light to identify AMPs is an effective good method, though it is not enough for thorough particle analysis. Data about AMP fluorescence could provide valuable information about their consumption by organisms, as well as the colour. The accumulation of MPs and MFs poses a serious threat to ecosystem health, as many organisms ingest them unintentionally, mistaking them for food sources (Devereux et al., 2021; Gomiero et al., 2018; Pukos et al., 2023; Sforzi et al., 2024). Once into the food webs, AMPs may affect higher trophic levels, potentially triggering cascading ecological consequences (Zhang et al., 2023a). Previous studies have shown that the color of anthropogenic microparticles (AMPs) can provide valuable information on ingestion patterns between various organisms, making this parameter ecologically relevant (Carpenter et al., 1972; Jahan et al., 2019; Lusher et al., 2013; Romeo et al., 2015; Ugwu et al., 2021). In marine biota, the majority of MPs identified were blue, black, white and transparent (Jahan et al., 2019; Lusher et al., 2013; Romeo et al., 2015; Ugwu et al., 2021). Instead, analyses on land birds revealed the assumption of mid-tone (green, red, blue, etc) AMPs (Zhao et al., 2016). In karst systems, blue and fluorescent AMPs were found in the digestive tract of specialized crustaceans of Italian caves and springs (Sforzi et al., 2024). In the same study, blue, transparent, and black particles were found with high amounts in all analyzed superficial and subterranean waters. In our study, transparent particles were found in the highest amounts, as in Cliff cave, US, and in different Italian caves (Balestra and Bellopede, 2023; Balestra et al., 2024a; Balestra et al., 2024b; Balestra et al., 2023; Hasenmueller et al., 2023). Scientific studies have also highlighted the relationship between MP color and their affinity for chemical pollutants, which may enhance their role as contaminant vectors (e.g. Frias et al., 2010; Karapanagioti et al., 2011).

MP pollution in karst systems, as well as MFs and other AMPs, pose a significant threat to water quality, ecosystem health and biodiversity, making this issue critical to conservation efforts. Monitoring these contaminants in dripping waters of underground karst systems is essential to assess the indirect impacts of human activity, improve understanding of pollutant sources and transport dynamics, and support the development of targeted conservation strategies for these vulnerable ecosystems.

Fieldwork in cave environments presents significant logistical and ecological challenges. Show caves, due to their accessibility, can serve as valuable sites for testing methodologies, monitoring pollutants and environmental processes, such as MP pollution and leaching. Increased efforts to sample and study karst systems are critical, and conservation strategies must recognize ecological connectivity between surface and subsurface karst environments. In this perspective, promoting collaborations with cavers to facilitate access to otherwise inaccessible subterranean environments can be instrumental in acquiring new and valuable data. Such efforts are essential to advance research in this field and to develop targeted protection and mitigation strategies for these isolated and ecologically sensitive systems.

4.33 Conclusions

This study provides new insights into MP pollution and other AMPs, especially natural and regenerated MFs, in karst systems, marking the first record of this kind of pollution in cave dripping waters. The results point out that surface human activity can have an indirect impact on even the dripping waters in underground environments.

This work encourages further investigations in subterranean karst environments, using also show caves to study pollution in these under-researched habitats. Although only five MPs were found in three samples, and MFs only in two samples, abundances in some sampling points are not low. Therefore, further investigations into cave dripping waters have to be done. The predominance of non-synthetic fibres, the shape and composition of identified MPs indicated textiles, illegal landfills in sinkholes, garbage, nearby urban activities, roads and railway as a likely source of pollution.

Given the strong surface–underground connectivity typical of karst environments, a broader monitoring strategy is needed to improve understanding of MP and other AMP sources, transport mechanisms and ecological implications. Long-term multidisciplinary investigations will be key to assessing the extent and impact of these pollutants' contamination, ultimately supporting the development of effective conservation strategies for karst ecosystems.

Chapter 5

Microplastic and microfibre pollution in speleological caves and springs.

Case Study: Analyses on submerged sediments and waters in aquatic environments of the Classical Karst Region with protected at EU level stygobionts

Origin of the Chapter
This chapter is adapted from the peer-reviewed articles:

- "Microplastic pollution calls for urgent investigations in stygobiont habitats: a case study from Classical Karst" authored by Balestra et al. (2024b), published in Journal of Environmental Management, Volume 356, April 2024, 120672, DOI: **https://doi.org/10.1016/j.jenvman.2024.120672**.
  The author of this dissertation was responsible for conceptualisation, methodology, validation, formal analysis, investigation, resources, data curation, writing – original draft, writing – review and editing, visualisation, project administration.
- "The problem of anthropogenic microfibres in karst systems: Assessment of water and submerged sediments" authored by Balestra et al. (2024a), published in Chemosphere, Volume 363, September 2024, 142811, DOI: **https://doi.org/10.1016/j.chemosphere.2024.142811**.
  The author of this dissertation was responsible for term, conceptualisation, methodology, validation, formal analysis, investigation, resources, data curation, writing – original draft, writing – review and editing, visualisation, supervision, project administration.

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5.1 Introduction

Research on MP and MF pollution in underground environments and karst systems is lagging behind.

Karst systems represent the most sensitive aquatic environment worldwide (Dossi et al., 2007). These habitats host organisms with interesting ecological adaptations, such as stygophiles (aquatic, facultative subterranean environment-dwelling organisms) and stygobionts(aquatic organisms that are obligate cave-dwelling) (Culver and Pipan, 2019; Mammola, 2019). However, the particular biology of these species, and the diversity of the aquatic environment in karst areas (groundwater, ponds, pools, stream and lakes), even if interconnected to each others, pose several challenges to conservation (Pipan et al., 2010). Subterranean waters are usually strongly depleted of trophic resources and local species richness of species communities is low (Barzaghi et al., 2017; Zagmajster et al., 2010). Subterranean aquatic environments do not host complex food webs, which makes predation risk limited for some organisms. Hosted communities are not very resilient and can be easily impacted by the variations of environmental conditions (Hose et al., 2022). However, in spring habitats subterranean and surface water features interplay, leading to the formation of complex environments (Cantonati et al., 2020), in which many species exploit both ecosystems during their life cycle (Barzaghi et al., 2017). Different animals provide ecosystem services in these habitats, especially invertebrate, whose movements helps to remix sediments, oxygen and organic materials (Mermillod-Blondin et al., 2023).

MP and MF research in karst environments is recent; this kind of pollution was still little studied in subterranean environment during this research, developed in 2022-2023 (Balestra and Bellopede, 2022b, 2023; Valentić et al., 2022), and lacking in karst ecotones.

The Classical Karst Region was the first studied karst area (Gunn, 2004), and extends across the border between NE Italy and SW Slovenia. This area is rich in peculiar habitats and species, including stygobionts, such as the olm, *Proteus anguinus* Laurenti, 1768, and various aquatic crustaceans like *Troglocaris planinensis* Birštein, 1948.

The olm is the only troglobiont (i.e. specialsit of underground environments) vertebrate present in Europe and is a completely aquatic species. It is endemic to the subterranean water flowing in the southern Slovenia, in the Italian Classical Karst, in western Croatia and in Bosnia and Herzegovina (Sket, 1997) and is a priority species in the EU Habitats Directive (92/43/EEC). The olm is the top predator of the karst aquifers thus it can be particularly sensitive to pollution in these environments, and represents a functionally relevant target for a first assessment of MP pollution impacts on groundwaters (Aljančič, 2019; Manenti et al., 2020). Olm occurrence in the Italian sector of the Classical Karts is confirmed by dozens of observations in different sites, although reported only in the grey literature (Mauri et al., 2018; Stoch, 2017 and references therein). Even if the olm is generally considered a classic example of a strict troglobiont organism, there is growing evidence that they can actively exploit the ecotones of underground environments, such as springs (Manenti et al., 2024).

Waters in the Classical Karst have been an important resource for the economy of this region (Zini et al., 2010), but intense exploitation and urbanization are making the waters reserves a highly vulnerable resource (Zini et al., 2010). Today, different zones of this area are protected, however, still even protected ecosystems are close to urban and industrial areas which could threat habitats and species.

The particular subterranean and surface habitats of the Classical Karst Region, the presence of stygobionts in the aquatic environments, and the water reserves, make these environments extremely interesting from an ecological point of view, and very susceptible to environmental changes and pollution, due to the open nature of the system (Balestra et al., 2023; Gillieson, 2011; Kurwadkar et al., 2020; Ruggieri et al., 2017; White, 1988). Therefore, monitoring these habitats is fundamental, especially with regard to the presence of invisible pollutants, such as MPs and MFs, which can damage habitats and species, and contaminate waters. Evaluating MP and MF occurrence in subterranean and surface waters can provide important insights for water management, allowing to combine in situ pollution data and key species conservation.

In this study, water and submerged sediment samples from aquatic subterranean and surface (spring) environments of the Italian sector of the Classical Karst Region were investigated. Monitored areas were differently exposed to anthropogenic factors and host diverse animal communities. The aim of this study were: i) to investigate, for the first time, the presence of MPs and MFs in highly vulnerable subterranean and surface aquatic environment of the Italian sector of the Classical Karst Region, ii) to examine MP and MF pollution in waters and submerged sediments of aquatic karst environments hosting the protected *Proteus anguinus*, and iii) to discuss MP and MF abundance and characteristics, potential risks and ecological effects that this kind of pollution could lead to these protected and susceptible habitats and species. In detail, investigations on the following questions were done: a) is there an accumulation of MP and MFs in submerged sediments or concentrations are higher in waters? b) does the MP and MF amount increases with the decrease in the size considered? c) are synthetic MFs less abundant than natural and regenerated ones?

5.2 The Study area

The Classical Karst Region (Kras) is a rocky limestone plateau of about 900 km^2 located between the NE part of Italy, along the eastern border of Friuli Venezia Giulia region, (Fig. 55), and the SW part of Slovenia, which run for about 15-20 km wide and 40 km long in the SW-NW direction (Visintin and Cucchi, 2010). The name “Classical Karst” comes from the first karst phenomena studies made in this area, from which the “karst” term derives (Gunn, 2004). The plateau is mainly composed by Cretaceous to Eocene carbonate rocks (Comeno/Komen Unit) (Cucchi et al., 1987; Placer, 1981) This area is intensely karstified, characterized by a large number of surface karst morphotypes and caves. In the Italian sector, more than 3500 caves and 80 big sinkholes are known (Zini et al., 2010). The major aquifers occurring in the Italian section are connected with Isonzo, Vipacco and

Timavo rivers, and with local subterranean waters. The Timavo River flows underground for >70-80 km, with frequent changes of direction in the preferential flows; only two days of travel time occur under high water level conditions (Zini et al., 2010).

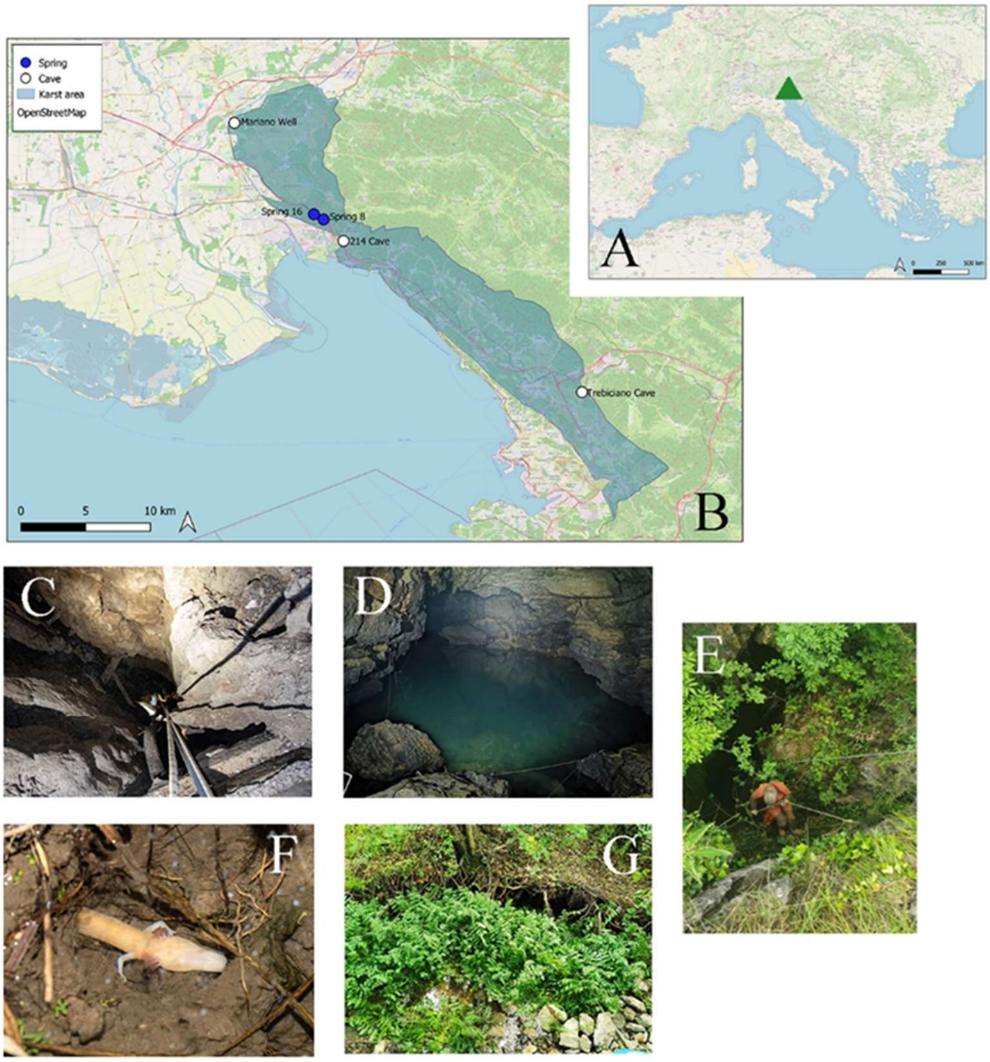


Fig. 55. Study area and sampling points in the Italian sector of Classical Karst, Friuli-Venezia-Giulia Region. A: Study area (Maps created with QGIS Desktop 3.12.1 with GRASS 7.8.2 using OpenStreetMap map, modified - openstreetmap.org/copyright); B: Karst area in light blue, springs in blue points and caves in white ones (Maps created with QGIS Desktop 3.12.1 with GRASS 7.8.2 using OpenStreetMap map, modified - openstreetmap.org/copyright); C: Mariano Well (photo V. Balestra); D: Trebiciano Cave (photo M. Galbiati); E: 214 Cave (photo M. Galbiati); F: Spring 8 with Proteus anguinus *(photo V. Balestra); G: Spring 16 (photo M. Galbiati). Image from Balestra et al. (2024a).*

Different habitats are present in the Italian sector, of which many are priority habitats according to the European Union classification, such as caves, limestone and scree floors, cliffs, karst lakes and the aquatic and riparian vegetation near the Timavo River. The Timavo River springs are one of the highest-discharge regions in the Mediterranean area (medium discharge of 40 m^3/s, maximum of 175 m^3/s) (Zini et al., 2010). The springs form two large karst depressions, partially filled by Doberdò and Pietrarossa Lakes, a Regional Nature Reserve, one of the few examples of karst pond lake in Europe, separated by a limestone ridge with numerous surface karst phenomena. Animal and plant biodiversity is accentuated by the presence of different natural environments. Karst system of this area host different stygobiont species, such as *Proteus anguinus* and various aquatic crustaceans such as *Troglocaris planinensis*.

Thanks to the high water quality and the abundance of the resources, the Classical Karst waters played an important role for the economy and social development of this region (Zini et al., 2010). This area was strongly altered by human activities, which irreversibly tampered the hydrology of the system (Fornasir, 1929; Gemiti, 2004; Marocco and Melis, 2009). Recently, urbanization, commercial and industrial activities increased too, making the waters reserves a highly vulnerable resource (Zini et al., 2010). Today, different zones of this area are protected, however, the ecosystem is open and close to urban and industrial areas, railways and highways, which could threaten habitats and species.

5.3 Method

5.3.1 Field sampling

Three subterranean (caves: Mariano Well, Trebiciano Cave and 214 Cave) and two surface (springs: Spring 8 and Spring 16) aquatic environments were selected in the Italian sector of the Classical Karst Region, based on the presence of stygobiont species previously reported in bibliography (Mauri et al., 2018), in particular *Proteus anguinus*, their geographical and biological relevance (Fig. 55). Preliminary investigations in the sampling areas confirmed the presence of stygobiont species (Manenti et al., 2024).

Monitoring was conducted in May 2022, at the beginning of the lean period. The subterranean sites are representative of the major aquifers occurring in the Italian sector of the Classical Karst Region; their access requires the use of caving equipment. Surface sites are small springs that flow from the ground, creating small pools.

214 Cave (Pozzo presso S. Giovanni di Duino, 214/226VG) is located in Duino Aurisina municipality at about 50 m a.s.l. It is a pit with a vertical drop of 47 m, with a water basin on the bottom, strictly connected with the nearby resurgences. This cave is known especially for the exhaustive studies done on the underground course of the Timavo River. A series of tests with dye were previously carried out to examine the arrivals in the resurgences: in flood periods, water arrived in the resurgences after 40 days, highlighting a marginal water table compared to the water courses feeding the springs. The bottom of the pit is closed by a bank of gravel. The cave is adjacent to railway tracks and is very close to the provincial road and the highway.

Trebiciano Cave (Grotta di Trebiciano, 3/17VG) is located in Trieste municipality, and develops for 1198 m, with a vertical drop of 329 m. The cave entrance is located at about 342 m a.s.l. in a wooded area. It is the best known cave among those existing in the Italian sector of the Classical Karst, both for the underground water flowing in its terminal part and the in-depth studies carried out in different periods. It played an important role in the Timavo River course investigations, being this cave the only intermediate point where the water that disappears in the San Canziano Cave, Slovenia, reappears, albeit for a short distance. From 1974, considerable economic resources and working time were dedicated to preserve and enhance the cavity, and an interesting underground laboratory was set up. Trebiciano cave is not considered a show cave and there are no guided excursions, however, it is an equipped cave, often visited by a lot of speleologists and researchers.

Mariano Well (Pozzo primo di Castelvecchio, 8186/6611VG) is located in the Sagrado municipality (Gorizia karst area). It develops for about 40 m, with a vertical drop of about 23 m, 15 m for reaching the surface of water. The cave entrance, at about 28 m a.s.l., is artificial and opens into the cellars of a building, as it used to collect water for drinking purposes. The original metal structures that went down into the water were demolished and removed in the past, due to their extreme degradation and dangerousness. Underwater explorations did not reveal obvious continuations for speleo-divers.

Spring 8 and 16 are permanent springs identified by preliminary investigations as emitters of a complex aquifer network which connects multiple surface habitats (including two karst lakes) of the Doberdò and Pietrarossa Regional Nature Reserve and also, partially, the subterranean sites sampled (Cucchi et al., 2008).

5.3.2 Zoological sampling

In the chosen sites, the occurrence of vertebrate and invertebrate aquatic predators and detritivores was visually recorded, distinguishing between species considered as strictly stygobionts and those known to live in surface waters. Stygobiont species recognition was based on already existing reports for the groundwater fauna of the study area (Stoch, 2017).

Samples of zooplankton and macro-zoobenthos were made too. Plankton collection was performed by filtering 10 L of water from each sampling site through standardized amount of clean cotton cloth. The organisms captured within the fabric were then released into 50 ml of filtered water and then stored in a fridge until being analysed under light microscopy. The detection and quantification of zooplankton was done using light microscopy with 100x magnification, while larger magnification was used to determine the taxon of the organisms. Analyses were performed on 5 ml of the already filtered and resuspended samples, which corresponds to 1 L of the original site water before filtering. Even if this method does not allow an exact quantification of zooplankton species, it provides good insight into relative differences between sites. Macrozoobenthos was sampled by dip-netting within a square of 20 x 20 cm that was positioned marked out at the substrate level. Collected animals were then identified into groups, counted, weighted for biomass, and subsequently released.

5.3.3 Microplastic sampling

Sediment and water bulk samples (Hidalgo-Ruz et al., 2012) were collected using pre-cleaned glass jars and nitrile gloves. In the subterranean sites, the jars were limited and wrapped with anti-impact material to transport them safely in the speleological bags. For each sampling area, a quantity between 500 and 1150 mL of surface water and a minimum of 250 g of superficial submerged sediments (upper 5 cm) were collected depending on the water and sediment pool availability (Table

23). Two samples (water of Spring 8 and sediment of Spring 16) were damaged during transportation, therefore, they were not analyzed.

For more detailed information on sampling methods see Chapter 3.

Table 23. Sample characteristics

Sampling area	Water sample [ml]	Sediment samples in water environment [g]	Water percentage in sediment samples
Spring 8	-	283.41	78.58 %
Spring 16	1100	-	-
214 Cave	1080	440.47	52.86 %
Trebiciano Cave	500	435.57	40.97 %
Mariano Well	1150	412.64	48.77 %

5.3.4 Laboratory analysis

Blank controls on milliQ water, H_2O_2 30% (Merck), ethanol absolute (VWR Chemicals), and NaCl solution (Carlo Erba NaCl + milliQ water) were done. The blank correction method used estimates the results of unknown samples by subtracting the contribution of the blank (Shruti and Kutralam-Muniasamy, 2023 and references therein). For more detailed info on contamination control, see Chapter 3.

Water samples were analyzed according to the method described in Balestra et al. (2023), improved for these samples, visually rich in organic materials. After being poured in glass beakers, samples were pre-treated through the application of 1:1 30% H_2O_2 solution, covered with an aluminum foil, and left to react for seven days under natural conditions. Each water sample was filtered through a 1.2-µm pore size glass fibre filter (Phenomenex, Ø 47 mm).

Submerged sediment samples were analyzed according to the methodology described in Balestra and Bellopede (2023). Dried sediments were pre-treated through the application of 1:1 30% H_2O_2 solution, and left to react for seven days under natural conditions. In relation to the quantity of dry sediments, trying to use as much material as possible, three sub-samples of 15 g were selected for each sample via coning and quartering, and put into beakers with 150 ml NaCl solution (200 g NaCl/0.6 L, $\rho = 1.2$). Subsequently, the supernatant was extracted with a glass pipet and filtered through a 1.2-µm pore size glass microfibre filter (Phenomenex, Ø 47 mm).

For more detailed information on water and sediment sample analysis, see Chapter 3.

5.3.5 Microparticle identification and characterization

Particles on filters were observed under a Leitz ORTHOLUX II POL-MK microscope equipped with a DeltaPix Invenio 12EIII 12 Mpx Camera, with and without a UV flashlight. It was established to analyze MPs and MFs up to 0.1 mm. Particles not clearly identifiable as MPs or MFs were not taken into consideration.

Particles were verified using theµFTIR+ATR. On each filter, 15% of MPs and 10% of MFs was analyzed. Particles on filters were transferred with a dissecting needle on a silver filter (GVS Life Sciences, Membrane Disk 47 mm, 0.8 µm pore size). Spectra were accepted only with a match degree ≥80% for MPs and ≥75 for MFs.

For more detailed information on microscopic and spectroscopic techniques used, see Chapter 3.

5.4 Results

5.4.1 Fauna occurrence

Our surveys confirmed that both subterranean and surface monitored sites host *Proteus anguinus* populations together with other species usually considered as strictly subterranean, such as the Crustacean Atyid *Troglocaris planinensis* and the Crustacean Spheromatid *Monolistra racovitzai* Strouhal, 1928 (Fig. 56, Table 24). In Trebiciano Cave we also detect the Italian chub, *Squalius squalus* (Bonaparte, 1837), which is a surface fish. Adult olms were detected in all sampling sites. In 214 Cave, some juveniles were observed too, as well as in Spring 16 with the occurrence of a larva. Plankton density varied between cave and spring sites (Table 25), as well as the variety of macrozoobenthic invertebrates sampled (Table 26).

Table 24. Results of visual encounter surveys on cave-dwelling and surficial predators and detritivores in both caves and springs.

Site name	Environment	Stygobiont predators	Stygobiont detritivores	Surficial predators	Surficial detritivores
Spring 8	surface	*Proteus anguinus*	*Troglocaris planinensis*	*Dendrocoelum lacteum*	*Asellus aquaticus* *Emmericia patula*
Spring 16	surface	*Proteus anguinus*	*Monolistra racovitzai*	*Esox lucius* *Polycelis nigra*	*Asellus aquaticus* *Emmericia patula*
214 Cave	subterranean	*Proteus anguinus*	*Troglocaris planinensis*	-	-
Trebiciano Cave	subterranean	*Proteus anguinus*	*Troglocaris planinensis*	*Squalius squalus*	-
Mariano Well	subterranean	*Proteus anguinus*	*Troglocaris planinensis*	-	-

Table 25. Results of plankton samplings in both caves and springs.

Site name	Ciliata (micro)	Gastrotricha (micro)	Rotifera (meso)	Hydrachnida (meso)	Ostracoda (meso)	Nauplius (meso)	Copepoda (macro)	N. micro	N. meso	N. macro
Spring 8	0	0	0	0	2	6	3	0	8	3
Spring 16	657	0	0	0	0	0	1	657	0	1
214 Cave	0	0	0	0	0	0	0	0	0	0
Trebiciano Cave	0	0	0	0	0	0	0	0	0	0
Mariano Well	0	0	0	0	0	0	0	0	0	0

Table 26. Results of dip-netting sampling.

Site name	N. squares sampled	Taxa and density (in parenthese)	Total weight (g)
Spring 8	1	*Polycelis nigra* (30), Gammaridae (10), Lumbricidae (1), *Emmericia patula* (6)	0.69
Spring 16	2	*Asellus aquaticus* (4), *Planorbis* sp. (2), *Planorbarius* sp. (1), Gammaridae (2)	0.12
214 Cave	10	*Troglocaris planinensis* (50)	24.2
Trebiciano Cave	10	*Troglocaris planinensis* (10), *Dendrocoelum* sp. (1)	5.19
Mariano Well	3	None	0

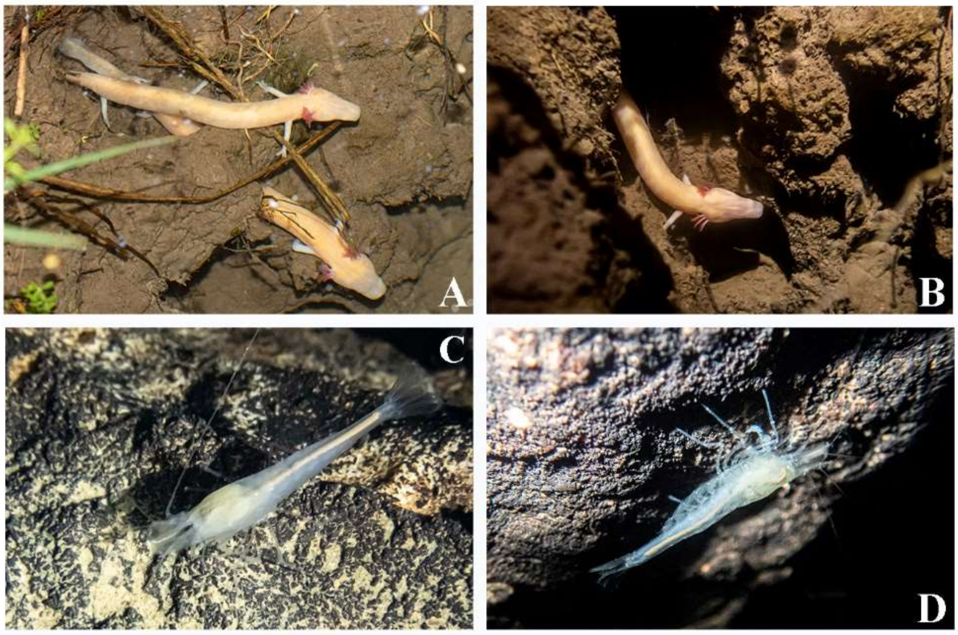


Fig. 56. Stygobiont fauna in surface and subterranean monitored environments. A: Proteus anguinus *in surface habitat (spring); B:* Proteus anguinus *in subterranean habitat (cave); C:* Troglocaris planinensis *in surface habitat (spring); D:* Troglocaris planinensis *in subterranean habitat (cave) (photos: V. Balestra). Image from Balestra et al. (2024b).*

5.4.2 Microplastic abundance and size

MPs were found in all water and sediment samples. Considering the value found in the blank samples, the possible contamination linked to the pre-existing material in the chemical products used during laboratory activities turned out to be about 10.2 items/L for water samples and 1.7 items/15g for sediment samples. In water, MP concentration ranged between 47.2 and 96.0 items/L, between 36.9 and 85.6 items/L with correction (Table 27). In sediments, MP concentration ranged between 888.9±101.8 items/kg and 2177.8±473.0 items/kg (average and standard deviation), between 775.6 and 2064.4 items/kg with correction (Table 28).

Table 27. Microplastic characteristics in water samples of aquatic environments in the Classical Karst.

Water sample	ml	MPs	MPs/L	MPs with correction	MPs/L with correction	Fibre	Microfibre	Fragment	Microfragment	Film	Microfilm	Pellet	Microbead	Foam	Microfoam
Spring 16	1100	56	50.9	44.6	40.5	13	30	0	12	0	0	0	1	0	0
214 Cave	1080	51	47.2	39.8	36.9	15	26	0	9	0	1	0	0	0	0
Trebiciano Cave	500	48	96.0	42.8	85.6	13	22	0	11	0	2	0	0	0	0
Mariano Well	1150	95	82.6	83.1	72.3	10	33	0	43	0	1	0	7	0	1
TOT		250		215.5		51	111	0	75	0	4	0	8	0	1
%		100		100		20.4	44.4	0.0	30.0	0.0	1.6	0.0	3.2	0.0	0.4

Table 28. Microplastic characteristics in sediment samples of aquatic environments in the Classical Karst.

Sediment sample	MPs/15g	MPs/kg	MPs/15g with correction	MPs/kg with correction	Fibre	Microfibre	Fragment	Microfragment	Film	Microfilm	Pellet	Microbead	Foam	Microfoam
Spring 8 - 1	13	866.7	11.3	753.3	2	8	0	3	0	0	0	0	0	0
Spring 8 - 2	12	800.0	10.3	686.7	3	5	0	3	0	0	0	1	0	0
Spring 8 - 3	15	1000.0	13.3	886.7	0	12	0	3	0	0	0	0	0	0
214 Cave - 1	32	2133.3	30.3	2020.0	4	22	0	4	0	1	0	1	0	0
214 Cave - 2	6	400.0	4.3	286.7	0	3	0	3	0	0	0	0	0	0
214 Cave - 3	19	1266.7	17.3	1153.3	4	12	0	1	0	1	0	1	0	0
Ttrebiciano Cave - 1	34	2266.7	32.3	2153.3	5	10	0	13	0	0	0	6	0	0
Ttrebiciano Cave - 2	39	2600.0	37.3	2486.7	2	8	1	7	0	0	0	21	0	0
Ttrebiciano Cave - 3	25	1666.7	23.3	1553.3	4	6	0	11	0	0	0	4	0	0
Mariano Well - 1	12	800.0	10.3	686.7	3	8	1	0	0	0	0	0	0	0
Mariano Well - 2	14	933.3	12.3	820.0	3	9	0	2	0	0	0	0	0	0
Mariano Well - 3	15	1000.0	13.3	886.7	0	10	0	4	0	0	0	1	0	0
TOT	236		215.6		30	113	2	54	0	2	0	35	0	0
%	100		100		12.7	47.9	0.8	22.9	0.0	0.8	0.0	14.8	0.0	0.0

The size distribution of collected MPs in water indicated that mini-MPs (MPs < 1mm) are the most abundant (79.6%) (Fig. 57A); MPs from 5 to 1 mm accounted for 20.4%. Five mesoplastics (5–25 mm) were found too: two in Spring 16, two in Trebiciano Cave and one in Mariano Well. Mariano Well data indicated percentages very different from other sampling points: about 10.5% of particles had a dimension >1 mm, about half of Spring 16 and one third of Trebiciano and 214 Caves.

Size average percentages of MPs in sediment samples appeared more homogeneous: MPs from 5 to 1 mm accounted for 13.6%, and MPs < 1 mm for 86.4% (Fig. 57B). Again, Mariano Well showed different percentages, though only slightly: about 17.1% of particles had a dimension > 1 mm, otherwise, the other sampling areas were between 12.2 and 14.0%. No mesoplastics were found in sediment samples.

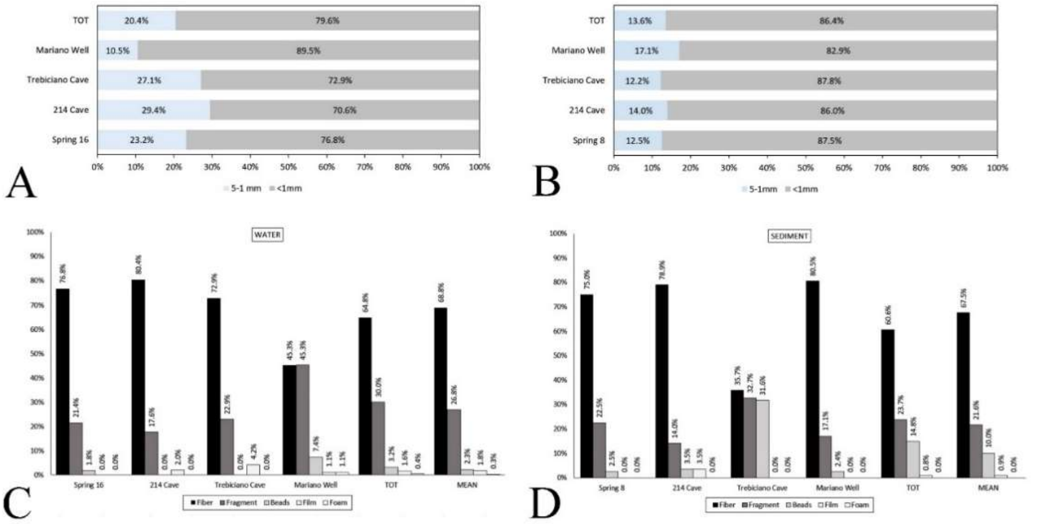


Fig. 57. Microplastic characterization in water and sediments of spring and cave aquatic environments of the Italian Classical Karst. A: Microplastic size in water samples; B: Microplastic size in sediment samples; C: Microplastic shape in water samples; D: Microplastic shape in sediment samples.

5.4.3 Microplastic shape

Fibre-shape dominated all water samples (64.8%), followed by fragments (30.0%), beads (3.2%), film (1.6%) and foam (0.4%) (Fig. 57C, 58). The values found for the waters of Spring 16, 214 Cave and Trebiciano Cave were similar, with high percentage of fibres (>73%), followed by fragments (18-23%). Other shapes had percentages less than 4%. Mariano Well sample had very different values, with fibres and fragments around 45%, and 7.4% of beads. Foam shape was found only in Mariano Well.

Fibre-shape dominated all sediment samples (60.6%), followed by fragments (23.7%), beads (14.8%) and film (0.8%) (Fig. 57D, 58). No foam was found. The values found for the sediments of Spring 8, 214 Cave and Mariano Well were similar, with high percentages of fibres (75.0-80.5%), followed by fragments (14.0-22.5%). Other shapes had percentages less than 3.5%. Instead, Trebiciano Cave sample had very different values, with fibres, fragments and beads respectively 35.7, 32.7 and 31.6%.

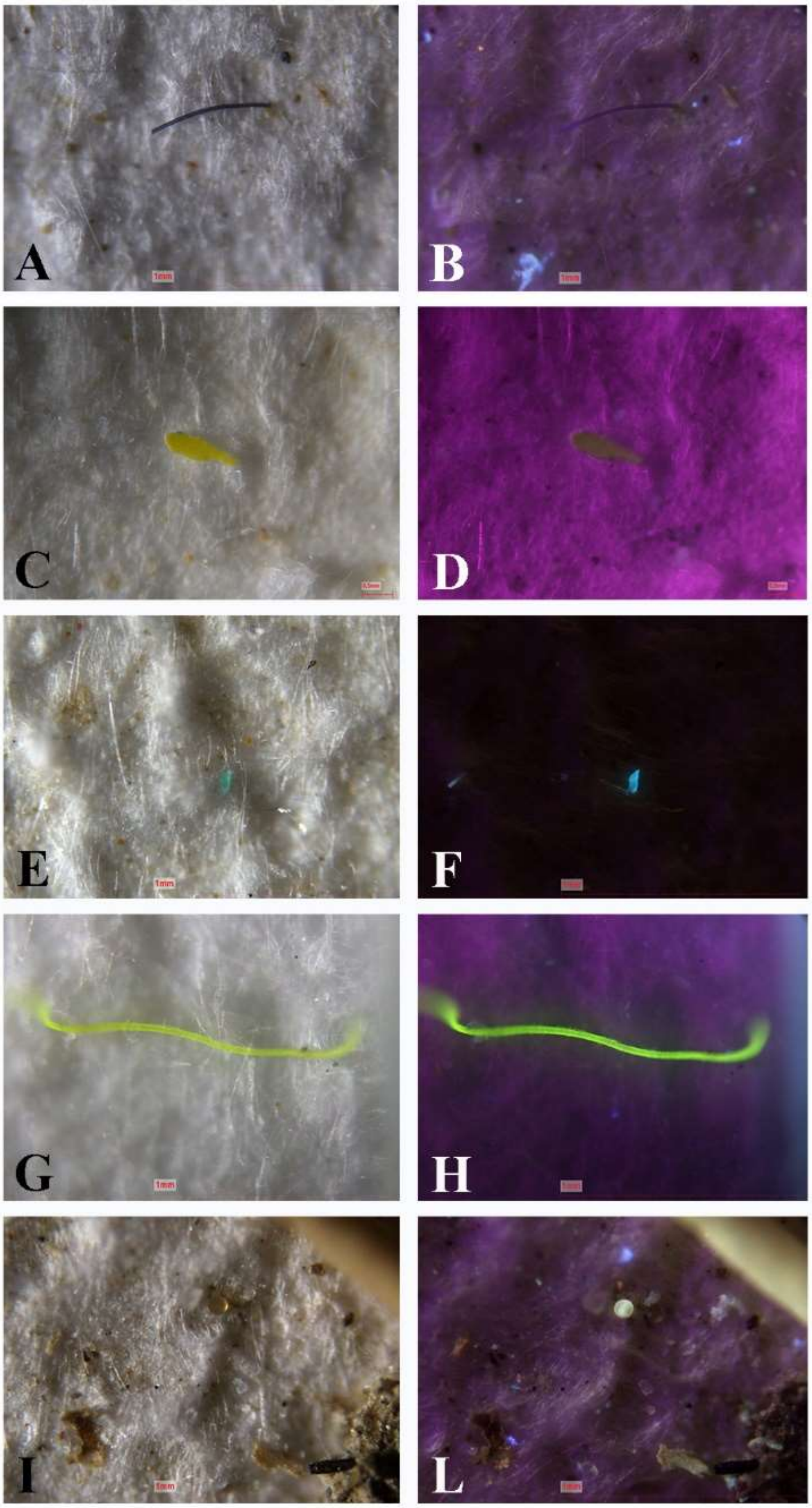


Fig. 58. Images of microplastics found in the Italian Classical Karst water environments under microscope, with and without UV light. A, B: blue fibre without fluorescence; C, D: yellow fragment without fluorescence; E, F: green fragment with blue fluorescence; G, H: yellow fibre with yellow fluorescence; I, L: amber sphere (microbead) with white fluorescence (Photos: V. Balestra). Image from Balestra et al. (2024b).

5.4.4 Microplastic fluorescence and colour

Figures 58 and 59 underline the importance of visual identification under microscope, in order to analyze non-fluorescent particles (about 35% in waters and 31% in sediments).

The fluorescent particle abundance percentages in water (Fig. 59A) were similar for each sampling area, with a slight increase in Trebiciano Cave (70.8%) and a minimum value in 214 Cave (60.8%). The major part of fluorescent particles had blue fluorescence (68.7%), followed by green (17.8%) and red one (6.7%) (Fig. 59C). A 6.7% of particles had other fluorescence colours. Spring 16 and Mariano Well MPs had very similar fluorescence colour percentages, Trebiciano Cave particles had only blue, green and red fluorescence, instead, 214 Cave had higher blue fluorescent particles (77.4%), low values of green fluorescent MPs (12.9%), and no red fluorescent ones. (Fig. 59C).

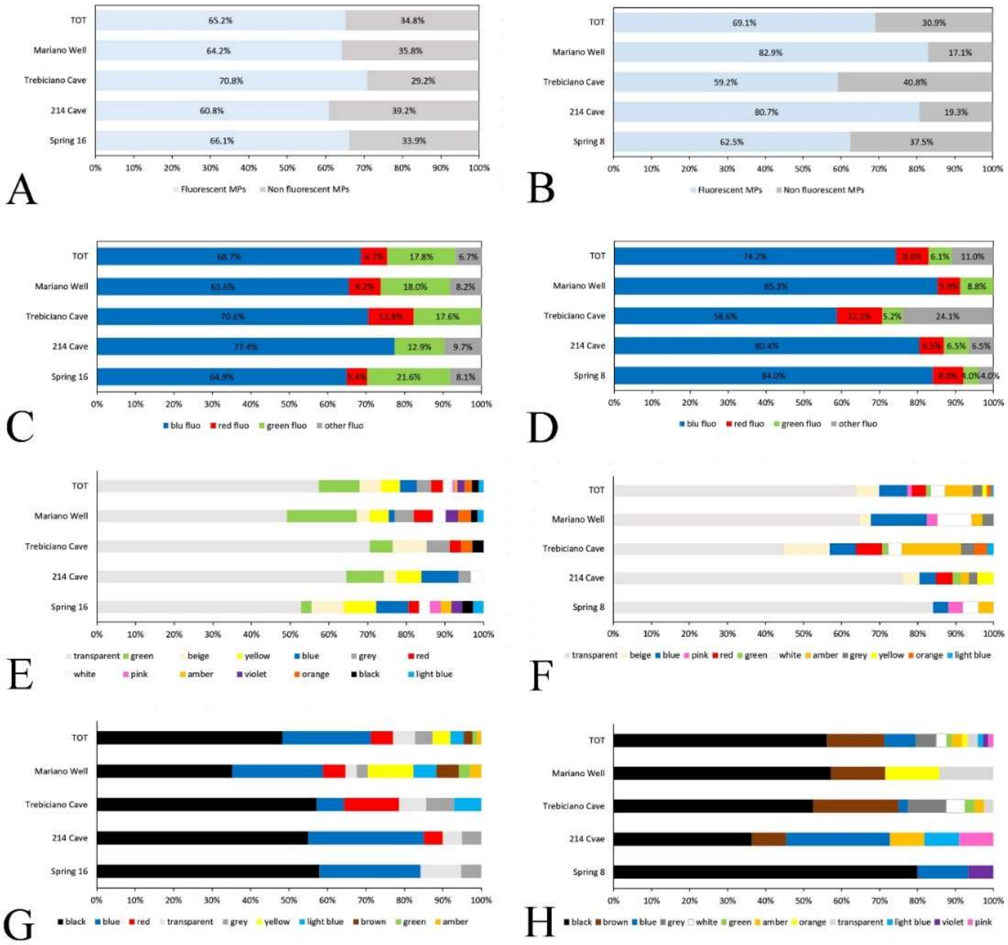


Fig. 59. Colour of examined microplastics. A: Fluorescent microplastic in water samples; B: Fluorescent microplastic in sediment samples; C: Fluorescence colour in water samples; D: Fluorescence colour in sediment samples; E: colours of fluorescent microplastics in water samples; F: colours of fluorescent microplastics in sediment samples; G: colours of non-fluorescent microplastics in water samples; H: colours of non-fluorescent microplastics in sediment samples. Image from Balestra et al. (2024b).

The fluorescent particle abundance percentages in sediments (Fig. 59B) were similar for 214 Cave and Mariano Well (>80%), while Spring 8 and Trebiciano Cave had about 60% of fluorescent MPs. The major part of fluorescent particles had blue fluorescence (74.2%), followed by red (8.6%) and green (6.1%) one (Fig. 59D). A 11% of particles had other fluorescent colors. MPs in Spring 8, 214 Cave and Mariano Well had similar fluorescence colours: more than 80% of fluorescent particles had blue fluorescence. Instead, Trebiciano Cave sample returned very different percentages: blue fluorescent particles were only 58.6%, and high values of different colors were present (24.1%) (Fig. 59D).

Of the fluorescent MPs found in water samples, 57.4% were transparent, followed by green (10.5%), and beige (5.6%) ones; particles with other colours had percentages between 0.6 and 4.9% (Fig. 59E). Non-fluorescent MPs were mainly black (48.3%), blue (23.0%), red (5.7%), and transparent (5.7%); particles with other colours had percentages between 1.1 and 4.6% (Fig. 59G).

Of the fluorescent MPs found in sediments, 63.8% were transparent, followed by blue (7.4%), amber (7.4%) and beige ones (6.1%); particles with other colours had percentages between 0.6 and 3.7% (Fig. 59F). Non-fluorescent MPs were mainly black (56.2%), brown (15.1%), blue (8.2%), and grey (5.5%); particles with other colours have percentages between 1.4 and 2.7% (Fig. 59H).

Analyzing data by sampling area, it is possible to observe that most common particles were transparent and black. However, some differences were present considering the other colours: percentages for each color were very different from each other, not all colours were present in each sampling area, the two springs had no brown MPs, and Spring 8 had less colours than the other sampling areas.

5.4.5 Characterization of microplastic by μFTIR-ATR

Water samples contained especially polyesters (15%) and copolymers (10%), followed by PVAc (polyvinyl acetate), PTFE (polytetrafluoroethylene), PAM (polyacrylamide), EVOH (ethylene vinyl alcohol), and epoxy resin (Fig. 60A). The 45% of particle spectra obtained during the μFTIR-ATR analysis on water samples had a match below 80%.

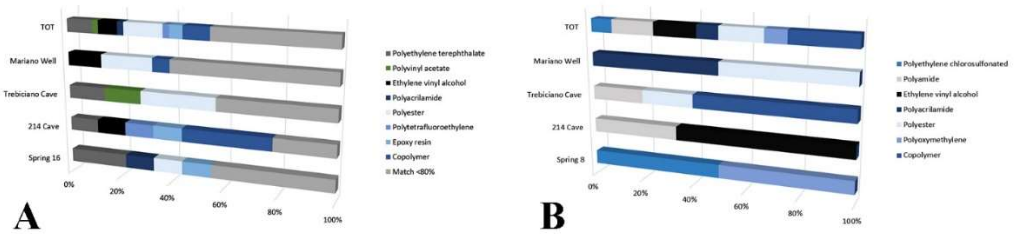


Fig. 60. Typology of examined microplastics. A: typology of microplastics in water samples; B: typology of microplastics in sediment samples. Image from Balestra et al. (2024b).

In sediments samples were found especially copolymers (25%), followed by PA (polyamide), EVOH, PAM, polyester, POM (polyoxymethylene or polyacetal), CSPE (polyethylene chlorosulfonated) (Fig. 60B). Particles spectra obtained during the μFTIR-ATR analysis were all counterbled with high matches. MPs found in waters and sediments showed different degradation degree (Fig. 60): MP spectra in water were much more difficult to examine with spectroscopy because particles were often contaminated by different materials and very degraded.

Examined MPs were randomly selected. Most of the examined MPs were fibres of different size and color. Examined fragments resulted epoxy resin, and were <1mm and transparent.

5.4.6 Microfibre abundance

Procedural blank indicated a possible contamination linked to pre-existing material in the chemical products used during laboratory activities (Tables 29, 30). Analyzed MFs of blank were mainly comprised of cellulose (Tables 29, 30).

Table 29. Estimates of blank contamination in water samples. ND=microfibres not clearly identifiable and microfibres with sprectrum match <75%.

Water	N. of analyzed blank samples	Analyzed quantity for each blank sample [ml]	Natural and regenerated fibres in blank sample (average)	Synthetic fibres in blank sample (average)	ND fibres in blank sample (average)	Amount of chemical used for analyzed sample [ml]	Amount of natural and regenerated [fibres/L]	Amount of synthetic [fibres/L]	Amount of ND [fibres/L]
milliQ water	4	100	20	0	0	20	4.0	0.0	0.0
H2O2 30%	4	100	18	1	0	1000	180.0	10.0	0.0
Absolute Ethanol	4	100	14	1	0	20	2.8	0.2	0.0
TOT							186.8	10.2	0.0

Table 30. Estimates of blank contamination in submerged sediment samples. ND=microfibres not clearly identifiable and microfibres with sprectrum match <75%.

Sediment	N. of analyzed blank samples	Analyzed quantity for each blank sample[ml]	Natural and regenerated fibres in blank sample (average)	Synthetic fibres in blank sample (average)	ND fibres in blank sample (average)	Amount of chemical used for analysed sample [ml]	Amount of natural and MMC [fibres/L]	Amount of synthetic [fibres/L]	Amount of ND [fibres/L]
milliQ water	4	100	20	0	0	20	4.0	0.0	0.0
H2O2 30%	4	100	18	1	0	15	2.7	0.2	0.0
Absolute Ethanol	4	100	14	1	0	20	2.8	0.2	0.0
NaCl solution	8	150	95	3	3	50	31.7	1.0	1.0
TOT			147	5	3		41.2	1.4	1.0

MFs were found in all water and submerged sediment samples, highlighting MF pollution in surface and subterranean habitats of the karst system (Tables 31, 32, Fig. 61). A mean concentration of 163.5 MFs/L was found in waters, and of 4776.7 MFs/kg in sediments. An accumulation of MFs in sediments was highlighted (Fig. 61). Most of the analysed MFs (>60%) were cellulosics (Fig. 61). Synthetic MFs were more abundant in waters, and were only 22.2% in waters and 14.7% in submerged sediments (corrected values) (Fig. 61). Degraded (Unknown) MFs were mainly present in submerged sediments (Fig. 61).

Table 31. Microfibre abundance in water samples of aquatic environments in the Classical Karst Region.

Filter Water	Examined amount [ml]	TOT [MFs/L]	Natural and regenerated [MFs/L]	Synthetic [MFs/L]	Unknown [MFs/L]
Spring 16	1100	110.4	60	28	23
214 Cave	1080	149.1	97	27	25
Trebiciano Cave	500	192.0	121	65	6
Mariano Well	1150	202.7	143	25	34
TOT		654	421	145	88
%			64.4	22.2	13.4
Mean		163.5	105.3	36.4	21.9

Table 32 Microfibre abundance (average of three subsamples) in submerged sediment samples of aquatic environments in the Classical Karst Region.

Filter Sediment	Examined amount [g]	TOT [MFs/Kg]	Natural and regenerated [MFs/Kg]	Synthetic [MFs/Kg]	Unknown [MFs/Kg]
Spring 8	15	4537.8	34.8	8.6	24.7
214 Cave	15	8982.2	91.8	13.6	29.3
Trebiciano Cave	15	2648.9	20.8	10.3	8.7
Mariano Well	15	2937.8	26.5	9.6	8.0
TOT			173.9	42.1	70.7
%			60.7	14.7	24.7
Mean		4776.7	43.5	10.5	17.7

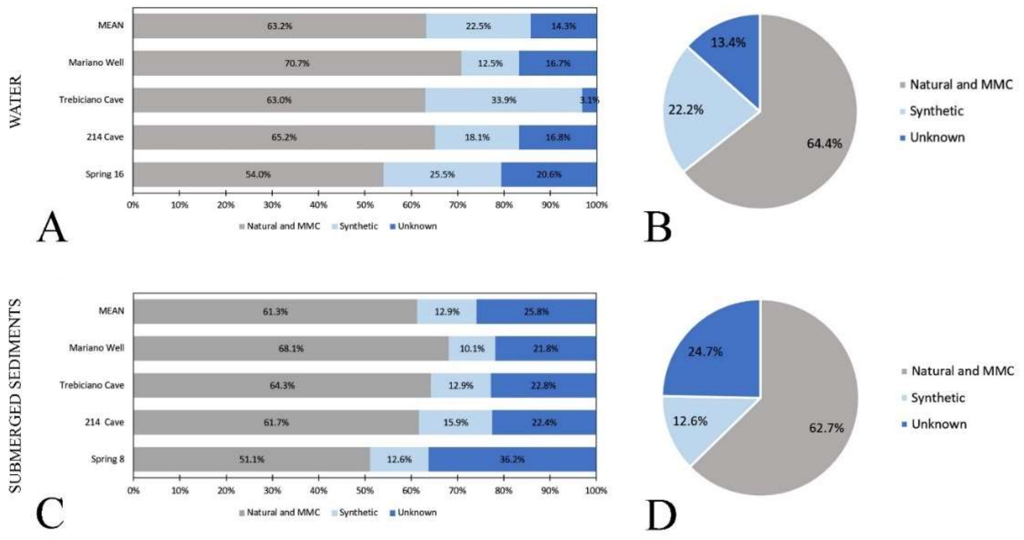


Fig. 61. Abundance of microfibres in water and submerged sediment samples in surface and subterranean aquatic environments of the Classical Karst Region. MMC = regenerated. A, C: Water samples abundances by sampling area; B, D: Total amount of microfibres in water. Image from Balestra et al. (2024a).

5.4.7 Microfibre size

The size distribution of collected MFs indicated that big MFs (1-5 mm) were less abundant and accounted for a mean of 21.6% in water samples and 16.3% in submerged sediments (Fig. 62). The highest percentages of larger fibres were found in Trebiciano Cave, both in water and submerged sediment samples. Percentages increased with the decrease of the considered size (Fig. 62).

Eight mesoplastics (5–25 mm) were found in water samples: three in Spring 16, two in 214 Cave, two in Trebiciano Cave, and one in Mariano Well. Three mesoplastics were found in submerged sediments: one in Spring 8, and two in 214 Cave.

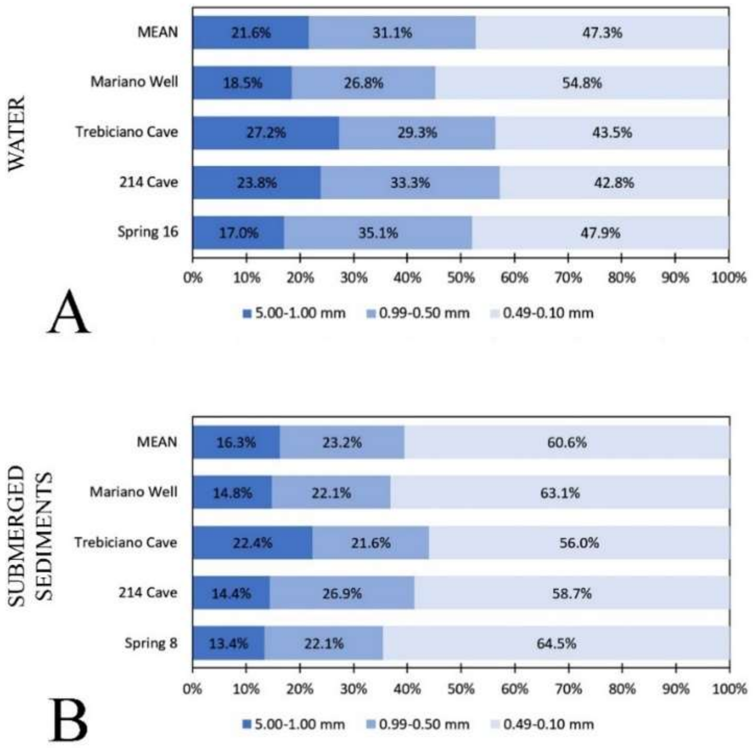


Fig. 62. Size of analyzed microfibres. A: Size of microfibres found in waters samples; B: Size of microfibres found in submerged sediment samples. Image from Balestra et al. (2024a).

5.4.8 Microfibre fluorescence and colour

Most MFs were fluorescent under UV light: a mean of 81.9% in waters and 93.0% in submerged sediments (Fig.s 63A, B, 64). Although high, these percentages highlight that as MF identification under UV light would neglected about 10-20% of MFs. Percentages of fluorescent MFs were similar for each sampling area, with a slightly lower values in Trebiciano Cave (Figs. 63A, B). Most of fluorescent MFs had blue fluorescence in both water and submerged sediments samples (>93%), followed by green and red one in waters, and red and green in submerged sediments (Fig.s 63C, D, 64). Other fluorescence colours were found with values less than 1%. Only in Trebiciano Cave percentages were slightly different in water samples (Fig. 64C).

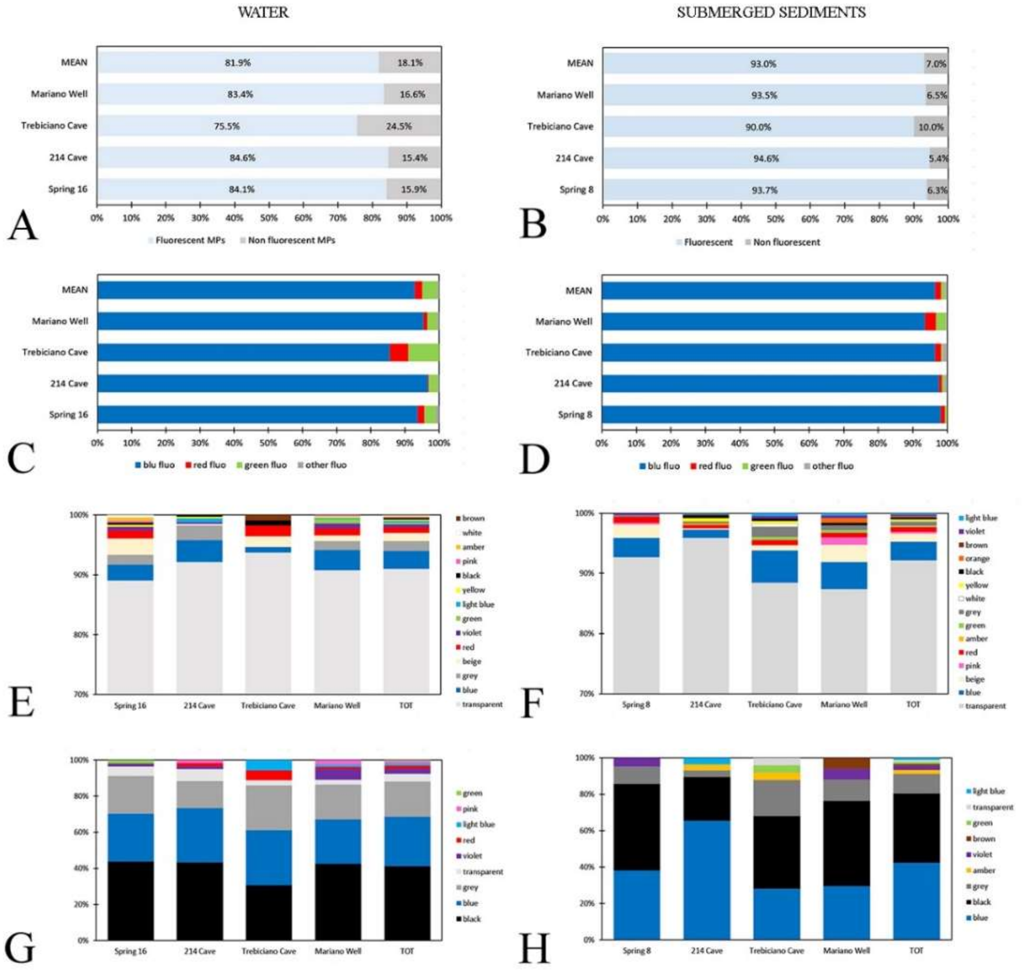


Fig. 63. Fluorescence and colours of analyzed microfibres. A: Percentages of fluorescent microfibres in waters; B: Percentages of fluorescent microfibres in submerged sediments; C: Fluorescence colour of microfibres in waters; D: Fluorescence colour of microfibres in submerged sediments; E: Colours of fluorescent microfibres in waters; F: Colours of fluorescent microfibres in submerged sediments; G: Colour of non-fluorescent microfibres in waters; H: Colour of non-fluorescent microfibres in submerged sediments. Image from Balestra et al. (2024a).

Of the fluorescent MFs, most were transparent (91.0% in waters, 92.2% in sediments), followed by blue ones (3.0% in waters, 3.1% in sediments); particles with other colours had percentages less than 1.6% (Fig.s 63E, F, 64). Non-fluorescent MFs were mainly black (41.3%), blue (27.2%), and grey (19.6%) in water samples, and blue (42.4%), black (38.0%), and grey (10.9%) in submerged sediment samples; particles with other colours had percentages between 0.4 and 4.3% (Fig.s 63G, H, 64).

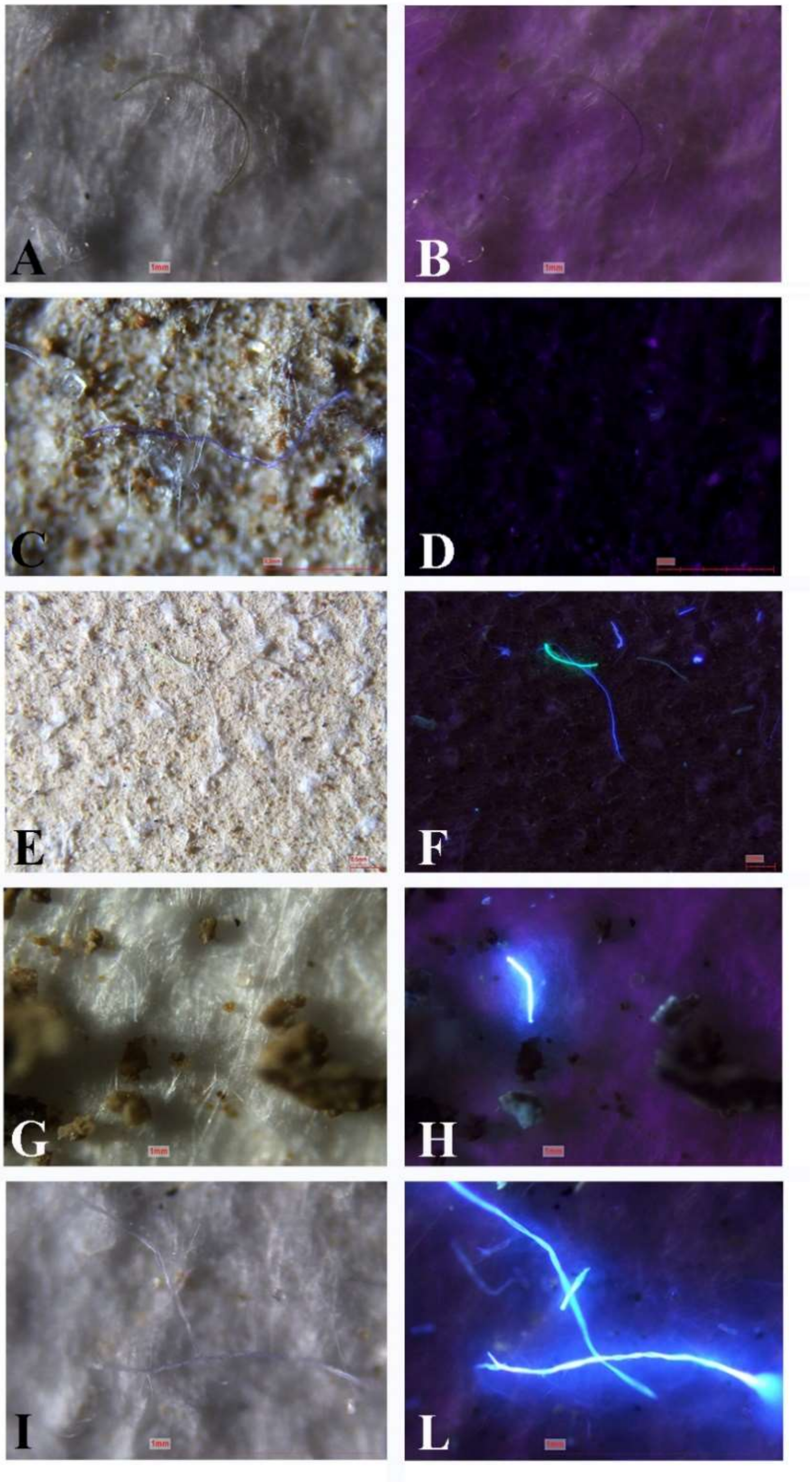


Fig. 64. Images of microfibres of anthropogenic origin found in the Italian Classical Karst aquatic environments under microscope, with and without UV light. A, B: green synthetic fibre without fluorescence; C, D: violet cellulosic (cotton) fibre without fluorescence; E, F: yellow synthetic fibre with green fluorescence and transparent cellulosic fibres with blue fluorescence; G, H: transparent synthetic fibre with blue fluorescence; I, L: transparent cellulosic fibres (cotton) with blue fluorescence (Photos: V. Balestra). Image from Balestra et al. (2024a).

5.4.9 Microfibre characterization by µFTIR-ATR

A half of the total analyzed fibres did not exceed a match of 75%, respectively 56.3% for water and 43.8% for submerged sediments samples (Fig. 65).

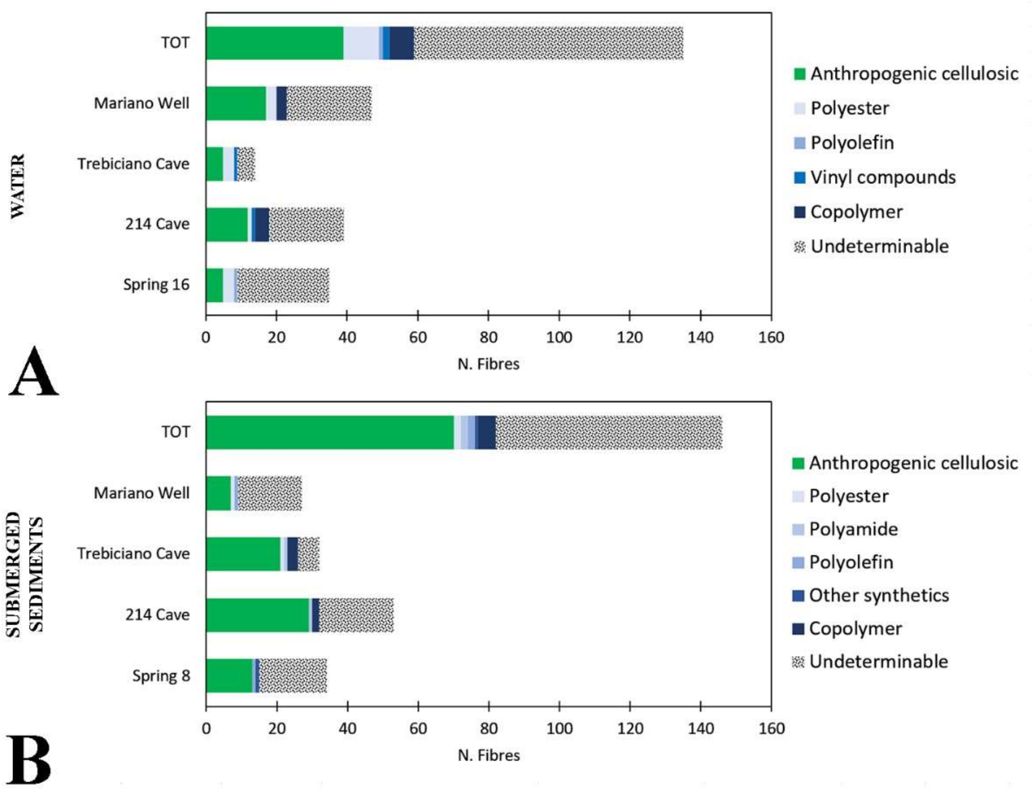


Fig. 65. Microfibres typologies. Green for natural and regenerated microfibres (e.g. cotton), blue shades for synthetic microfibres, texture for undeterminable microfibres (degraded microfibres and microfibres with FTIR library spectrum match <75%). Image from Balestra et al. (2024a).

µFTIR-ATR characterization revealed that 28.9% of the analyzed fibres in waters and 47.9% in submerged sediments were anthropogenic cellulosic (natural and regenerated). Except a pair of ramie fibres, all analyzed natural fibres were found seemed to be cotton. Cotton was the most frequent match (20% of all examined fibres in water and 24% in submerged sediments), followed by regenerated fibres, such as Cupro/Bemberg, Tencel/Lyocel and cellulose acetate. Different methyl cellulose and hydroxypropil methyl cellulose MFs were found too. Only 14.8% of fibres in waters and 8.2% in submerged sediments were synthetic. Most plastic fibres were polyester and copolymers. Water samples contained 7.4% polyester, especially PET, 5.2% copolymer, 1.5% vinyl compounds such as PTFE and PVAc, and 0.7% polyolefin such as PAM. Submerged sediments contained 3.4% copolymer, especially EVOH, 1.4% polyester, 1.4% polyolefin such as PAM and PE-Chlorosulfonated, 1.4% polyamide, and 0.7% other synthetics.

5.5 Discussion

5.5.1 Microplastic pollution

MPs were found in all examined water and sediment samples, highlighting intense MP pollution in surface and subterranean freshwater karst habitats. Comparisons with other karst areas are challenging since methodologies and analyses are different and can affect the results. Moreover, the size of MPs taken into account, the sampling point, and environmental conditions may vary MP concentrations over time. However, some assumptions on the MP amounts in karst environments could be made (Table 33).

Table 33. Microplastic concentrations found in previous work.

	Typology	**Considered size**	**MP concentration**	**References**
Bossea Cave system, Italy	Springs	5-0.1 mm	23 and 29 items/L	Balestra et al. (2023)
Timava Springs, Slovenia	Springs	5-1 mm	9.52±<0.01 items/m³	Valentić et al. (2022)
Springs in Illinois, USA	Springs	5-0.5 mm	Maximum concentration of 15.2 items/L, mean of 7.9 items/L	Panno et al. (2019)
Wells in Illinois, USA	Groundwaters	5-0.5 mm	Maximum concentration of 4.4 items/L, mean of 2.8 items/L)	Panno et al. (2019)
Wells and bore wells in coastal south India	Groundwaters	5-0.1 mm	median concentration of 4.2 items/L, maximum concentration of 10.1 items/L	Selvam et al. (2021)
NW Germany	Groundwaters	5 mm-20 µm	5.6 items/L	Mintenig et al. (2019)
Alluvial unconfined aquifer in Victoria, Australia	Groundwaters	500-20 µm	average concentration of 38 ± 8 items/L	Samandra et al. (2022)
Bossea Cave, Italy	Cave waters	5-0.1 mm	12 to 54 items/L, mean of 28 items/L	Balestra et al. (2023)
Kačna cave, Slovenia	Cave waters	5-1 mm	16.67±0.01 items/m³	Valentić et al. (2022)
Škocjan Caves system, Slovenia	Cave waters	5-1 mm	9.55±16.64 items/m³	Valentić et al. (2022)
Postojna Cave, Slovenia	Cave waters	5-1 mm	1.00±1.48 items/m³	Valentić et al. (2022)

MPs found in the Classical Karst Region highlighted that MP abundance in surface and subterranean waters is higher than most of underground waters investigated so far (Table 30), with the exception of Bossea Cave (Balestra et al., 2023) waters and ab alluvial aquifer monitored in Australia (Samandra et al., 2022). The size range of MPs detected in these works is different and the size range observed in our study is often wider, therefore, may be detected higher MP concentrations. Moreover, the examined waters are stagnant for long periods, and this can determine an accumulation of pollutants over time. Karst lakes are the surface manifestation of the karst subterranean water discharge (Culver and Pipan, 2014); in the lean period the water level decreases limiting water exchanges and aquatic habitats to channels and pools, creating peculiar environments. Water level in the examined cave areas is subject to seasonal variations, and water resources are limited to pools and large ponds in different periods of the year. Water level variations and connections between surface and subterranean systems in the area are likely to exacerbate the impact of microplastics in karst aquifers, being involved multiple food chains (including the terrestrial one) and habitats.

To the author knowledge, very few works examined MP pollution in sediments collected in subterranean karst environments and no one in the aquatic ones, therefore, comparisons were possible only with not aquatic karst environments. In sediments of three NW Italian show caves, were found 3823-4695 MPs/kg along the tourist path, and 1600-3186 MPs/kg in the speleological areas (MPs from 5 to 0.1 mm) (Balestra and Bellopede, 2023). The MP amount found in Trebiciano Cave was similar to those found in the speleological areas of the Italian show caves, while in 214 Cave, Mariano Well and Spring 8 the abundances were lower. In the Škocjan region, Slovenia, about 6667 items/m^3 were found in Kačna Cave sediments and 60,000 items/m^3 in Škocjan Caves system (Valentić et al., 2022), very low values compared to those found in the Classical Karst. Considering caves as conservative environments (Chiarini et al., 2022), even though the water levels may vary during the year, is reasonable to suppose an accumulation of pollutants in sediments of aquatic karst environments over time.

Percentages of MPs from 5 to 1 mm found in the Classical Karst waters were similar to those found in the Bossea Cave system (Italy) which accounted for 17.1% of the total MPs found (Balestra et al., 2023), while they were lower than those found in the waters collected in Guizhou Province, China, which MPs from 5 to 3 mm accounted for 44.20% (An et al., 2022). Percentages of MPs from 5 to 1 mm found in the sediment of the Classical Karst aquatic environments were less than those found in the non-aquatic sediments in the Italian show caves, which accounted for 25.3% in Toirano Caves, 27.5% in Borgio Verezzi Cave, and 27.3% in Bossea Cave (Balestra and Bellopede, 2023).

According to previous studies (e.g. Balestra and Bellopede, 2022b, 2023; Balestra et al., 2023; Panno et al., 2019; Valentić et al., 2022), the major part of MPs in the examined karst environments were fibres, both in liquid and solid matrices. About 60% of textile production worldwide is synthetic (Barrows et al., 2018; Boucher and Friot, 2017), suggesting pollution in this area could be linked mainly to synthetic textiles deterioration. Fragments had a relevant percentage too (30.0% in water and 23.7% in sediments). Italian show cave sediments along the tourist paths had a 5.5-12.1% of fragments (Balestra and Bellopede, 2023). In Bossea Cave karst system water, Italy, a 4.3% of fragments were found (Balestra et al., 2023). However, Classical Karst values about fragments were less than those found in the unconfined groundwater aquifer monitored in Victoria, Australia (94%) (Samandra et al., 2022). Fragments could have been produced from waste deterioration in the environment or nearest human activities. The monitored springs are located near important high speed roads, and 214 Cave develops near the railway track, therefore, MPs pollution in this sampling areas could be linked to the transit of vehicles, road markings, road wear, tire, train and track degradation, dust and garbage (Andersson-Sköld et al., 2020; Burghardt and Pashkevich, 2023). Abundant garbage was found near the train railway, highway and roads close to all examined areas. Mariano Well is located inside a urban complex with concrete constructions for drinking water collection in its initial stretch, and its waters resulted the most polluted by fragments. Therefore, most of the pollution in the well probably comes from the surface anthropogenic activities, which reach the subsoil with the infiltration of water into the ground. Another possible source of pollution could be leak from domestic water pipes or sewerage, that can percolates underground. The inevitable mixing of waste water with rainwater does not allow for any general assessment on the basis of water balances. Therefore, it is difficult to estimate the sewerage losses. Examining the other MP shapes found in this well, it is possibile to observe a high number of microbeads (7.4%) too. Microbeads are often used in care and beauty products (Anagnosti et al., 2021), such as scrubs, toothpaste, and cosmetics, reinforcing the hypothesis of sewerage losses. An interesting value of microbeads was also found in the sediment sample of Trebiciano Cave (31.6%), as well as the highest percentage of fragments (32.7%) among the sediment samples. Trebiciano Cave is an equipped cavity, visited by speleologists and researchers from 1974, therefore, this kind of pollution could be linked to the frequent presence of people, and to the surface human activities and garbage, which could be transported into the cavity by the waters of the inner stream.

As reported in previous research (Balestra and Bellopede, 2022b, 2023; Balestra et al., 2023), most of the MPs found were fluorescent under UV light (65% in water and 69% in sediments). These percentages were lower than those found in waters (85%) and sediments (88%) of Bossea Cave, Italy, and in sediments of Borgio Verezzi Cave (74%), Italy. Instead, percentages found in the Classical Karst were higher than that observed in sediments of Toirano Caves, Italy (60%) (Balestra and Bellopede, 2023; Balestra et al., 2023). These data show as the identification of MPs by UV light can be a good method, however, it is not sufficient for all particle analysis. MP fluorescence could provide information on consumption by organisms too. For examples, fluorescent pellets, fibres and fragments of anthropogenic origin were found in stygofauna collected from three groundwater bodies in Italian karst areas (Sforzi et al., under review).Ecosystem health is heavily affected by MP accumulation in the environment and some organisms wrongly mistake MPs for trophic resources consuming them (Gomiero et al., 2018; Sforzi et al., under review). Ingestion of MPs induces different deleterious effects on organisms, such as pathological alteration or blockages of digestive processes. MP colour can provide information on consumption by organisms (Carpenter et al., 1972; Jahan et al., 2019; Lusher et al., 2013; Romeo et al., 2015; Ugwu et al., 2021), making this step of analysis fundamental for ecological and environmental research. In marine biota, most of MPs particles show a blue colour (32.9%), followed by white (24.7%), black (18.8%) and transparent (16.5%) particles (Ugwu et al., 2021). In fish and marine mammals, the predominant colour of MPs found were blue, black, transparent, and white. Different colours were found in MPs ingested by birds, where transparent colour are the majority (55.6%), followed by blue (33.3%) and brown (11.1%). Some studies identified black plastics as the most often ingested ones by the analyzed sea animals (Jahan et al., 2019; Lusher et al., 2013); in Romeo et al. (2015) transparent and white MPs occurred in the stomach of all analysed top sea predators, whereas blue particles and yellow ones were found only in some of them. In Sforzi et al. (under review), some anthropogenic particles of blue colour were found in the digestive tract of crustaceans of karst caves and springs, suggesting the potential presence of MPs in the fecal pellets. In this study, transparent, black and blue particles were found in high amounts in all examined superficial and subterranean sampling areas; white particles were present in all sampling area, even if only in small percentages. Therefore, further investigations could be done in the future to better understand if the aquatic organisms of the Classical Karst, especially stygobionts, consume MPs mistaking them for trophic resources. MP colour and fluorescence could be not of particular interest for stygobiont ingestion, being these animals mostly blind, however, they may be important for consumption by surface organisms and animals less adapted to the hypogeal environment. In addition, MP colour can provide indication about the chemical pollutants which can bind to them by contaminating them (e.g. Frias et al., 2010; Karapanagioti et al., 2011). In marine environments, high levels of pollutants were found on yellow and black particles (Frias et al., 2010; Karapanagioti et al., 2011). Black particles were found in all sampling areas with high amounts, and different yellow MPs were observed in Spring 16, 214 Cave and Mariano Well. Most of the non-fluorescent MPs found in the Classical Karst was black (48.3% in water and 56.2% in sediment), and different yellow particles in fluorescent MPs were found in water samples (4.9%). Future investigations should be carried out to better understand the presence of pollutants related to MPs in those environments.

At the moment of the research, in 2023, MP typology comparisons with other karst systems were possible only between few areas (An et al., 2022; Balestra and Bellopede, 2023; Balestra et al., 2023; Romano et al., 2023; Valentić et al., 2022): PET and PA have been found frequently, together with PE (polyethylene), PP (polypropylene), and PS (polystyrene), PVA (polyvinyl alcohol), EVOH, PVC (polyvinyl chloride), acrylic adhesive, PU (polyurethane), PAM, EVA (ethylene vinyl acetate), EPDM (ethylene propylene diene rubber), PMMA (polymethylmethacrylate), PBT (polybutylene terephthalate), PVFM (polyvinyl formal), PTFE, copolyester, TPV (thermoplastic vulcanizates) and EBA (ethylene butyl acrylate). Many of these plastics are commonly used in the textile industry, validating the primary hypothesis on the origin of synthetic fibres from the textiles.

5.5.2 Microfibres pollute karst systems

A high amount of MFs was found in all water and submerged sediment samples, highlighting an intense pollution in the aquatic habitats of the karst system. MFs had higher abundances into sediment compared to water, providing a relevant insight into the role of karstic sediment for the storage of MF pollution in subterranean and surface karst environments, as suggested by Hasenmueller et al. (2023). Baraza and Hasenmueller (2023) highlighted an increase in anthropogenic microparticle transport during and after discharge peaks, suggesting that flood events can trigger resuspension of particle in sediments of karst systems. The presence of anthropogenic MFs in the karst system is concerning because they are harmful for organism (Athey and Erdle, 2022; Rochman et al., 2013); polluted sediment could increase the risk of ecotoxicity for stygobiotic species that live in these habitats, especially if detritivores. To our knowledge, this is the first study analyzing MF pollution in submerged sediments in a karst system. Monitoring of submerged sediments should be taken into account in water matrix studies in order to better understand the amount and the way of accumulation of MFs in sediments, especially for karst systems, being precious subterranean water reserves.

Few studies were conducted on MPs in karst system before this research (e.g. Balestra and Bellopede, 2022b, 2023; Balestra et al., 2024b; Balestra et al., 2023; Valentić et al., 2022), generally in show caves and nearby environments; to the author knowledge, anthropogenic materials (5mm - 4 µm) were only detected in one American karst system (Baraza and Hasenmueller, 2023; Hasenmueller et al., 2023). The lack of studies on natural and regenerated MFs in karst systems represents a significant gap in current micropollutant research because of the known ecotoxicity of these materials (Athey and Erdle, 2022 and references therein). A great effort must be done to sample in non-touristic caves and in connecting environments such as springs, to better understand the transport of these pollutants along the karst systems. Collecting larger volumes of samples in caves and springs is challenging, but monitoring multiple areas can allow measuring the extent of contamination. Long-term monitoring will be necessary to verify seasonal variation and accumulation of MFs over time.

Comparisons with other studies are difficult because a standardized methods for MF detection and characterization in environmental samples does not exist, MF monitoring in karst system are rare, and MF studies in karst sediments are scarce. Several publications do not report natural and/or regenerated MFs, some studies chemically digest natural MFs, and others excluded natural materials during spectroscopic analysis or simply from final reports (Athey and Erdle, 2022). Moreover, the size of the examined particles, sampling areas, monitoring period, and environmental conditions may vary pollutants concentrations over time.

Considerations with another karst system could be done only with part of the data reported in Hasenmueller et al. (2023) and Baraza and Hasenmueller (2023), which analyzed water and sediment samples from Cliff Cave, a show cave in the United States with limited visitor access, and a perennial spring issuing from the cavity. Inside the cave, a concentration of suspected anthropogenic microparticles of 7.1±2.1 particles/kg (average ± SD) in water, and 842.7±166.4 particles/kg in sediments were found, of which 91% were fibres and 59% clear (Hasenmueller et al., 2023). In water, 58% of particle were regenerated and only 1% was plastic, while in sediments, 31% of particle was regenerated and 29% plastic (Hasenmueller et al., 2023). Spring waters issuing from that cave contained a mean of 9.2 particles/L during baseflow, increased to 81.3 particles/L during floods, of which 85.8% were fibre-shape, and 76.8% were cellulosic, predominantly clear (Baraza and Hasenmueller, 2023).

In a different environment, Suaria et al. (2020a) detected fibre pollution in oceanic surface waters of 617 locations with fibre concentrations from 0.02 to 25.8 fibres/L (considered size: 0-15 mm) was found. Also in this case, the major part of the fibres were natural (91.8%), of which 79.5% cellulosic, with cotton as the most frequent (50% of all fibres); only 8.2% fibres were synthetic, of which 6.2% polyester.

MF abundances in these studies were lower than those found in the CKR, however, it is possible to notice that other data were similar to those found in this study: most particles were fibres, clear, and not synthetic, and an accumulation of pollutants in sediments was highlighted. Different studies reported that non-synthetic MFs were more abundant that synthetic ones in natural environments, and mostly detected cellulosic fibres, especially cotton (Athey and Erdle, 2022 and references therein). As about 64% of textile production worldwide is synthetic (Textile Exchange, 2022), there is a discrepancy between the MF composition detected in natural environments and the global production of synthetic textiles, which is worth additional in-dept research.

Except a pair of probable ramie fibres, all natural fibres analyzed by µFTIR-ATR resulted to be cotton, which was also the most frequent fibres found among those identified with spectroscopic analyses (20% of all examined fibres in water and 24% in submerged sediments). The regenerated MFs comprised Cupro/Bemberg, Tencel/Lyocel and cellulose acetate MFs, commonly used in textile production, methyl cellulose and hydroxypropil methyl cellulose.

Cellulose is versatile polymer and chemical modification of cellulose allow to produce strong, low cost, reproducible, recyclable and biocompatible cellulose derivatives, therefore, cellulosic materials are increasingly used (Lavanya et al., 2011).

In textile industry, cellulose ethers can be used as sizing, leveling, and thickening agents of textile pulp. Methyl cellulose does not occur naturally, and is synthetically produced by heating cellulose with chemicals (Lavanya et al., 2011). It is used as a thickener in foods, supplements, cosmetics, care products, glue and binder, construction materials, and as sizing in the production of papers and textiles as it protects the fibres from absorbing water or oil (Lavanya et al., 2011). Hydroxypropyl cellulose (HPC) is highly demanded by different end-use sectors, especially pharmaceutical, personal care, and foodstaff; in textile industry. HPC demand increased recently, due to the rising demand in textiles and employing eco-friendly sizing agents (https://www.reportsanddata.com/report-detail/hydroxypropyl-cellulose-market; accessed: 02-03-2024 15:58). In addition to textiles, cellulose acetate is usually used for cigarette filters production, together with plasticized additives (Belzagui et al., 2021). Common commercially non-flushable and flushable wipes are often made of synthetic fibres and cellulose, or cellulose alone (Ó Briain et al., 2020).

Most detected synthetic MFs are commonly used in textile production, such as polyester, especially PET, polyamide, polytetrafluoroethylene and some copolymers. In this work, polyesters and copolymers were the most present synthetic fibres. Polyester production alone represent the 54% of the global total fibre production for textiles (Textile Exchange, 2022), and, due to its properties and density, it sinks very quickly and shows weathering resistance (Šaravanja et al., 2022). Other polymers are used in textiles production during pretreatment, dying processes, coatings, as binder in non-wovens, textile sizing, auxiliaries, and finishing, such as EVOH, PVAc or Polyacrylamide.

Different sampling points, such as 214 Cave or the two Springs, are located near roads and railways, which are potential sources of pollution. Natural and synthetic fibres are commonly used in asphalt mixtures, acting as an asphalt stabilizer to decrease the drain-down effect, and as a reinforcing additive to enhance the mechanical performance of asphalt mixtures (Guo et al., 2023). Natural fibres employed in asphalts are generally plant-based (e.g. bamboo, coconut/coir, jute and sisal) or mineral fibres, while synthetic fibre are mainly polyamides, polyolefins, especially polypropylene and polyester (Guo et al., 2023). The construction of roads is often improved by the use of geotextiles, with considerable advantages, including the increase in the lifetime of the road structure. Geotextiles are used in railway construction too, to improve the stability and performance of track beds and embankment structures. Geotextiles can be non-woven, woven, or knitted, and they can contain natural or synthetic fibres. The polymers most used to manufacture geotextiles include polypropylenes (PP), polyesters, especially PET, polyamides, and polyethylenes. Even geomembranes are commonly used in this field, composed by different kind of synthetic materials and copolymers. Fibres were commonly used in concrete construction too.

Both direct and indirect human activities contribute to the pollution in karst habitats, including activities in caves, albeit probably to a small extent. Cave suits are made from resinated cotton to nylon, or technical materials such as polyamide and Cordura, but undertunics are often in polyester, although they do not rub directly on the rock. However, it is reasonable to think that the number of cavers in one year of activity could be not compared to the extent of external human activities and therefore, pollution. Moreover, except Trebiciano cave, the other ones are not frequented by cavers so much because they ended in waters. Rubbish degradation near roads and railways can be a source of pollution too.

Most of the MF pollution is probably related to the hydrodynamic regime of the aquifer, the geology of the karst area, and the local meteorological conditions. The examined system is only the final part of a kilometric karst system starting from Slovenia, which manages huge water supplies. These enormous flows carry large quantities of material from outside and rework sediments previously deposited in the system. An increase in anthropogenic microparticle transport during and after discharge peaks were highlighted in Baraza and Hasenmueller (2023), suggesting that flood events can trigger resuspension of particle in sediments of karst systems. Atmospheric deposition and precipitations play a fundamental role in micropollutant deposition, as highlighted for MPs (e.g. Allen et al., 2019; Liu et al., 2019b). This kind of pollution is strongly related to the soil characteristics (Zhou et al., 2021): contamination occur because of the micropollutants transport throughout the soil pores and rock fractures, which can accumulate in subterranean waters and environments (Chia et al., 2021; Fahrenfeld et al., 2019; Frei et al., 2019; Lwanga et al., 2017; McGechan, 2002; Viaroli et al., 2022; Wanner, 2021).

We assume that both atmospheric and flood depositional processes may have introduced anthropogenic MFs in aquatic surface and subterranean habitats, as well as the presence of highways, roads and railway tracks near the examined areas. Human activities in caves may have contributed too, but their impacts probably are negligible considering the water inputs involved. MF infiltration through fractures and soil can lead to an accumulation of micro-pollution in karst systems, posing a risk to water quality and biodiversity conservation. Considering subterranean habitats as conservative environments (Chiarini et al., 2022), the possible hazards for these habitats and resources become even more evident and alarming.

MF fluorescence and colour can provide information on the MF consumption of organism (Carpenter et al., 1972; Jahan et al., 2019; Lusher et al., 2013; Romeo et al., 2015; Ugwu et al., 2021) and possible associations with other pollutants (Frias et al., 2010; Karapanagioti et al., 2011). Several researches highlighted that organisms consume anthropogenic materials, such as MPs and anthropogenic cellulose fibres, with toxic effects (Anbumani and Kakkar, 2018; Athey and Erdle, 2022; Gomiero et al., 2018; Kim et al., 2021a; Remy et al., 2015; Zhang et al., 2023a), even in karst and underground environments. Some studies demonstrated associations of pollutants with black and yellow MPs (Frias et al., 2010; Karapanagioti et al., 2011), therefore, in depth investigations should be done even in the field of not synthetic fibres.

Most of the MFs found in the CKR were fluorescent under UV light (81.9% in water and 93.0% in submerged sediments). Analysis under UV light allows to identify a high number of MFs, but are not sufficient: non-fluorescent particles are generally coloured and could be consumed by organism, and a part of the non-fluorescent MFs found in the CKR was black, color which could be index of presence for other pollutants.

It should be taken into account that the use of chemicals for the OMR may decrease the fluorescence, partially degrading them. However, OMR used in our analysis were done for a week only, limiting also particle degradation and additive release.

Many studies focus only on abundance and shape of pollutants, still color and fluorescence can provide important information. Further investigations are required to understand if organisms that live in the karst habitats consume MFs, especially aquatic and stygobionts species, and if MFs are linked to the presence of other pollutants.

Research on MFs is challenging, and the analysis of natural and regenerated MFs in environmental matrices is more difficult compared to synthetic ones, because the methodologies used to detect and characterize MFs were originally designed for MPs (Athey and Erdle, 2022). The amounts of micro-pollutants found in the used chemicals and solutions tested in this research, highlight the importance to filter all products before laboratory analysis. A greater awareness of how much these materials can pollute the samples is necessary, for boh scientist and producers, which should intervene as soon as possible on the quality of their products. Organic matter removal with certain chemicals may result in the partial or complete degradation of non-synthetic MFs (Athey and Erdle, 2022; Treilles et al., 2020). Digestion with H_2O_2 is the most common method used in the MFs analysis (Athey and Erdle, 2022), but it can affect mechanical properties and IR spectra, and increases the fragility of some kind of fibres: the brittleness could potentially lead to fibre fragmentation, resulting in counting errors and overestimation (Treilles et al., 2020).

5.5.3 General considerations

Pollution is major cause of modification of wildlife habitats, determining the loss of sensitive species. Anthropogenic activities, atmospheric depositions and infiltrations through fractures and soil can lead to MP and MF pollution in karst systems, posing a risk to water quality and biodiversity conservation of these particular ecosystems. From ciliates to detritivores and predators, the direct or indirect consumption of MPs available in the environment has been highlighted (e.g. Devereux et al., 2021; Lusher et al., 2013; Pennati et al., 2022). These data suggest that both surface and subterranean aquatic species in karst system are potentially exposed to MP and MF pollution. Even in relatively simplified ecosystems like subterranean water, MPs and MFs could affect multiple levels of the trophic web, and impact species with important regulatory functions across the ecosystem, such as the olm. Usually, the quantification of contaminants/pollutants affecting apex, long-lived predators can represent an effective method for detecting their transfer through food webs. As the olm is the apex predator in Classical Kast aquifers, evaluating the level of MP and MF occurrence in the habitats where olm occur represents a first step to assess the biological effects on organisms depending from groundwaters and, potentially, also humans. Taking into account previous work on aquatic fauna, it is reasonable to think that stygobionts exposed to MPs and MFs could suffer detrimental impacts such as disrupted feedings, compromised reproductivity or organs damage, especially in the gastrointestinal system (Anbumani and Kakkar, 2018; Zhang et al., 2023a). MPs in stygobionts digestive tract and gut were recently detected in cave and spring habitats, thanks to this thesis research (Sforzi et al., under review), but biological response to MP exposure in this habitats is unknow. Further studies assessing MP and MF content in the stomachs of apex predators such as the olm will be useful to elucidate MP and MF impacts in aquatic subterranean and surface environments. Groundwater and spring fauna plays an important role in the sediment transport and the cycle of nutrient, positively contributing to the overall health of the aquatic ecosystem (Mermillod-Blondin et al., 2023). MPs and MFs along food chains could have implications until higher trophic levels, disrupting the above-mentioned processes, with cascading ecological consequences (Zhang et al., 2023a). MPs and MFs occurring in subterranean environments may impact also the boundaries of subterranean environments, including fragile ecotones like springs.

MP and MF monitoring is essential to define the health status of the environment, and, consequently, to establish conservation strategies. Ecological connections between habitats have to be taken into account, such as species displacements between surface and subterranean environments. Habitats are frequently subjected to partial conservation measures which rarely take into account the connections between ecosystems and the movement of species between them. These connections must be considered when planning comprehensive measures of protection. Assessing and promoting changes at the political and social levels that encourage real plastic reduction strategies and provide adequate education following the environmental sustainability principles are needed for the management and conservation of water resources, protected habitats and species. MP and MF pollution monitoring in karst areas must become a priority for the habitat and species conservation and the water resources management, implementing analyses on a larger number of aquatic surface and subterranean habitats.

5.6 Conclusion

These first results improved knowledge on MP and MF pollution in natural environments, drawing attention on karst water quality, and the potential risks that could affect protected habitats and species. This first investigation in the Italian sector of the Classical Karst Region, hosting different protected and stygobiont species, documented the presence of MPs and MFs in surface and subterranean waters, drawing attention on the potential exposition of stygobiont species (but not only) to MP and MFs pollution, which could attain multiple levels of the trophic resources.

The concentration of MPs in monitored waters and sediments was highly variable, with most of particles being transparent and fluorescent under UV light. Vice versa, not-fluorescent particles were mainly black or dark. Fibre-shape dominated the samples, suggesting that synthetic textiles are the main source of microplastic pollution in this area. Other possible sources of pollution could be linked to nearby human activities, providing useful references for further research.

Most studies on MF pollution in natural environment focused on synthetic fibres only, neglecting a major component of MF pollution: cellulosic fibres. These results improved knowledge on MF pollution in karst systems, showing the co-occurrence of natural, regenerated and synthetic fibres. MFs were frequent in karst systems, from caves to springs, and were distributed in both water and sediment reservoirs, with potential impacts on habitat, species and water quality. MF abundances were particularly high in submerged sediments, suggesting a major role of sediments for the storage of microparticle pollution in subterranean and surface environments.

MP and MF monitoring is a key step to understand the health status of environment and to establish management strategies. Future studies are needed to better understand MP and MF sources and transport in karst areas, verifying the potential effects on ecosystems and aquatic organisms. Long-term monitoring, considering more subterranean and surficial sites, will help understanding the impact of MP and MF pollution on habitats.

Surface and underground environments in karst areas are closely connected, therefore, greater efforts should be made to establish more comprehensive measure of protection. Extensive karst system, especially when they develop among more nations, should be managed at the international level, with monitoring plans covering the entire drainage (Canedoli et al., 2022).

Chapter 6

Unexplored caves

Case Study: Microplastic and microfibre pollution in unexplored caves of Abruzzo Region, Italy

Origin of the Chapter

This chapter is adapted from the peer-reviewed article "Explorations in the dark continent: did microplastics and microfibres get here before us?" authored by Balestra and Bellopede (2025), published in Science of The Total Environment, Volume 977, May 2025, 179328, DOI: **https://doi.org/10.1016/j.scitotenv.2025.179328.**
The author of this dissertation was responsible for conceptualisation, methodology, validation, formal analysis, investigation, resources, data curation, writing – original draft, writing – review and editing, visualisation, project administration.

Permissions

6.1 Introduction

Anthropogenic microparticle (AMP) pollution (MPs and natural and regenerated MFs) in karst habitats can be linked to direct and indirect human activities, and is strictly related to the hydrodynamic regime of the aquifer, air circulation, geology, karstification degree, and the local meteorological conditions. Direct contamination in subterranean environments is linked to human presence, such as tourists in show caves or speleologists and researchers in the other ones. The indirect contamination in these environments can come from litter, wastewater, agriculture activities, transports, industrial production, soil and surface water pollution, and atmospheric deposition (e.g. Allen et al., 2019; Liu et al., 2019b; Zhou et al., 2021). Once in the soil, MPs can be reduced in dimension and transported through soil pores and rock fractures, accumulating in subterranean waters and environments (Chia et al., 2021; Fahrenfeld et al., 2019; Frei et al., 2019; Lwanga et al., 2017; McGechan, 2002; Viaroli et al., 2022; Wanner, 2021). MFs origin and transport mechanisms could be very similar to MP ones, however, these studies are at the early stage.

Throughout history, humans have explored every place on the surface of the planet, even the most remote and inaccessible, also with the help of different technologies capable of exploring these environments for them. The same does not apply to the subterranean world, in which passages still completely unknown, since there are no tools and technologies for these kind of explorations. At the moment, the only way to increase knowledge about these environments is to explore them by yourself, therefore, the underground world remains the last frontier of terrestrial exploration. Evaluating MPs and natural and regenerated MFs occurrence in unexplored subterranean environments provide important insights for the management and conservation of karst areas, highlighting how indirect pollution can contaminate even places never explored by humans before.

In this research, sediment samples from five unexplored caves of the Abruzzo Region, Italy, were investigated. The aims of this study were: i) to examine, for the first time, the presence of MPs and natural and regenerated MFs in unexplored caves; if present, ii) to discuss AMP abundance and characteristics, potential risks and ecological effects that this kind of pollution could lead to karst environments, even indirectly.

This work wanted to be the first assessment of AMP pollution in unexplored environments, encouraging more researchers and speleologists to sample also during explorations, despite all the difficulties of progression in unknown environments, helping environmental studies on pollution, with the aims of promoting appropriate conservation measures for karst areas.

6.2 The Study area

Examined caves are located in the village of San Vito, in the Valle Castellana municipality, Abruzzo Region, Italy (Fig. 66). Valle Castellana is part of the Gran Sasso and Monti della Laga National Park. The valley ranges from medium low altitudes (400 m) to high peaks that exceed 2400 m a.s.l. San Vito village is located at about 684 m a.s.l. The soil is predominantly of marly-sandstone, however, travertine is present in some areas, in which caves are present. The area is characterized by a mountain climate, with snowy winters and mild summers. The diversity of altitude, the abundance of water, and the variety of microclimates present favored the expansion of forests and dense woodlands in the past.

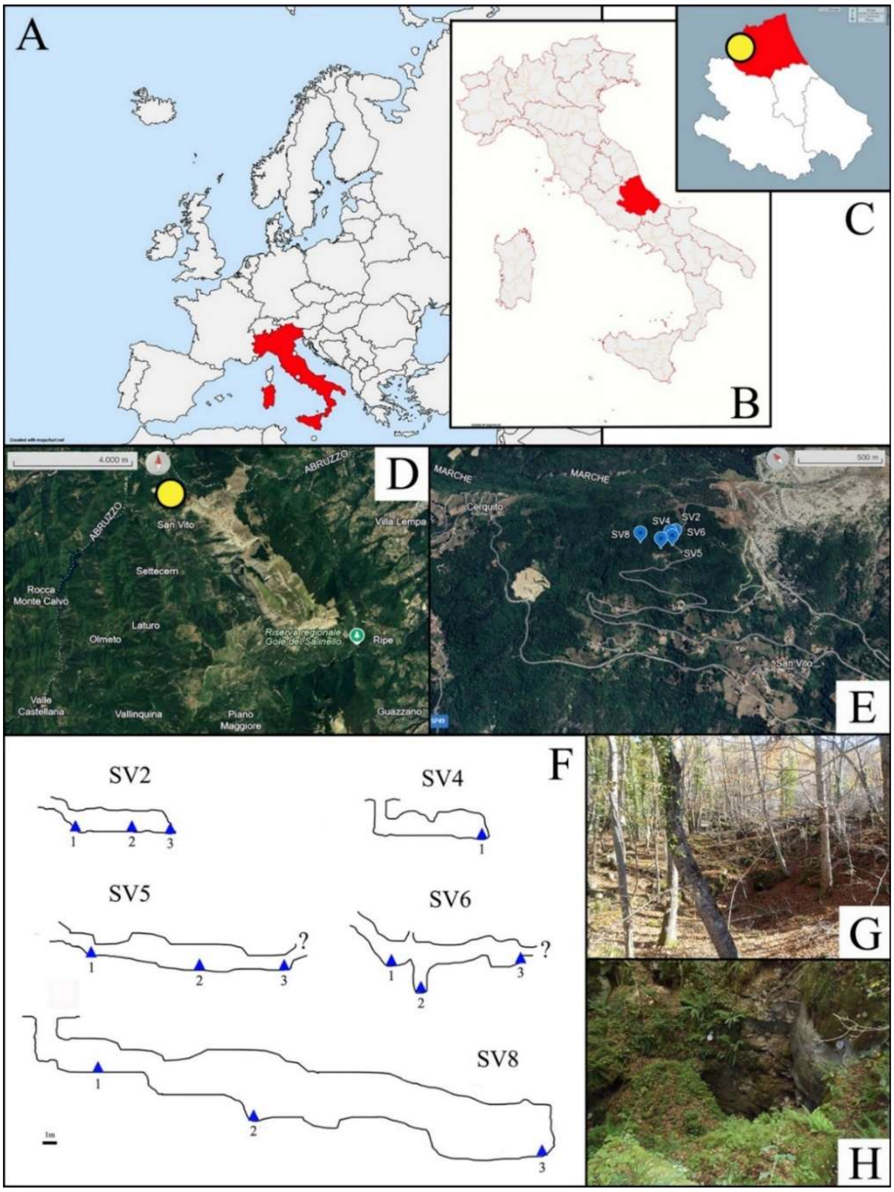


Fig. 66. Study area, caves and sampling points. A, B: Study area (maps from mapchart.net/italy, modified and mapchart.net/italy, modified); C: Abruzzo Region, Italy, with yellow point for the studied area (Map from https://d-maps.com/carte.php?num_car=21530&lang=it, modified); D: Detailed images of the area with yellow point for the studied area (map from https://earth.google.com/, Imagery ©2009 Google, Airbus, GeoBasis-DE/BKG (©2009), modified [access 2024-11-11]); E: Detailed images of the area with blue placemark for the monitored caves (Map from Google, Airbus (©2009), modified [access 2024-11-15]); F: Cave surveys (sections) with sampling points (blue triangles); G, H: photos from the monitored area near the caves (photos by E. Licocci). Image from Balestra and Bellopede (2025).

6.3 Method

6.3.1 Field sampling

Five unexplored caves were selected for this study (Fig. 66E, F). For San Vito caves 2, 5, 6, 8, three sampling points at different distance from the entrance were defined, one for San Vito cave 4 (Table 34), based on the environmental characteristics of the caves. Sampling points were called from 1 to 3 starting from the entrance to the deepest areas of the caves (Fig. 66F). Monitoring was conducted in September 2022.

Table 34 – Information regarding the monitored caves and their respective sampling points.

Cave	Horizontal development [m]	Development	Sampling points	Sampling point distance from the entrance [m]
SV2	10	Horizontal	SV2-1	2.5
			SV2-2	7
			SV2-3	10
SV4	10	Semi-vertical	SV4-1	10
SV5	22	Horizontal	SV5-1	3
			SV5-2	13
			SV5-3	20
SV6	16	Horizontal	SV6-1	3
			SV6-2	10
			SV6-3	16
SV8	45	Vertical	SV8-1	7
			SV8-2	20
			SV8-3	45

San Vito 2 cave (Fig. 66F) has a predominantly horizontal development, with a entrance of about 4.5 m characterized by the presence of a dry wall that partially protects the entrance. The entrance opens after a small descent of about 2 m, characterized by the foliage of the beech trees. The first sampling point (SV2-1) was located in the entrance hall, the second (SV2-2) after a narrow passage, under a small rock jump, and the last (SV2-3) at the end of the cave, at the foot of a ascent that connects San Vito 2 cave with the nearby San Vito 3 cave. Bones of small animals were found inside the cave.

San Vito 4 cave (Fig. 66F) has a semi-vertical development with a pit-entrance of about 3 m. The first room was unfortunately invaded by garbage. The only sampling point (SV4-1) was located at the end of a second room, distant about 10 m from the entrance.

San Vito 5 cave (Fig. 66F) has a predominantly horizontal development of about 22 m with a slightly sloping entrance of about 2 m. Although the presence of the provincial road (SP) San Giacomo-San Vito at about 10 m from the cave entrance, the entrance is not immediately visible, and is about 1.8 m wide by 1.5 m high. The cave develops along a fracture, with collapsed boulders. The first sampling point (SV5-1) was located after the slide at the entrance, rich in foliage and earth falling from the surface. In this area, a cave salamander and some arthropods were observed. The second sampling point (SV5-2) was located in the central part of the cave, and the third (SV5-3) at the end. The end of this cave is located under the SP. During the sampling, the soil sampled was barely wet, such as all the cave.

San Vito 6 cave (Fig. 66F) has a predominantly horizontal development of about 16 m with a slightly sloping entrance of about 5 m high, at the end of which was located the first sampling point (SV6-1). The entrance is located near the SP San Giacomo - San Vito. In the middle of the cave a pit of about 4 m is present (Sampling point SV6-2). The last sampling point was located at the end of the cave (SV6-3).

San Vito 8 cave (Fig. 66F) is located in a vegetated zone rich in ferns, mosses and other plants typical of highly humid areas, in a ex-travertine quarry, closed about 40 years ago. The cave entrance is about 4 m high and 1.7 m wide. The cave has a vertical development, characterized by about four wells. Sampling were done immediately under the first well (SV8-1), the third well (SV8-2), and at the end of the cave (SV8-3), characterized by the presence of mud, but without flowing water during the sampling period in September.

Following the precautionary principle and evaluating the environmental characteristics of the studied cave, the amount of collected samples was limited to about 400-600 g of shallow sediments (upper 5 cm) for each sampling area, according to the availability. Sediment bulk samples (Hidalgo-Ruz et al., 2012) were collected by the Speleo Club Teramo cavers during the cave explorations. Before sampling, speleologists were trained on methodology and sampling through training courses and the drafting of a protocol. Samples were named with SV (San Vito), the number of the cave (2, 4, 5, 6, 8) and the number of the sampling point (from 1 to 3). The jars were limited and coated with anti-impact material to transport them safely in the speleological bags.

For more detailed information about sampling, see Chapter 3.

6.3.2 Laboratory analysis

Sediment samples were analyzed following the procedures outlined by Balestra et al. (2024a). Dried sediments were weighed and subjected to organic matter removal (OMR) using a 1:1 30% H_2O_2 solution and left to react for seven days under natural conditions. Depending on the amount of remaining dry sediments in each sample, three sub-samples of 15 g each were selected using the coning and quartering method. Samples were filtered through a 0.2-μm pore size ANODISC 47 microfibre filter (Cytiva, Ø 47 mm).

For more detailed information about the method, see Chapter 3.

6.3.3 Microplastic and microfibre identification and characterization

A combination of microscopic and spectroscopic techniques was used in this work. Initially, microparticles on filters were observed under a Leitz ORTHOLUX II POL-MK microscope equipped with a DeltaPix Invenio 12EIII 12 Mpx Camera. A further detailed comparison was made for the MF analysis, observing details showed in longitudinal and x-sectional microscopic images of natural, regenerated and synthetic fibres (e.g. Khan et al., 2017). Microparticles ranging from 5 to 0.1 mm were analyzed. Microparticles that could not be definitively identified as AMPs were excluded from the analysis. Spectroscopic analyses were performed on 40% of the microparticles found on the filters, from each sampling area, using a μFTIR+ATR. Only spectra with a match degree ≥ 70% were accepted.

For more detailed information on microscopic and spectroscopic techniques used, see Chapter 3.

6.4 Results

6.4.1 Abundance and composition

MPs were not found in the major part of the sediment sub-samples, however, in three sub-samples were present (Table 35). Caves SV4 and SV8 had no MPs, in the other monitored caves MPs were present only in one sampling point, and in relatively small amounts (2-4 items for 15g of sediments) (Table 35).

Table 35 – Anthropogenic microparticle (AMP) abundances for each sub-sample of sediment from unexplored caves, and estimates for one kilo of sediments. AMPs were divided into synthetic (microplastics) and non-synthetic (natural and regenerated microfibres).

Filter Water	Natural and Regenerated [items/15g]	Synthetic [items/15g]	N.D. [items/15g]	TOT [items/15g]	Natural and Regenerated [items/kg]	Synthetic [items/kg]	N.D. [items/kg]	TOT [items/kg]
SV2 1.1	0	0	0	0	0	0	0	0
SV2 1.2	0	0	0	0	0	0	0	0
SV2 1.3	0	0	2	2	0	0	133	133
SV2 2.1	0	0	0	0	0	0	0	0
SV2 2.2	52	3	0	55	3467	200	0	3667
SV2 2.3	0	0	0	0	0	0	0	0
SV2 3.1	0	0	0	0	0	0	0	0
SV2 3.2	0	0	0	0	0	0	0	0
SV2 3.3	0	0	0	0	0	0	0	0
SV4 1.1	0	0	0	0	0	0	0	0
SV4 1.2	0	0	0	0	0	0	0	0
SV4 1.3	54	0	0	54	3600	0	0	3600
SV5 1.1	0	0	0	0	0	0	0	0
SV5 1.2	0	0	0	0	0	0	0	0
SV5 1.3	0	0	0	0	0	0	0	0
SV5 2.1	0	0	0	0	0	0	0	0
SV5 2.2	0	0	0	0	0	0	0	0
SV5 2.3	100	4	0	104	6667	267	0	6933
SV5 3.1	6	0	0	6	400	0	0	400
SV5 3.2	0	0	0	0	0	0	0	0
SV5 3.3	49	0	0	49	3267	0	0	3267
SV6 1.1	0	2	0	2	0	133	0	133
SV6 1.2	0	0	0	0	0	0	0	0
SV6 1.3	19	0	0	19	1267	0	0	1267
SV6 2.1	41	0	0	41	2733	0	0	2733
SV6 2.2	0	0	0	0	0	0	0	0
SV6 2.3	0	0	0	0	0	0	0	0
SV6 3.1	0	0	0	0	0	0	0	0
SV6 3.2	0	0	0	0	0	0	0	0
SV6 3.3	0	0	0	0	0	0	0	0
SV8 1.1	0	0	0	0	0	0	0	0
SV8 1.2	0	0	0	0	0	0	0	0
SV8 1.3	0	0	0	0	0	0	0	0
SV8 2.1	0	0	0	0	0	0	0	0
SV8 2.2	0	0	0	0	0	0	0	0
SV8 2.3	0	0	0	0	0	0	0	0
SV8 3.1	0	0	0	0	0	0	0	0
SV8 3.2	34	0	0	34	2267	0	0	2267
SV8 3.3	0	0	0	0	0	0	0	0
TOT	355	9	2	366	23667	600	133	24400
%	97.0	2.5	0.5	100.0		163.9	36.4	6666.7

In addition to MPs, taking into account also natural and regenerated MFs of anthropogenic origin, all monitored caves were polluted, with more contaminated sampling sites (Fig. 67, Tables 36 and 37). Taking into account the average amounts of microparticles found for each cave, SV4 was the most polluted cave, and SV8 was the least one (Table 36). Considering that examined caves were unexplored, the quantities of AMPs found in the sampling points were considerable, with an estimate ranging from 0 to 2311.1 items/kg (Fig. 67 and Table 37).

Table 36. Anthropogenic microparticle (AMP) abundances for unexplored cave (averages). AMPs were divided into synthetic (microplastics) and not synthetic (natural and regenerated microfibres).

Cave	Natural and Regenerated [items/15g]	Synthetic [items/15g]	N.D. [items/15g]	TOT [items/15g]	Natural and Regenerated [items/kg]	Synthetic [items/kg]	N.D. [items/kg]	TOT [items/kg]
SV1	5.8	0.3	0.2	6.3	385.2	22.2	14.8	422.2
SV4	18.0	0.0	0.0	18.0	1200.0	0.0	0.0	1200.0
SV5	17.2	0.4	0.0	17.7	1148.1	29.6	0.0	1177.8
SV6	6.7	0.2	0.0	6.9	444.4	14.8	0.0	459.3
SV8	3.8	0.0	0.0	3.8	251.9	0.0	0.0	251.9
MEAN	10.3	0.2	0.0	10.5	685.9	13.3	3.0	702.2

Table 37. Anthropogenic microparticle (AMP) abundances in sediment samples of unexplored caves, for each sampling point (averages). AMPs were divided into synthetic (microplastics) and not synthetic (natural and regenerated microfibres).

Filter	Natural and Regenerated [items/15g]	Synthetic [items/15g]	N.D. [items/15g]	Mean [items/15g]	St dev	Natural and Regenerated [items/kg]	Synthetic [items/kg]	N.D. [items/kg]	Mean [items/kg]	St. dev
SV2 1	0.0	0.0	0.7	0.7	0.9	0.0	0.0	44.4	44.4	62.9
SV2 2	17.3	1.0	0.0	18.3	25.9	1155.6	66.7	0.0	1222.2	1728.5
SV2 3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
SV4 1	18.0	0.0	0.0	18.0	25.5	1200.0	0.0	0.0	1200.0	1697.1
SV5 1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
SV5 2	33.3	1.3	0.0	34.7	49.0	2222.2	88.9	0.0	2311.1	3268.4
SV5 3	18.3	0.0	0.0	18.3	21.8	1222.2	0.0	0.0	1222.2	1454.8
SV6 1	6.3	0.7	0.0	7.0	8.5	422.2	44.4	0.0	466.7	568.3
SV6 2	13.7	0.0	0.0	13.7	19.3	911.1	0.0	0.0	911.1	1288.5
SV6 3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
SV8 1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
SV8 2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
SV8 3	11.3	0.0	0.0	11.3	16.0	755.6	0.0	0.0	755.6	1068.5

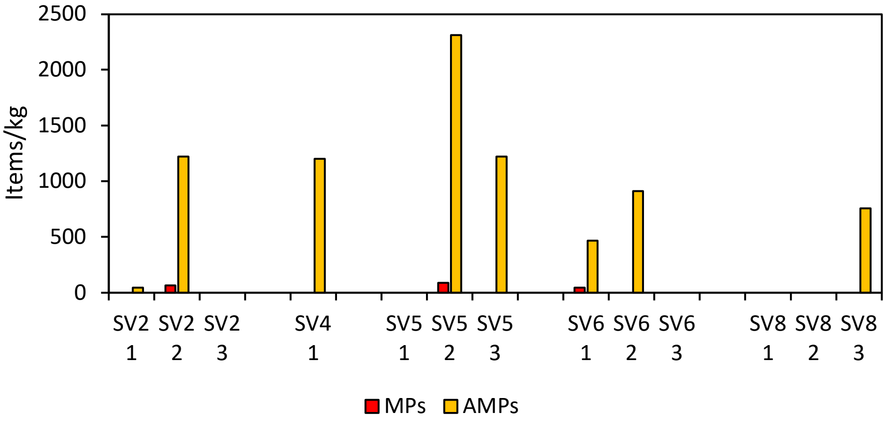


Fig. 67 – Microplastic (MP) and anthropogenic microparticle (AMP) abundances for each sampling point. Image from Balestra and Bellopede (2025).

Microscopic analysis showed that 97.1% of AMPs were natural and regenerated materials, 2.2% synthetics, and 0.7% not clearly distinguishable (Fig.s 68A, 69). Of the natural and regenerated MFs, 34.1% were cotton, ranging from 21.5% to 58.1% in caves SV4 and SV2 respectively. All other materials were cellulosic, except for one woolen MF found in cave SV5.

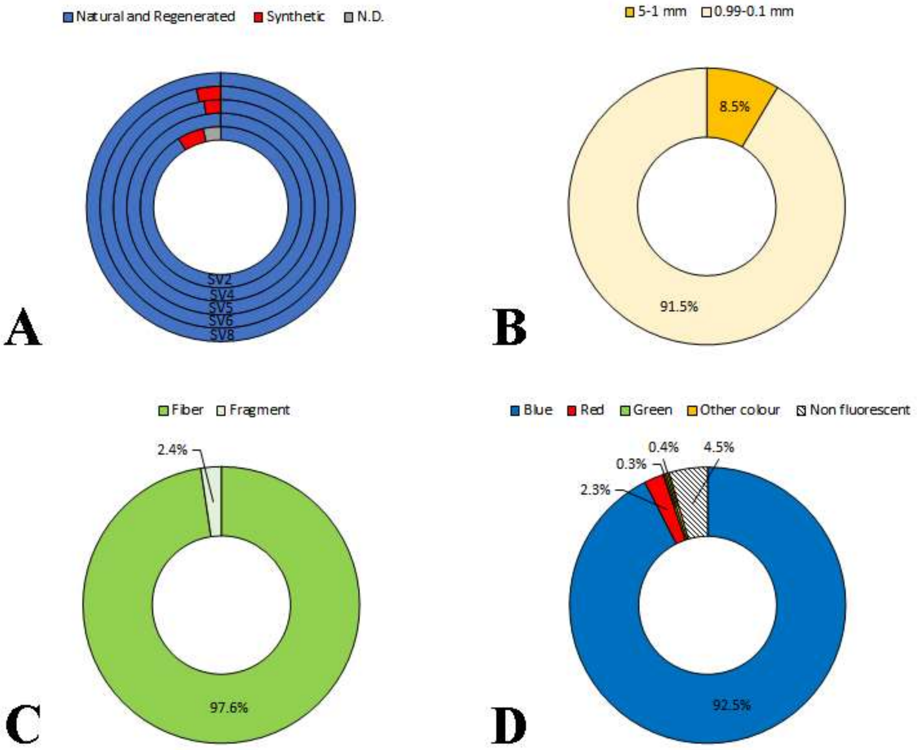


Fig. 68 – Anthropogenic microparticle characteristics found in sediment samples from unexplored caves (mean of the collected data). A: microparticle composition; B: microparticle size; C: microparticle shape; D: microparticle colour of fluorescence. Image from Balestra and Bellopede (2025).

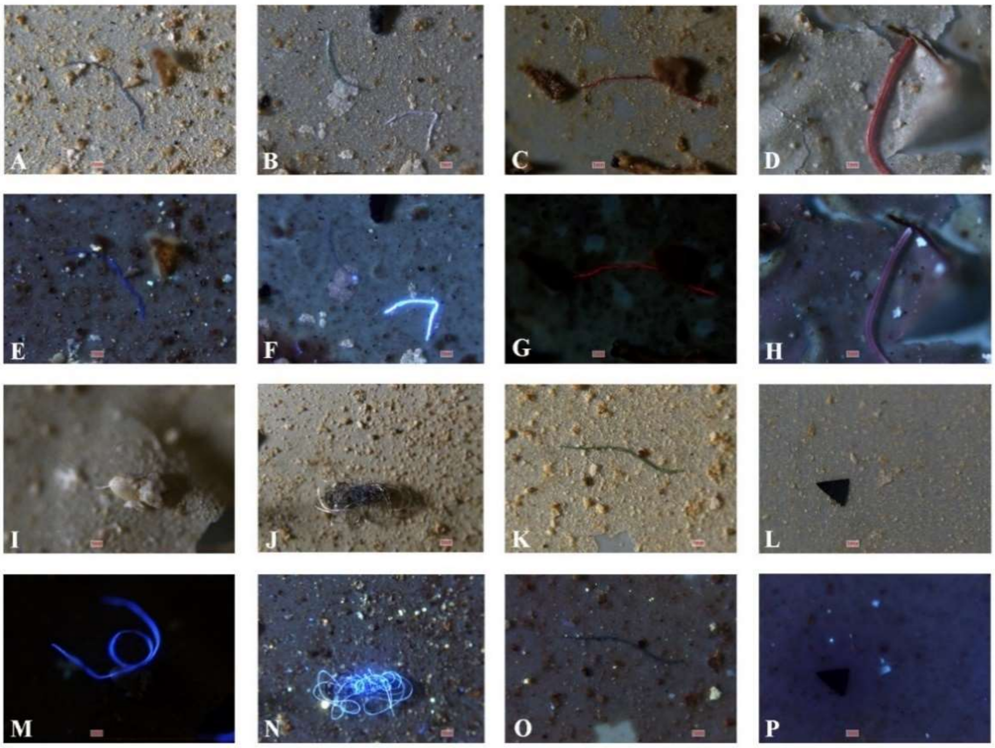


Fig. 69 – Anthropogenic microparticles under microscope, with and without UV light. A, B, C, D, I, J, K, L without UV light; E, F, G, H, M, N, O, P with UV light. A-E: cotton blue fibre with blue fluorescence under UV light; B-F: light green synthetic fibre non fluorescent under UV light, and transparent cellulosic fibre with blue fluorescence under UV light; C-G: red cotton fibre with red fluorescence under UV light; D-H: red synthetic fibre with no fluorescence under UV light. I-M: transparent synthetic fibre with blue fluorescence under Uv light; J-N: ball of fibres of different composition, colour, and fuorescence; K-O: green cotton fibre not fluorescent under UV light; black synthetic fragment not fluorescent under UV light. Image from Balestra and Bellopede (2025).

µFTIR-ATR analysis were useful to confirm microscopic analysis. Unfortunately, AMPs found in the sediments showed a high degradation degree and were often contaminated by different matter, therefore, spectroscopic analysis were not always possible, resulting in spectra with low matches. Analyzed AMPs (40%) with good spectra confirmed that the major part of the particles were cellulosic MFs, transparent, with size less than 1 mm. Of the synthetic AMPs, analyses confirmed the presence of polyethylene terephthalate (PET), polyethylene clorinated (CPE or PE-C) and silicon grease.

6.4.2 Size and shape

Size average percentages of analyzed microparticles were similar for all caves (Table 38). AMPs < 1 mm were the most abundant (91.5%); big AMPs (5-1 mm) accounted for only 8.5% (Fig. 68B). Three meso-MFs (5–25 mm) were also found: one in SV5 cave, sampling area 3, and two in SV8 cave, sampling areas 1 and 2.

Fibre-shape dominated all samples (97.6%), followed by fragments (2.4%) (Fig.s 68C, 69 and Table 38).

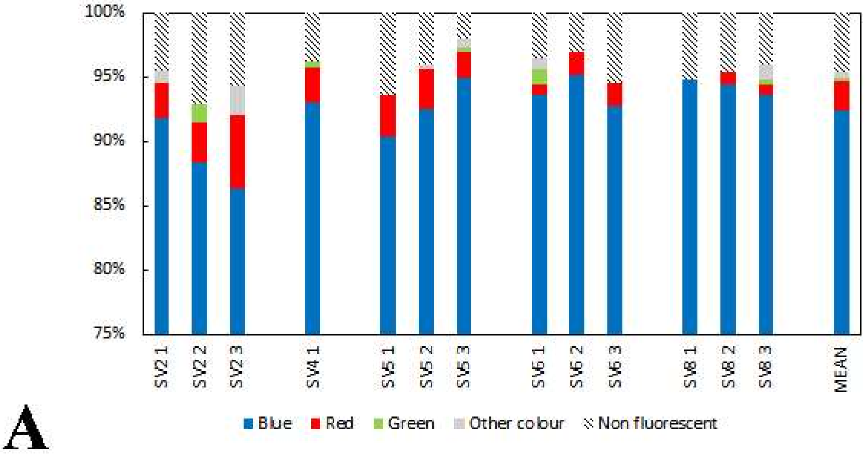
Table 38. AMP characteristics in sediment samples of unexplored caves.

	Size		Shape					Fluorescence		Colour of fluorescence			
Filter	5-1 mm	0.99-0.1 mm	Fibre	Fragment	Film	Bead/ Sphere	Foam	Fluorescent	Non fluorescent	Blue	Red	Green	Other colour
SV2	8.0	92.0	95.9	4.1	0.0	0.0	0.0	94.2	5.8	88.9	3.8	0.5	1.1
SV4	7.6	92.4	96.2	3.8	0.0	0.0	0.0	96.2	3.8	93.0	2.7	0.5	0.0
SV5	8.6	91.4	98.4	1.4	0.0	0.2	0.0	95.8	4.2	92.6	2.8	0.1	0.3
SV6	9.0	91.0	98.7	1.3	0.0	0.0	0.0	96.0	4.0	93.8	1.5	0.4	0.3
SV8	9.6	90.4	98.6	1.4	0.0	0.0	0.0	95.4	4.6	94.3	0.6	0.1	0.4
MEAN	8.5	91.5	97.6	2.4	0.0	0.0	0.0	95.5	4.5	92.5	2.3	0.3	0.4

6.4.3 Fluorescence and colour

The 95.4% of the analyzed AMPs were fluorescent under UV (Fig.s 68D, 69, and Table 35). Of the fluorescent particles, the major part had blue fluorescence (92.5%) (Fig.s 68D, 69, 70A and Table 35). The non-fluorescent AMP abundance percentages in sediments were quite similar for each sampling area (average of 4.5%), with the exception of SV2 cave, which has even higher values (5.8%) (Fig. 59A and Table 35).

Of the fluorescent AMPs found 91.8% were transparent, followed by red (2.4%), and blue (1.5%) ones; particles with other colours had percentages between 0.3 and 1.0% (Fig. 69, 70B). Non-fluorescent AMPs were mainly black (58.6%), transparent (9.0%), brown (8.4%), and grey (7.2%); particles with other colours had percentages between 1.3 and 6.2% (Fig. 69, 70C). However, the percentages vary greatly from cave to cave, and also between different sampling sites within the same cave.



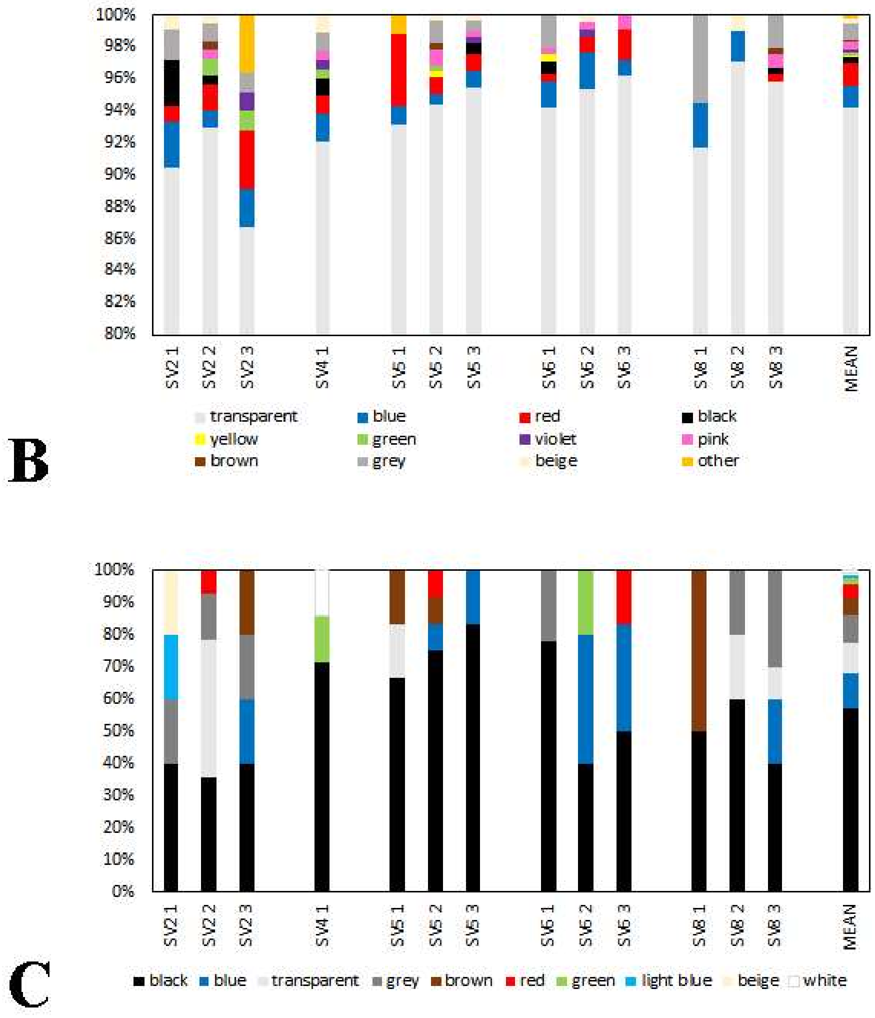


Fig. 70. Anthropogenic microparticle (AMP) fluorescence and colour for sampling site. A: Fluorescence; B: Colour of fluorescent AMPs; C: Colour of non-fluorescent AMPs. Image from Balestra and Bellopede (2025).

6.5 Discussion

6.5.1 The dark continent is polluted by anthropogenic microparticles

MPs were found only in the sediments of three sampling point (SV2-2, SV5-2 and SV6-1) with small amount: Cave SV4 and SV8 had no MPs (Table 32). However, taking into the account also natural and regenerated MFs of anthropogenic origin, all monitored caves resulted polluted, and with more contaminated sampling sites (Fig. 67 and Table 32). Considering that examined caves were unexplored, the quantities of AMPs found in the sampling points were comparable to values found in known caves (e.g. Balestra et al., 2024a), with an estimate ranging from 0 to 2311.1 items/kg (Fig. 67 and Table 34). The most polluted cave resulted SV4, the least one SV8 (Table 33). Cave SV4 had a lot of garbage inside, in the first room, therefore, the high AMPs amount could be directly linked to the litter inside the cave. Cave SV8 is located in a more wooded area respect to the other caves, and it is also the most distant from road and villages, suggesting that the proximity to human activities has a major influence on pollution in the caves.

Comparisons with other unexplored caves are not feasible at the moment, as this research is the first conducted in unexplored underground environments. Furthermore, making comparisons between studies of MPs and MFs is generally challenging, as differing methodologies and analytical approaches can influence the results. Variables such as the size of the microparticles considered, the sampling location, and environmental conditions may also lead to fluctuations in concentrations over time. Nevertheless, some assumptions can be made regarding the presence of MPs and MFs in karst subterranean environments (Table 39).

MPs found in the Abruzzo unexplored caves highlighted that MP abundance is lower than show caves sediments (Balestra and Bellopede, 2022b, 2023; Hasenmueller et al., 2023) and submerged sediments in the caves of the Italian Classical Karst area investigated so far (Balestra et al., 2024a; Balestra et al., 2024b) (Table 4). However, they resulted higher than those found in the examined Italian marine caves (Bergamin et al., 2024; Romano et al., 2023) and in the caves of the Slovenian Classical Karst (Valentić et al., 2022) (Table 4). Considering also MFs, the abundances of AMPs are higher, more similar to that found in the speleological area of Bossea cave, Italy (Balestra and Bellopede, 2022b), or in Cliff cave, a US show cave with limited visitor access (Baraza and Hasenmueller, 2023; Hasenmueller et al., 2023). It must be specifically takes account that the size range of detected microparticles in these works is different, as well as used methodologies, and examined caves have totally different environmental (submerged sediments in fresh and sea water, sediments after a flood, sediments away from water), climatological, and anthropogenic sources (tourism or not, presence of roads, railway and villages near the caves or wild areas) conditions. Moreover, someone include anthropogenic MFs in the abundances, others separate MPs from MFs.

Microscopic analysis showed that 97.1% of AMPs were natural and regenerated materials, 2.2% synthetics, and 0.7% not clearly distinguish. Of the natural and regenerated MFs, 34.1% resulted cotton, with averages for cave ranging from 21.5% in cave SV4 to 58.1% in cave SV2. All other materials were cellulosic, except a wool MFs found in cave SV5. μFTIR-ATR analysis generally confirmed the microscopic ones, highlighting a prevalence of transparent cellulosic fibres, but, unfortunately, spectra were not good, due to the AMPs degradation degree, and especially by different materials contamination on microparticles and filters. Analysis on synthetic AMPs showed the presence of PET, PE-C, and silicon grease. PET is commonly used in the textile industry, while PE-C is used in cable and rubber industries. Silicone grease is not strictly considered plastic, but rather a substance derived from silicone polymers, which are a type of synthetic material, therefore, someone include this particles in MPs research. MP typology comparisons with other karst areas are possible only with few works (An et al., 2022; Balestra and Bellopede, 2023; Balestra et al., 2024a; Balestra et al., 2024b; Balestra et al., 2023; Romano et al., 2023; Valentić et al., 2022), where polyesters, poliammide (PA), and copolymers have been found frequently; PE-C and silicon grease were less reported. Inside the Cliff cave, US, 31% of particles was cellulosic and 29% plastic (Hasenmueller et al., 2023), values quite different from our study.

Table 39. Microplastic and microfibre concentration found in cave sediments in previous works.

Cave	Sampling information	Considered size	MP/MF concentration	References
Bossea cave Piedmont, Italy Show cave area	Surface sediments (5 cm) along the show cave path	5-0.1 mm	From 2500 to 8700 items/kg	Balestra and Bellopede (2022b)
Bossea cave Piedmont, Italy	Surface sediments (5cm) in a speleological area of the cave	5-0.1 mm	1600 items/kg	Balestra and Bellopede (2022b)
Toirano caves Liguria, Italy	Surface sediments (5 cm) along the show cave path	5-0.1 mm	3823 items/kg (average)	Balestra and Bellopede (2023)
Toirano caves Liguria, Italy	Surface sediments (5cm) in a speleological area of the cave	5-0.1 mm	3186 items/kg	Balestra and Bellopede (2023)
Borgio Verezzi cave Liguria, Italy	Surface sediments (5 cm) along the show cave path	5-0.1 mm	4695 items/kg (average)	Balestra and Bellopede (2023)
Borgio Verezzi cave Liguria, Italy	Surface sediments (5cm) in a speleological area of the cave	5-0.1 mm	2933 items/kg	Balestra and Bellopede (2023)
214 Cave Friuli-Venezia-Giulia, Italy	Speleological caves, submerged sediments	5-0.1 mm	1267 MPs/kg	Balestra et al. (2024b)
214 Cave Friuli-Venezia-Giulia, Italy	Speleological caves, submerged sediments	5-0.1 mm	8982 MFs/kg	Balestra et al. (2024a)
Trebiciano cave Friuli-Venezia-Giulia, Italy	Speleological caves, submerged sediments	5-0.1 mm	2178 MPs/kg	Balestra et al. (2024b)
Trebiciano cave Friuli-Venezia-Giulia, Italy	Speleological caves, submerged sediments	5-0.1 mm	2649 MFs/kg	Balestra et al. (2024a)
Mariano Well Friuli-Venezia-Giulia, Italy	Speleological caves, submerged sediments	5-0.1 mm	911 MPs/kg	Balestra et al. (2024b)
Mariano Well Friuli-Venezia-Giulia, Italy	Speleological caves, submerged sediments	5-0.1 mm	2938 MFs/kg	Balestra et al. (2024a)
Bue Marino cave, Sardinia, Italy	Surface sediments (2 cm) in a speleological area of the marine part of the cave	25 mm-8 µm	From 10 to 27 items/kg	Romano et al. (2023)
Argentarola cave, Tuscany, Italy	Surface sediments (2 cm) of the marine caves	5-0.1 mm	From 5 to 12 MPs/kg	Bergamin et al. (2024)
Planina cave, Slovenia	Sediments	5-1 mm	0 MPs/kg	Valentić et al. (2022)
Postojna cave, Slovenia	Sediments	5-1 mm	0 MPs/kg	Valentić et al. (2022)
Škocjan caves system, Slovenia	Sediments	5-1 mm	60,000 MPs/m3 (circa 35 al kg)	Valentić et al. (2022)
Kačna cave, Slovenia	Sediments	5-1 mm	6667 MPs/m3 (circa 3.7 al kg)	Valentić et al. (2022)
Jama 1 v Kanjaducah, Slovenia	Sediments	5-1 mm	0 MPs/kg	Valentić et al. (2022)
Cliff cave, Missouri, US	Sediments during a flood	5 mm - ? µm	8423 ± 166 particle/kg	Hasenmueller et al. (2023)

However, our values are similar to that found in other environments, for example in the oceanic surface waters, in which the major part of the analyzed MFs were natural (91.8%), of which 79.5% cellulosic and 50% of all fibres cotton; only 8.2% fibres were synthetic, of which 6.2% polyester (Suaria et al., 2020a). In natural environments, different research reported that non-synthetic MFs were more abundant that synthetic ones, and especially cellulosic fibres, principally cotton (Athey and Erdle, 2022 and references therein). Given that approximately 67% of textile production worldwide in 2023 was synthetic (Textile Exchange, 2024) there is a divergence between the MF composition identified in natural environments and the global production of synthetic textiles, requiring more in-depth research. These data could confirm the presence and accumulation of anthropogenic cellulosic fibres in the environment over time. However, in a karst system, pollution may spread more slowly compared to other environments, particularly when transported by water, due to the specific type of karst present in the area. As a result, pollutants may accumulate in rock fractures within the system, reaching the cavity at a later time.

Another interesting discussion topic regards the position of the AMPs respect to the cave entrance and morphology. In caves SV2, SV5 and SV6, the major part of the AMPs was found in the middle of the cave. This could be linked to the morphology of these caves, which have a predominantly horizontal development, white large entrances or slightly sloping ones, favoring the displacement of materials. Moreover, cave SV6 has a pit in the middle of the cave in which materials can accumulate. In SV4, SV5 and SV8 caves the inner part resulted polluted, while in SV2 and SV6 caves, AMPs were not found. Cave SV4 has a semi-vertical development, and the first area was polluted by litter, therefore, AMPs could be transported into the inner part by gravity, water and air circulation. Cave SV8 is the only vertical cave, characterized by a series of wells, which favor the falling of matter and pollutants towards the inner areas. Moreover, the presence of mud at the end of this cave, highlight also the presence of water in some periods, which could transport and accumulate materials in the inner parts.

According to previous studies (Balestra and Bellopede, 2022b, 2023; Balestra et al., 2024a; Balestra et al., 2024b; Hasenmueller et al., 2023; Valentić et al., 2022), the major part of AMPs in the examined karst environments were fibres. Fragments found in our study had not a relevant percentage respect other previously examined caves (e.g. Balestra and Bellopede, 2023).

Being unexplored caves, pollution in the karst subterranean environments comes from the surface anthropogenic activities, atmospheric depositions, and infiltrations through fractures and soil. The high presence of fibres in the unexplored caves suggests that pollution in this area could be linked mainly to textiles deterioration. MFs could be transported in this area by atmospheric deposition, reaching the caves by gravity, water and/or air transport, or could be linked to geo-textiles deterioration from the roads built near some caves, such as SV5. Fragments may have originated from the breakdown of waste in the environment or from nearby human activities. Some of the caves monitored are located near the provincial road, so the presence of AMPs may be linked to traffic, road markings, road wear, tire degradation, as well as dust and litter (Andersson-Sköld et al., 2020; Burghardt and Pashkevich, 2023). Our values were similar to that found inside the Cliff cave, US, in which 91% were fibres (Hasenmueller et al., 2023).

As reported in recent research (Balestra and Bellopede, 2022b, 2023; Balestra et al., 2024b; Balestra et al., 2023), most of the AMPs found were fluorescent under UV light (95.5%). These percentage was higher than those found in the sediments of Bossea (88%), Toirano (60%), and Borgio Verezzi (75%) caves, Italy, but similar to that found in the Italian sector of the Classical Karst, considering also MF pollution (mean of 93%) (Balestra et al., 2024a). These data indicate that using UV light to identify AMPs is an effective method, though it is not enough for a thorough particle analysis. The fluorescence of AMPs could also provide valuable information about their consumption by organisms. For example, fluorescent AMPs were found in stygofauna sampled from three groundwater bodies in Italian karst regions (Sforzi et al., 2024). The health of ecosystems is significantly impacted by the accumulation of AMPs, as some organisms mistakenly ingest them, confusing them for food sources (Devereux et al., 2021; Gomiero et al., 2018; Pukos et al., 2023; Sforzi et al., 2024). AMPs can lead to a range of harmful effects on organisms, including pathological changes, disruptions in digestive processes, and reproductive deficits (Assas et al., 2020). Entering in the food chains, AMPs could have implications that extend to higher trophic levels, leading to cascading ecological effects (Zhang et al., 2023a). In subterranean karst environments, AMPs may also affect the boundaries of these ecosystems, including vulnerable ecotones (Balestra et al., 2024b). As shown in previous research on MPs in biota of different environments, the colour of AMPs can give useful information on consumption by organisms (Carpenter et al., 1972; Jahan et al., 2019; Lusher et al., 2013; Romeo et al., 2015; Ugwu et al., 2021). This makes this step of analysis important for ecological and environmental research. Regarding AMPs ingestion by fauna, in marine biota, most of MPs particles found were blue, white, black, and transparent (Ugwu et al., 2021). Some studies found that black plastics were the most commonly ingested by marine animals (Jahan et al., 2019; Lusher et al., 2013), other ones showed that transparent and white MPs are the most found in the stomach of some top sea predators (Romeo et al., 2015). Instead, mid-tone (green, red, blue, etc) AMPs were found in terrestrial birds (Zhao et al., 2016). In karst areas, blue AMPs were found in the digestive tract of specialized crustaceans of Italian caves and springs (Sforzi et al., 2024). In the same study, blue, transparent, and black particles were found with high amounts in all analyzed superficial and subterranean waters. In this study, transparent particles were found in the highest amounts in the sediments of all examined caves, as in Cliff cave, US, in the Italian show caves, or in the caves monitored in the Classical Karst, Italy (Balestra and Bellopede, 2022b, 2023; Balestra et al., 2024a; Balestra et al., 2024b; Hasenmueller et al., 2023). Different other colours were present in the examined unexplored caves, especially blue and red. Many studies are present on aquatic organisms, but they are less common in terrestrial ones, and studies on hypogeal animals are still very poor. Future research could further explore whether hypogeal organisms, particularly troglobionts, mistakenly consume AMPs as food sources. AMP fluorescence and colour can be significant for their ingestion by epigeal fauna or organisms less adapted to the subterranean environment. Scientific observations were made even regarding the relationship between MP color and the chemical pollutants that can bind to them, leading to contamination (e.g. Frias et al., 2010; Karapanagioti et al., 2011). For instance, high levels of pollutants were found on yellow and black particles in marine environments (Frias et al., 2010; Karapanagioti et al., 2011). In our caves, black particles were the most common between the non-fluorescent AMPs, and present in all sampling points, however, not-fluorescent AMPs were only 4.5% (Fig. 70B, C). Yellow particles were sporadic only in two sampling points (Fig. 70B).

Although the sampling in caves was carried out according to the chosen methodology, it should be considered that contamination, albeit limited, may be occurred during sampling, because of the material of the speleological clothes. However, speleologists wore speleological suits made of polyamide (PA) and cordura, a type of rayon, red and black. Helmets have outer shell in acrylonitrile butadiene styrene (ABS) or polycarbonate (PC) and inner foam in expanded polystyrene (EPS) or expanded polypropylene (EPP). The harnesses are composed of PA and polyvinyl chloride (PVC). The speleological bags are made of PVC or thermoplastic polyurethane (TPU), and gloves are generally made of PE, polypropylene (PP), PA with nitrile coatings, a synthetic rubber. The ropes are made of PA or polyester (PE). None of these materials were found in the analyzed samples.

Monitoring of AMP pollution in unexplored environments is a key element in understanding how and when human activity also indirectly affects uncontaminated environment, helping environmental studies to better understand sources and transport on pollutant, with the aims of promoting appropriate conservation measures for karst areas. Working in extreme and dark environments is not easy, and the difficulties of progression in unknown environments can be really hard. Large volumes of samples are not recommended in fragile ecosystems, and collecting samples and transporting them can be challenging. However, underground environments are rarely studied, and the dark continent remains the last frontier in terrestrial explorations, consequently, any new information is crucial for a better understanding of the ecosystems themselves, and possible threats. Greater effort in sampling underground environments is essential, and even during exploration.

6.6 Conclusion

These results contribute to a better understanding of MP and MF pollution in natural environments, highlighting the potential risks to subterranean ecosystems.

This study is the first that documents the presence of MPs and MFs in unexplored subterranean environments, demonstrating that human activities indirectly impact also the dark continent. This work wanted to be the first assessment of AMP pollution in unexplored environments, encouraging more researchers and speleologists to sampling also during explorations, despite all the difficulties of progression in unknown environments. Little is done in these environments, consequently, any new information is crucial to better understand these ecosystems and possible threats.

While the concentration of MPs found was really low or absent in the monitored caves, the presence of MFs in sediments was higher and more variable. Most of the particles were transparent and fluorescent under UV light, while non-fluorescent particles were predominantly black or dark. Fibre-shaped particles were the most common, suggesting that textiles are a primary source of pollution in this area, probably linked to nearby human activities and roads, or atmospheric deposition.

Surface and underground environments in karst areas are closely interconnected, so greater efforts should be made to implement more comprehensive protection measures. Expanding analyses to include a wider range of surface and subterranean habitats is essential. Future research is needed to better understand the sources and transport of MPs and MFs in karst areas, as well as their potential effects on ecosystems and organisms. Long-term monitoring will help clarify the impact of direct or indirect AMP pollution on karst habitats.

Chapter 7

Microplastic and microfibre pollution in deep aquifers

Case Study: Deep aquifers of Cuneo Province

7.1 Introduction

Groundwaters are vital resources for the environment and the society, especially as potable water and support in agricultural areas. Growing evidence suggests that MPs infiltrate and accumulate in groundwaters, ranging from 0.1 to 6,832 items/L (Hoang et al., 2025 and references therein; Re, 2019). However, groundwaters are poorly studied, especially deep and confined aquifers.

MPs may enter through recharge zones, preferential flow paths, or via leakage from overlying contaminated strata (Hoang et al., 2025 and references therein). Once introduced, MPs can be transported through interconnected pore spaces or fractures, depending on their size and characteristics (Hoang et al., 2025 and references therein). Into the aquifers, MPs may undergo colonization and aging, becoming carriers of pollutants such as heavy metals and organic microcontaminants (Ren et al., 2021), and degrading into smaller items or nanoplastics. Owing to their low flow velocity and the prolonged residence time of groundwater, MPs may persist within aquifers for extended periods. The hydro chemical properties of groundwater are key factors governing the distribution, abundance, and mobility of MPs (Hoang et al., 2025 and references therein).

Deep waters and confined aquifers are often used for human consumption, therefore, MP pollution can raise concerns for water quality, as conventional filtration systems may not remove them.

Confined aquifers are groundwater reservoirs bounded above and below by low-permeability layers, which isolate them from direct surface influence. Water within these systems is under hydrostatic pressure, often exceeding atmospheric levels. When a well penetrates such an aquifer, the internal pressure may cause water to rise naturally, and, in some cases, to flow freely at the surface, forming artesian wells. These artesian conditions result from recharge occurring at higher elevations, where the permeable layer is exposed. Even though artesian waters are often very old and protected, they are not immune to seepage, especially where geology is complex or human activity modified the natural stratigraphy. Confined and artesian aquifers are particularly vulnerable to persistent contaminants, as their limited exchange and slow flow rates can favor long-term accumulation. Therefore, monitoring became essential.

Checking the reviews, at the moment, less than 20 papers analyzed MPs in groundwaters (Hoang et al., 2025; Lee et al., 2024; Sumam et al., 2025; Viaroli et al., 2022). However, cave waters were not usually add in these works, even if they are groundwaters. Studies on groundwater remain poor, and research on karst systems are lacking.

Given the susceptibility of groundwater resources to contamination, anthropogenic stressors, and climate variability, alongside their essential ecological functions and significance as a primary source of potable water, it is imperative to implement strategies focused on subsurface environment protection and sustainable management of water resources (Balestra et al., 2023; Re, 2019). Consequently, systematic investigations are required to elucidate groundwater system dynamics, evaluate environmental health conditions, and identify potential pollutants and their sources.

The aims of this study are therefore i) to investigate, for the first time, MP and MF pollution in Italian deep aquifers, and in artesian aquifers, ii) if present, to discuss abundances and characteristic of MPs in these deep waters, iii) to verify possible correlations between AMP abundances and aquifers' depth, water chemical-phisical parameters, main iones and metals, iv) to analyze possible source of pollution. Moreover, at the conclusion of this thesis, to test the methodology used and the possibility of infiltrations in certain areas.

The investigated particles were defined as those ranging from 5 mm to 1 μm in size, and composed partially or entirely of synthetic, semi-synthetic and chemically modified natural polymers. In the MP definition were specifically considered only synthetic materials.

7.2 The study area

All samples were collected in the Cuneo province, Piedmont Region, NW Italy (Fig. 71). In this area, below the Quaternary alluvial cover and in the collinarian areas, various aquifer systems have been identified in the sedimentary successions of Plio-Pleistocene and Oligo-Miocene origin. Deep aquifers of these successions show a distinct geochemistry and, generally, lower permeability respect to surface quaternary aquifers. Permeability is variable and generally between 10^{-4} and 10^{-7}m/s, depending on the lithological composition and the cracking degree of the rocks.

These aquifers are mainly present in two macro-areas with different hydrogeological and structural characteristics: Langhe and Monregalese (Monregalese and Cebano hills included), and Roero, where aquifers are often intercepted in the plain via deep wells, for drinking water, irrigation and industrial uses (Civita et al., 2011).

Different geochemical facies and associated water quality were identified in this area (Valle, 2025). Identified facies and sub-facies related to our sampling points where reported in Table 40.

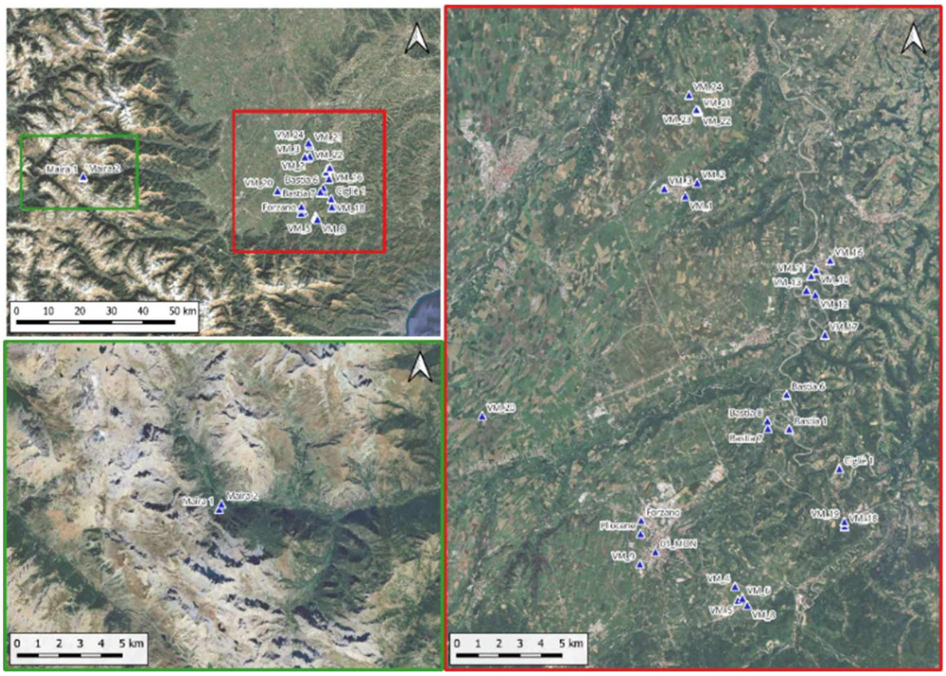


Fig. 71. Sampling points for water analyses in deep aquifers of the Cuneo Province, Piedmont, Italy.

7.3 Method

7.3.1 Field sampling

Twenty-eight water samples were collected in twenty-eight different deep aquifers and springs in the Cuneo province, Italy. The main information relating to the sampling and sampling points were shown in Table 40.

Water bulk samples (Hidalgo-Ruz et al., 2012) were collected with nitrile gloves and glass jars pre-cleaned with bi-filtered EMSURE ethanol absolute (Supelco, Sigma-Aldrich) and MilliQ water. Waters were sampled directly from the mouth of the water intakes, therefore, blank control during sampling was not carried out. Once sampled, glass jars were immediately closed with the cap. Samples were refrigerated at 6 ◦C until analysis in the laboratory.

Table 40. Sampling point information.

Sample ID	**Municipality**	**Date of sampling**	**Aquifer deep (m)**	**Year of construction of the water intake**	**Information**	**Altitude (m a.s.l.)**	**Main Facies**	**Sub-facies or Secondary Facies**
VM1	Benevagienna	13/08/2024	80-90	anni '90	Irrigated agricultural well. Water taken from a PVC tank. Some pipe sections are in PE.	351	BC	SM
VM2	Benevagienna	13/08/2024	200	-	Water taken from a tank. Stainless steel pipe and autoclave.	320	BA	

VM3	Benevagienna	13/08/2024	170	anni '70	Water taken from a tank. Some pipe sections are in PE.	352	SC	BAM
VM4	Vicoforte di Mondovì	27/08/2024	>100	-	Not artesian weel.	578	BAC	SM
VM5	Vicoforte di Mondovì	27/08/2024	198	-	70 m deep pump. Galvanized iron pipe. Some pipe sections are in PE.	542	BCM	
VM6	Vicoforte di Mondovì	27/08/2024	120	-	The well is salient and could even be artesian albeit with very modest flow rates. Some pipe sections are in PE.	524	BCM	
VM8	Vicoforte di Mondovì	27/08/2024	106	-	Artesian well, sample taken with pump on. Clean water.	523	BCM	
VM9	Mondovì	27/08/2024	180	-	Deep well used for garden irrigation. Very clean water. Some pipe sections are in PE.	440	BCM	
VM10	Farigliano	27/08/2024	200	Anni '50	Artesian well. Fountain in the garden of a house.	247	BA	
VM11	Farigliano	27/08/2024	200	anno '54	The well is no longer artesian but is still fed by a highly pressurized aquifer. Sample taken from the sink of a private individual's kitchen	264	BA	
VM12	Farigliano	27/08/2024	200	Anni '40-'50	The well is no longer artesian but is still fed by a highly pressurized aquifer. Sample taken from the sink of a private garden	267	BA	
VM13	Farigliano	27/08/2024	200	Anni '40-'50	Artesian well. Sample taken directly from the pipe.	258	BA	
VM16	Farigliano	19/09/2024	150	Anni '50	Aqueduct well	307	BA	
VM17	Clavesana	19/09/2024	120	-	Aqueduct well	387	BACM	
VM18	Niella Tanaro	19/09/2024	130	-	Aqueduct well	476	BAM	SC
VM19	Niella Tanaro	19/09/2024	130	-	Aqueduct well	453	BCM	

VM20	Rocca dei Baldi	19/09/2024	40	-	Aqueduct well	419	Non valutata	SI
VM21	Benevagienna	31/10/2024	150	-	Aqueduct well	338	BA	SCM
VM22	Benevagienna	31/10/2024	150	-	Aqueduct well	338	BA	SCM
VM23	Benevagienna	31/10/2024	150	-	Aqueduct well	337	BAC	SM
VM24	Narzole	31/10/2024	150	-	Aqueduct well	334	BA	SCM
Maira 1	Acceglio	18/07/2025	0	-	Maira River spring. Water from a deep water circulation. Sample taken from a concrete overflow.	1623	-	-
Maira 2	Acceglio	18/07/2025	0	-	Maira River spring. Water from a spring which flows from the ground, rich in travertine.	1600	-	-
Bastia 1	Bastia Mondovì	04/08/2025	180-200	After the Second World War	Artesian well. Sample taken from a fountain in a private yard in the center of the village. Metal pipe.	297	BA	
Cigliè 1	Cigliè	04/08/2025	250	After the Second World War	Artesian well. Metal pipe	313	BCA	
Bastia 6	Bastia Mondovì	04/08/2025	180-200	After the Second World War	Artesian well. Sample taken from a fountain in a private garden. Metal pipe.	283	BA	
Bastia 7	Bastia Mondovì	04/08/2025	180	After the Second World War	Artesian well. Sample taken from a fountain near a road. Metal pipe.	293	BCA	
Bastia 8	Bastia Mondovì	04/08/2025	214	After the Second World War	Artesian well. Sample taken from a fountain near a house. Metal pipe.	297	BCA	

BA: bicarbonate-alkaline
BAC: bicarbonate-alkaline-calcium
BACM: bicarbonate-alkaline-calcium-magnesian
BAM: bicarbonate-alkaline-magnesian
BC: bicarbonate-calcium
BCA: bicarbonate-chloride-alkaline
BCM: bicarbonate-calcium-magnesian
BMC: bicarbonate-magnesian-calcium
SC: sulphate-calcium
SCM: sulfate-calcium-magnesian
SM: sulphate-magnesian

7.3.2 Laboratory analysis

Deep water samples were analyzed following the procedures outlined by Balestra et al. (2024b), adapted. Samples were subjected to OMR using an amount of 30% hydrogen peroxide solution equal to half the sample. Samples were shaken (50 revs/min) for 2 hours at 45°C in a Shaker Water Bath (SBS30, Bibby Stuart Scientific). Once cooled, waters were then filtered through a 1.2 μm pore size silver filter (GVS Life Sciences, Membrane Disk 47 mm) to enhance IR spectroscopy analysis, using a glass vacuum pump. Filters were placed in glass petri dishes, covered with aluminum foil, and dried in an oven at 40°C until completely dry.

For more detailed information about method and contamination control, see Chapter 3.

7.3.3 Microparticle identification and characterization

A combination of microscopic and spectroscopic techniques was used in this work. Initially, microparticles on filters were observed under a Leitz ORTHOLUX II POL-MK microscope equipped with a DeltaPix Invenio 12EIII 12 Mpx Camera. A further detailed comparison was made for the MF analysis, observing details showed in longitudinal and x-sectional microscopic images of natural, regenerated and synthetic fibres (e.g. Houck, 2009; Khan et al., 2017). Microparticles ranging from 5 to 0.1 mm were analyzed. Microparticles that could not be definitively identified as AMPs were excluded from the analysis. Spectroscopic analyses were performed on the total of suspected MPs found on the filters, from each sampling area, using a μFTIR+ATR. Only spectra with a match degree ≥ 70% were accepted.

For more detailed information on microscopic and spectroscopic techniques used, see Chapter 3.

7.3.4 Statistical analysis

Spearman correlation was used to verify possible correlations between AMP abundances, aquifers' depth, water chemical-physical parameters and metals described in Valle (2025).

7.4 Results

7.4.1 Abundance and composition

AMPs were found in all sampling points, while MPs were found in 15 out of 28 aquifers (Fig. 72, Table 41), with totally different values. AMPs ranged from 0 to 231.5 AMPs/L (Fig. 72C, Table 41). Microscopic analysis showed that 93.2% of total AMPs were natural and regenerated materials, only 2.3% synthetics, and 4.5% not clearly distinguish (Fig. 72A, Table 41).

All possible MPs in each filter were analyzed by μFTIR with ATR. No MPs were found in the blank control. MPs ranged from 0 to 6.3 MPs/L (Fig. 72D). Vinyl compounds (28.6%) and copolymers (25.0%) were the main types of MPs found in the waters of deep aquifers and spring, followed by polyesters (14.3%), polyamides (10.7%), polyolefins (3.6%), and other synthetics (10.7%) (Fig. 72B, Tab. 42). The 7.1% was not clearly determinable. All kinds of MPs detected were shown in Table 42.

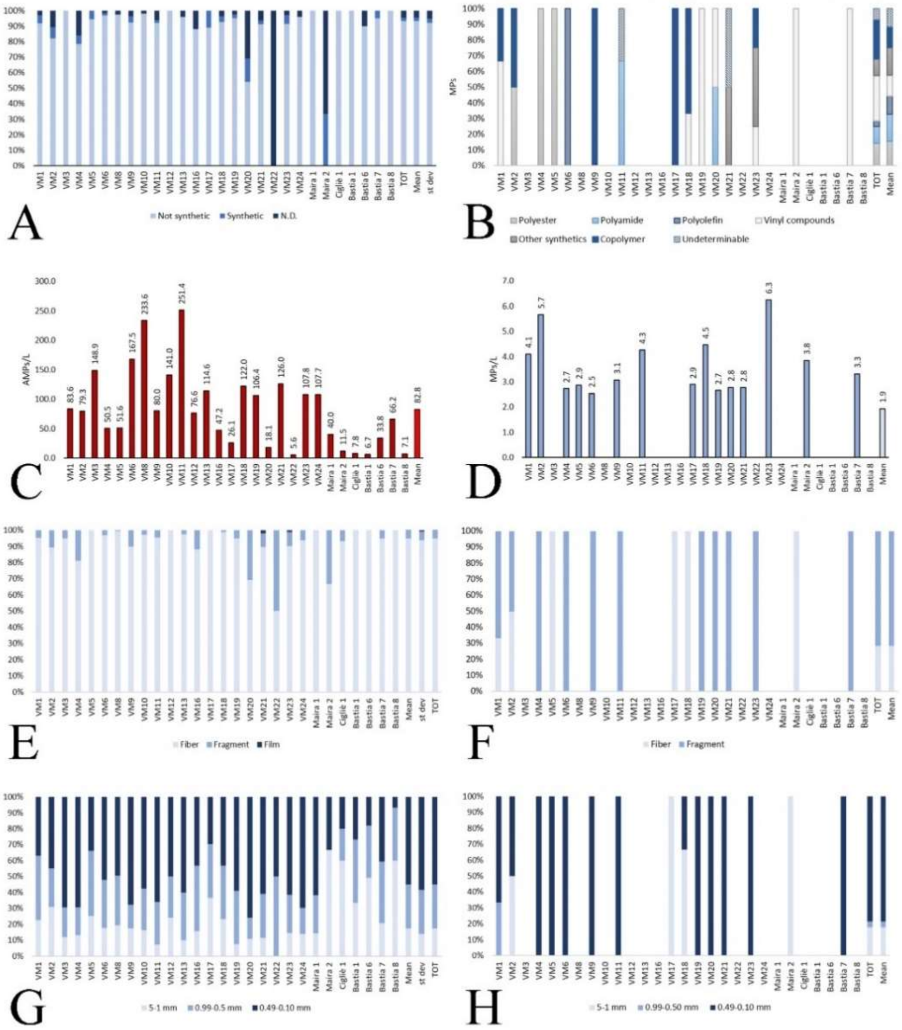


Fig. 72. Anthropogenic microparticle (AMP) abundances and characteristics in Italian deep aquifers. A: AMP composition; B: MP typologies; C: AMP abundance; D: MP abundance; E: AMP shape; F: MP shape; G: AMP size; H: MP size.

Table 41. Microparticle of anthropogenic origin found in Italian deep aquifers, with focus on microplastics (synthetic)

Filter Water	ml	Natural and regenerated	Synthetic	N.D.	TOT	Natural and Regenerated/L	Synthetic/L	N.D./L	TOT/L
VM1	730	56	3	2	61	76.7	4.1	2.7	83.6
VM2	706	23	2	3	28	65.2	5.7	8.5	79.3
VM3	618	46	0	0	46	148.9	0.0	0.0	148.9
VM4	732	29	2	6	37	39.6	2.7	8.2	50.5
VM5	698	17	1	0	18	48.7	2.9	0.0	51.6
VM6	395	64	1	1	66	162.4	2.5	2.5	167.5
VM8	672	153	0	4	157	227.7	0.0	6.0	233.6
VM9	325	24	1	1	26	73.8	3.1	3.1	80.0
VM10	391	54	0	1	55	138.5	0.0	2.6	141.0
VM11	704	163	3	11	177	231.5	4.3	15.6	251.4
VM12	692	53	0	0	53	76.6	0.0	0.0	76.6
VM13	672	74	0	3	77	110.1	0.0	4.5	114.6
VM16	720	15	0	2	17	41.7	0.0	5.6	47.2
VM17	690	8	1	0	9	23.2	2.9	0.0	26.1
VM18	672	76	3	3	82	113.1	4.5	4.5	122.0
VM19	752	38	1	1	40	101.1	2.7	2.7	106.4
VM20	718	7	2	4	13	9.7	2.8	5.6	18.1
VM21	722	83	2	6	91	115.0	2.8	8.3	126.0
VM22	710	0	0	2	2	0.0	0.0	5.6	5.6
VM23	641	63	4	2	69	98.4	6.3	3.1	107.8
VM24	678	70	0	3	73	103.2	0.0	4.4	107.7
Maira 1	200	8	0	0	8	40.0	0.0	0.0	40.0
Maira 2	260	0	1	2	3	0.0	3.8	7.7	11.5
Cigliè 1	256	2	0	0	2	7.8	0.0	0.0	7.8
Bastia 1	300	2	0	0	2	6.7	0.0	0.0	6.7
Bastia 6	296	9	0	1	10	30.4	0.0	3.4	33.8
Bastia 7	302	19	1	0	20	62.9	3.3	0.0	66.2
Bastia 8	282	2	0	0	2	7.1	0.0	0.0	7.1
TOT		1158	28	58	1244	2160.0	54.2	104.5	2318.7
Mean		41.4	1.0	2.1	44.4	77.1	1.9	3.7	82.8
st dev		41.7	1.2	2.4	43.8	62.6	2.0	3.6	64.3
%		93.1	2.3	4.7	100.0	93.2	2.3	4.5	100.0

Table 42. Microplastic characteristics and typology found in Italian deep aquifers.

Sample	Type	Size [mm]	Colour	Fluorescence	Typology
VM1	fragment	0.19	transparent	blue	acrylic
VM1	fragment	0.24	blue	blue	PVC
VM1	fibre	0.66	red	red	EVA
VM2	fragment	0.15	transparent	blue	PES
VM2	fibre	1.68	black	no	copolymer
VM4	fragment	0.11	transparent	blue	PET
VM4	fragment	0.16	blue	no	PET
VM5	fibre	0.32	blue	no	PET
VM6	fragment	0.27	yellow	no	polyethylene chlorosulfonated (CSM)
VM9	fragment	0.1	green	no	copolymer
VM11	fragment	0.19	transparent	green	PA
VM11	fragment	0.16	blue and red	no	n.d.
VM11	fragment	0.17	white and blue	no	PA
VM17	fibre	2.27	red	red	EVA
VM18	fibre	0.2	transparent	blue	ABS
VM18	fibre	1.08	orange	orange	styrene ethylene butylene (copolymer)
VM18	fibre	1.26	blue	no	PS
VM19	fragment	0.14	white	blue	PVC
VM20	fragment	0.1	green	green	PA
VM20	fragment	0.14	red	red	ABS
VM21	fragment	0.1	blue	blue	n.d.
VM21	fragment	0.16	black	no	PAC
VM23	fragment	0.1	blue	blue	polyvinylidene fluoride (PVDF)
VM23	fragment	0.13	green	blue	PAC
VM23	fragment	0.29	yellow	green	ABS
VM23	fragment	0.34	black	no	PU
Maira 2	fibre	1.12	red	red	acrylic
Bastia 7	fragment	0.1	red	red	PAM

7.4.2 Shape and size

Only fibres, fragments and films were found in the water samples. Fibres represented major part of the AMPs present in the analysed waters (94.9%) followed by fragments (4.9%), and films (0.2%) (Fig. 72E). No beads, spheres and foams were found.

Big AMPs (5-1 mm) accounted for only 17.3%, while 27.8% from 0.99 to 0.5 mm, and 54.9% from 0.49 to 0.1 mm, highlighting the increase of micropollutants with the decrease in the size considered (Fig. 72G).

Only MP fragments (71.4%) and fibres (28.6%) were found (Fig. 72F).

Big MPs (5-1 mm) accounted for 17.9%, while 3.6% from 0.99 to 0.5 mm, and 78.6% from 0.49 to 0.10 mm, highlighting again the increase of MPs with the decrease in the size considered (Fig. 72H).

Shape and size of some microparticles are shown in fig. 73.

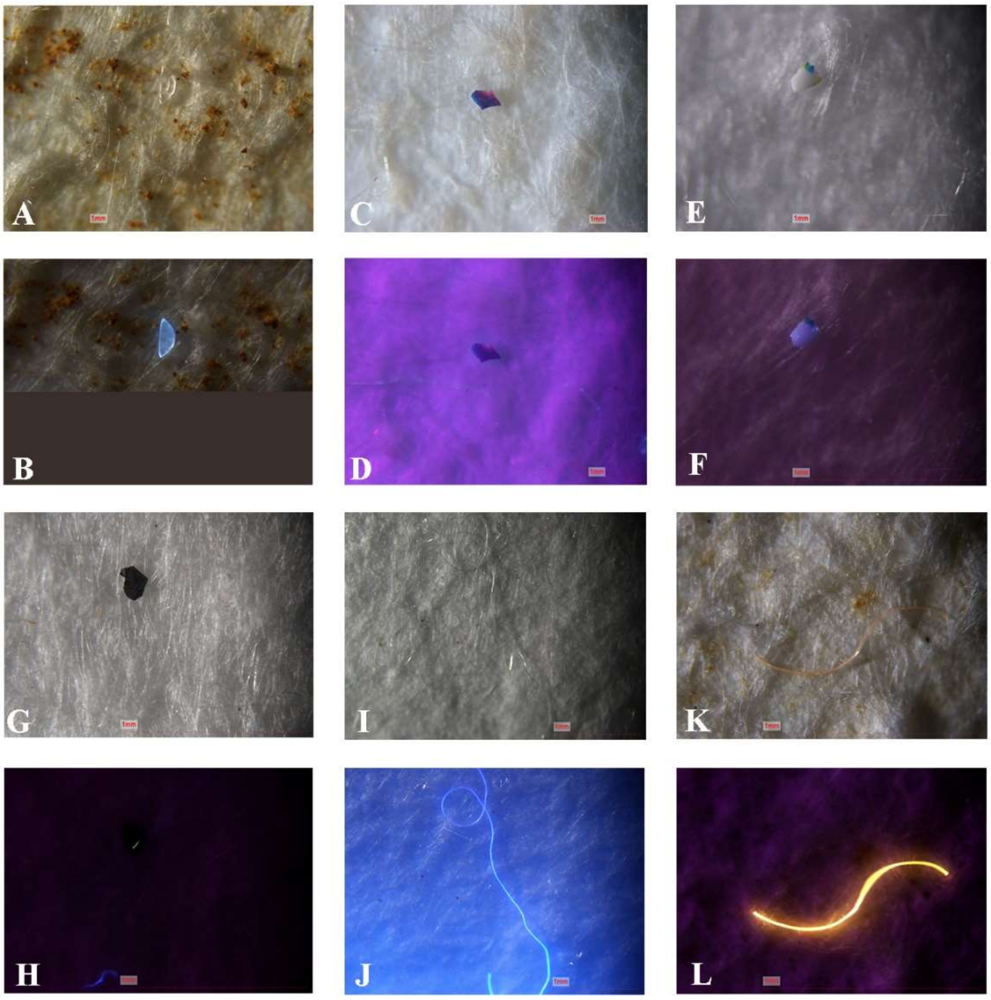


Fig. 73. Microplastics under microscope, with and without UV light. A,B: transparent fragment with blue fluorescence; C,D: blue fragment with no fluorescent and surface degradation; E,F: white fragment with blue fluorescence and surface biofouling; G,H: black fragment with no fluorescence; I,J: transparent fibre with blue fluorescence; orange fibre with red fluorescence.

7.4.3 Fluorescence and colour

The highest AMP abundance was fluorescent under UV light (75.8%) (Fig. 74A), showing especially a blue fluorescence (67.9%), followed by the red one (6.4%) (Fig. 74C). Of the fluorescent particles (Fig. 74E), 81.9% were transparent/clear, followed by blue (5.8%); other colours accounted for less than 3% of the total fluorescent ones. Non-fluorescent MPs (Fig. 74G) were mainly blue (46.8%), black (24.1%), and grey (19.5%); other colours accounted for less than 2.4% of the total non-fluorescent ones.

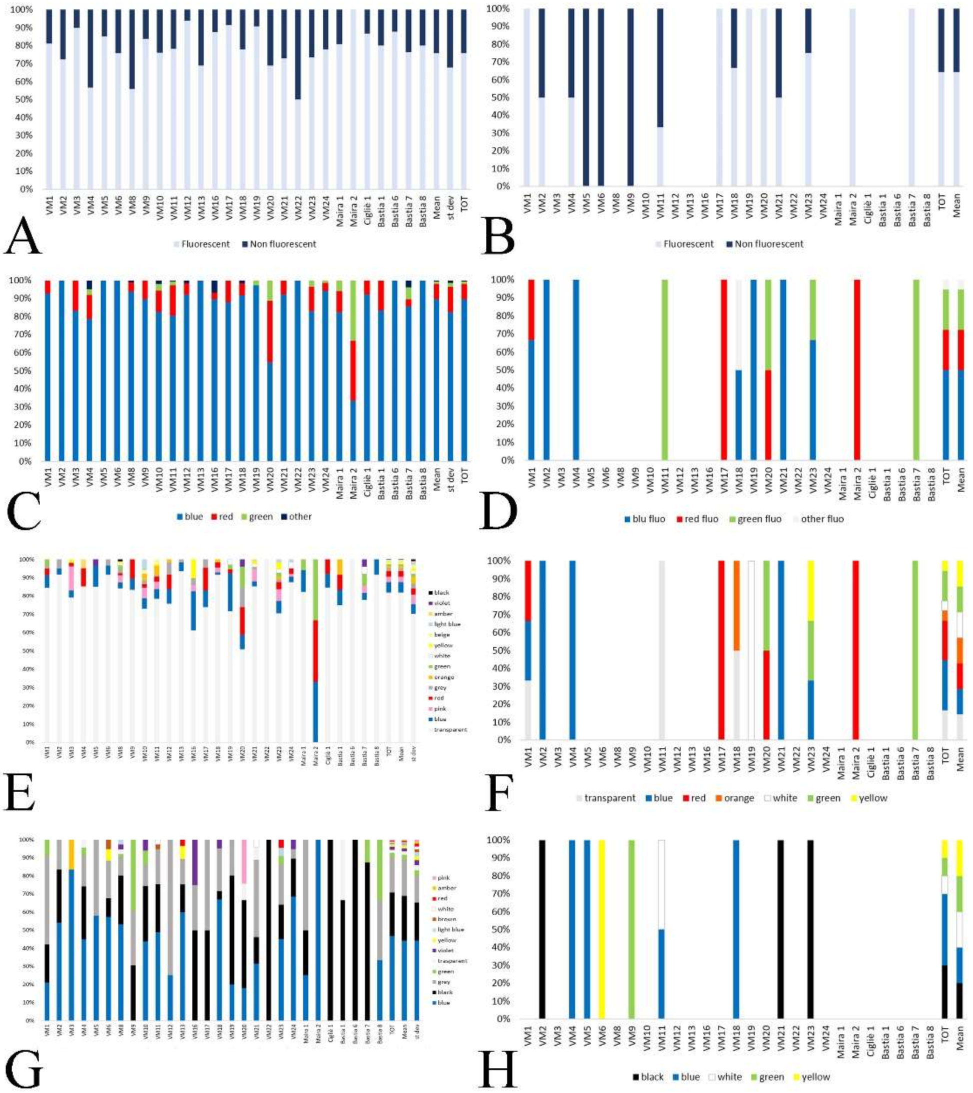


Fig. 74. Fluorescence and colour of anthropogenic microparticles (AMPs) and, in detail, microplastics (MPs) found in Italian deep aquifers. A: AMP fluorescence; B: MP fluorescence; C: AMP colour of fluorescence; D: MP colour of fluorescence; E: colours of fluorescent AMPs; F: colours of fluorescent MPs; G: colours of non-fluorescent AMPs; F: colours of non-fluorescent MPs.

The highest MP abundance was fluorescent under UV light (64.3%) (Fig. 74B), showing especially a blue fluorescence (32.1%) (Fig. 74D). Of the fluorescent MPs, 27.8 % were blue, 22.2% red, 16.7% transparent/clear, and 16.7% green; other colours (white, orange, and yellow) accounted for 5.6% each (Fig. 74F). Non-fluorescent MPs were mainly blue (40%) and black (30%); other colours (white, green, and yellow) accounted for 10% each (Fig. 74H).

Colours and fluorescence of some particles were shown in fig. 73.

7.4.4 Possible correlations

No statistically significant correlation was found between AMP abundances and water aquifer depth (Table 43).

Table 43. Spearman's correlation (rs/p) between AMP abundances and aquifer depth.

	Natural and Regenerated/L	Synthetic/L	TOT/L	Aquifer depth
Natural and Regenerated/L		0.52926	3.91E-24	0.99498
Synthetic/L	0.12409		0.29497	0.18845
TOT/L	0.99083	0.20516		0.80341
Aquifer depth	-0.0012456	-0.25605	-0.049263	

A moderate positive statistically significant correlation between AMPs, NR and MP abundances and quantities of Magnesium iones, and a moderate negative statistically significant correlation between AMPs and NR abundances and Nitrates iones are shown in Fig. 75.

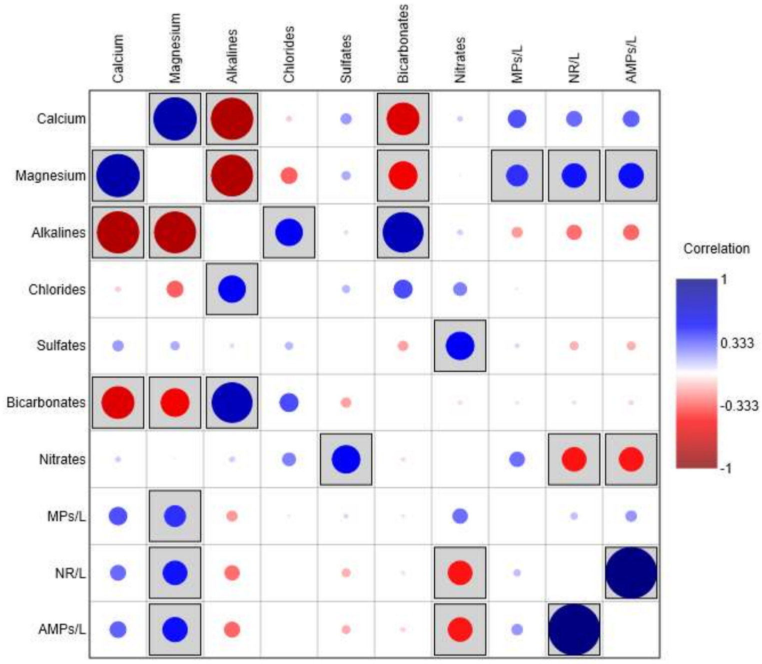


Fig. 75. Correlation between anthropogenic microparticle (AMPs) abundances, with focus on microplastic (MP) and natural and regenerated material (NR), and main iones.

A moderate negative statistically significant correlation between MP abundances and pH of analyzed waters was shown in Fig. 76.

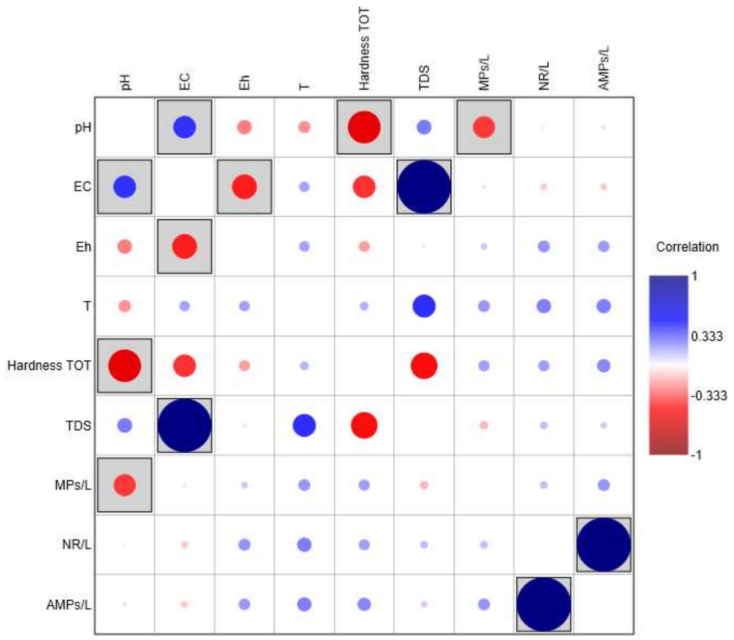


Fig. 76. Correlation between anthropogenic microparticle (AMPs) abundances, with focus on microplastic (MP) and natural and regenerated material (NR), and physical parameters.

A moderate negative statistically significant correlation between MP abundances and Cr of analyzed waters was shown in Fig. 77.

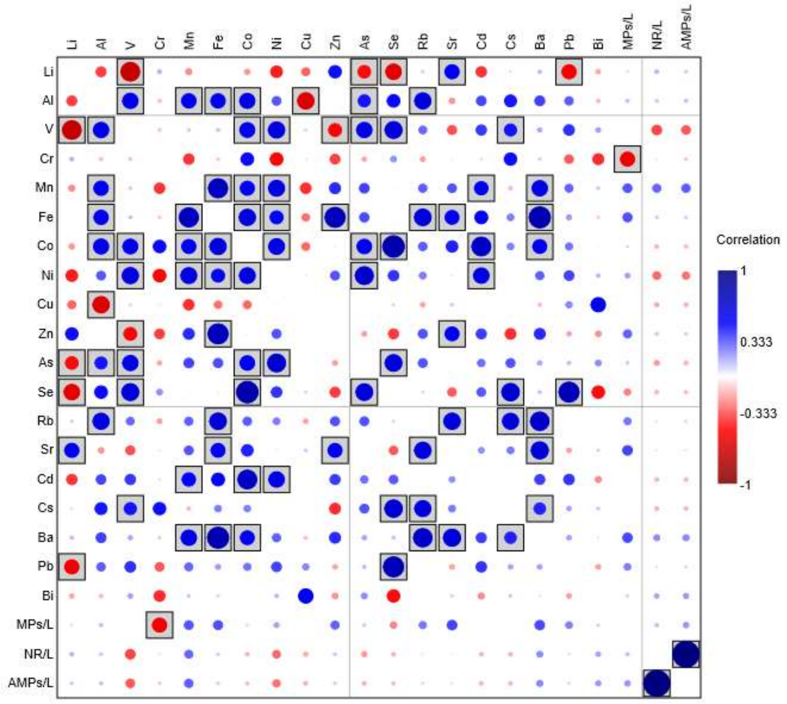


Fig. 77. Correlation between anthropogenic microparticle (AMPs) abundances, with focus on microplastic (MP) and natural and regenerated material (NR), and metals.

7.5 Discussion

MPs were found in 15 out of 28 aquifers, however, MPs were not found in the analyzed artesian aquifers, with the exception of Bastia 7, located near a provincial road. MPs ranged from 0 to 6.3 MPs/L. At the moment, a very limited number of studies have been carried out in groundwater and even fewer in confined and artesian aquifers. The main data obtained so far from groundwater studies were summarized in Table 41. Previous studies have found the most disparate values, from 0 to thousands items/L (table 41), due to different geographical areas, surface anthropic activities and used methodologies for MP analyses. Our values are similar to those found in karst aquifers of China (An et al., 2022; Shu et al., 2023), coastal aquifers of India (Selvam et al., 2021), groundwaters of Germany (Mintenig et al., 2019), Hungary (Sa'adu and Farsang, 2022), or Poland (Tarasewicz et al., 2025). However, the values found in these Italian deep aquifers were lower than those found in an alluvial unconfined aquifer in Australia (Samandra et al., 2022), in groundwater under a bedrock island in China (Gong et al., 2023), or in shallow and unconfined aquifer in India (Patterson et al., 2023). In previous work in cave groundwaters and alluvial aquifers in Italy, higher amounts of MPs were shown respect to those found in this study (Balestra et al., 2024b; Balestra et al., 2023; Sforzi et al., 2024). However, how is possible to see in Table 41, considered MP size vary from study to study, and also used methods, therefore, comparisons are really hard.

Vinyl compounds and copolymers were the main types of MPs found in this study, followed by polyesters, polyamides, polyolefins, and other synthetics. PET, PA, PVC and ABS where the most present plastics in the analyzed Italian deep aquifers, as reported in previous studies (Table 44).

Nothing is yet known about the transport of MFs in underground aquifers.

Abundances of MPs and MFs found in this study were correlated with aquifers' depth, water chemical-physical parameters, iones and metals. However, no important correlations were found with most of the data analyzed. Moderate correlations were found only between MP abundance, water pH, Cr, and Magnesium iones, and between AMP abundance and Magnesium and Nitrates iones. However, these data are preliminary, therefore, correlations have to be understood better. Correlations between the presence of MPs and heavy metals are known in literature, because MPs can adsorb some of them, thanks to their characteristics (Li et al., 2019; Selvam et al., 2021; Zhou et al., 2019). Instead, pH can influence both the solubility of certain metals and the surface charge of MPs. Moreover, relations between MP and MF pollution and certain iones in water are currently unknown.

From the chemical data, the waters of the Bastia Mondovì aquifer resulted old, probably with an age that can be estimated at a few tens of year. MPs were not found in these waters, with the exception of Bastia 7, therefore, as well as validating the methodology used, these data would also confirm the estimate, assuming at least about thirty-forty years. However, it is very difficult to understand the real age of these waters even using stable isotope tests, because they may have infiltrated when Tritium activity was higher than today, due to the effects of nuclear tests in the atmosphere. In this case, taking into account that the activity of the Tritium has returned to being very low, the risk of finding waters with Tritium higher than that of the actual rains is high, confirming that these waters are old, but not the precise age.

Table 44. MP pollution comparison of groundwaters from different areas of the world.

Country	**Abundance [items/L]**	**Considered size**	**Type**	**Sample type**	**Reference**
Australia	16-97 Mean: 38±8	18-491um	PP, PE, PVC, PS	Alluvial unconfined aquifer, capped groundwater monitoring bores (depth 2-29 m)	(Samandra et al., 2022)
China	0-4	1-5000 um	PS, PP, PET	Groundwater in Yulong River. Karst area	(Shu et al., 2023)
China	2.33-9.50 Mean: 4.5	1 um – 5 mm	PS, PE, PET, PA, PP, PVC, other	Karst cave groundwater in Guizhou Province	(An et al., 2022)
China	3-23.5 Mean: 10.38	50-5000 um	PE, PET, PVC, and PA	14 groundwater wells in Hainan, around municipal solid waste landfill	(Li et al., 2025)
China	34-64 Mean: 47.5	500-5000um	PES, PP, rayon, PE, olefin, acrylic acid	Groundwater under a bedrock island	(Gong et al., 2023)
China	4-7	-	PA	-	(Ganesan et al., 2019)
China	4-72 Mean: 29	0.1-2500 um	PA, PE, PP, PVC, PS	Groundwater, wells	(Shi et al., 2022)
Germany	0-7 Mean: 5.6	20 µm – 5 mm	PA, PE, PES, PVC, EP	-	(Mintenig et al., 2019)
Hungary	0-5 Mean: 2.3	<5 mm	-	Groundwater (1 m perched depth)	(Sa’adu and Farsang, 2022)
India	9-39 Mean: 29.73±3.27 Mean in bore wells: 32.9±4 Mean in open wells: 23.9±3.56	0.5-5 mm	PE, PP, PA, PS, PEU, PTFE, PES, CP	Shallow and unconfined aquifer of Tuticorin. 15 wells reaching the phreatic aquifer: 8 bore wells (3.7-6.1 m depth) and 7 open wells (4.3-7.3 m depth)	(Patterson et al., 2023)
India	2-80	10-500 um	PA, PVC, PE	Groundwater, 1 and 2 km distances from the municipal solid waste disposal sites. Corresponding borewell water depth ranges from 3 m to 30.48 m at the surrounding landfill sites	(Manikanda et al., 2021)
India	Max: 10.1 Mean: 4.2	0.1-5 mm	PA, PE, PES	Costal aquifer, wells and bore wells	(Selvam et al., 2021)
India	3.6-27.2 Mean: 12	<5 mm	-	Wells	(Priya et al., 2023)
Indonesia	0.25-0.95	0.069-4.459 mm	-	Dug wells	(Natsir et al., 2021)
Indonesia	1.8-2.3	-	-	Two sampling points in three caves. Underground river water in South Malang	(Suprayogi et al., 2024)
Iran	0-1.3 Mean: 0.48	<=500um	PE, PET, PS	10 well samples obtained from an alluvial aquifer in the Shiraz semi-arid region	(Esfandiari et al., 2022)
Italy	12-54 Mean: 28	0.1-5 mm	PE, PVA, PES, EVOH, PVC	Karst aquifer, Bossea cave, low-flow conditions	(Balestra et al., 2023)
Italy	47.2-96	0.1-5 mm	PET, PES, EVOH, PVAc, PTFE, EP, copolymers	Karst cave waters in the Classical Karst Regione. Trebiciano cave, 214 cave, Mariano well	(Balestra et al., 2024b)
Italy	18-911	5 um – 5 mm	EVA, PA, PAN, PE, PET, PP, copolymers, antropogenic cellulose, polisaccaride gums	Waters from two cave and two alluvial aquifers	(Sforzi et al., 2024)

Korea	Wet season: 0.014-0.554 Dry season: 0.042-1.026		PP, PET, PE	Shallow to deep aquifers (well depths: 3–120 m) and cave water of an agricultural region. Igneous fragmented rock, carbonate weathered rock, unconsolidated sediment, limestone cave	(Jeong et al., 2023)
Korea	0.006-0.192	20-100 um	PP, PE, PET, PVC, PS, PA, ABS, PU	Wells (13.7-143.2 m)	(Kim et al., 2023)
Mexico	10-34 Mean: 18.3	63-1002 um	HEC, iPP, PVC, LDPE	Highly permeable and affected by anthropogenic activities costal aquifer, capped boreholes	(Alvarado-Zambrano et al., 2023)
Netherland	Groundwater: 0.231-1.584 Groundwater after riverbank filtration: 0.519-2.223	20 um – 5 mm	PMMA, PP, PS, PTFE, polysulfone, silicone, rubbers, PVC, PET, PE, PE Chlorinated, PA, Polyacetal	Groundwater from one well, combined water from several wells	(Bäuerlein et al., 2022)
Poland	Max: 14.1 Mean: 1.3±2.1	20-5000 um	PU, PA, PE, PES	Groundwater in Biebrza Valley, one of Europe's largest and most ecologically significant wetland areas. The primary groundwater is related mainly to Quaternary deposits, consisting of alternating permeable sands and gravels, glacial tills, silts, and clays. 102 wells (1-11 m depth)	(Tarasewicz et al., 2025)
Russia	2	30-570 um	PE, PU, PC, PI, copolymers, phenol-formaldehyd (PF) resins, Polyterpene (TR) resins	Podolsk-Myachkovsky aquifer near the Zvenigorod Biological Station. Well 56 m deep, one sample	(Filimonova et al., 2025)
Slovenia	<0.02	1-5 mm	PP, PET, PU, PAM, PE, ethylene propylene diene rubber (EPDM), polymethyl methacrylate. (PMMA), PA, EVA, other	Cave waters, Karst aquifers. Škocjan cave system, Postojna cave, Kačna cave	(Valentić et al., 2022)
UK	0-113 Mean: 4.9	>25 um	PE, PET, PP	Chalk and sandstone aquifer. Raw water	(Johnson et al., 2020)
USA	9.2-81.3	4- >5000 um	Acrylonitrile, PE, PS, PA, PP, anthropogenic cellulose	Waters from the spring issuing from the main entrance of Cliff Cave, Missouri	(Baraza and Hasenmueller, 2023)
USA	Max: 4.4 Mean: 2.8	0.5-5 mm	PE	Karst aquifer, wells, low-flow conditions	(Panno et al., 2019)

Most of the analyzed waters are in agricultural or urban contexts, so there may be polluted by agricultural activities and products, the presence of roads and urban human activities. Human-derived activities, atmospheric fallout, and the percolation of particles through soils and fractured rocks can introduce MP pollutants into deep aquifers too, threatening both water quality and the ecological integrity of these distinctive environments. High levels of MPs in agricultural soils were shown in previous studies, due to the extensive use of agricultural plastics (Corradini et al., 2019; Kim et al., 2021b). However, MP groundwater contamination in agricultural areas is still limited (Jeong et al., 2023; Samandra et al., 2022; Severini et al., 2022), highlighting the need of increase monitoring.

In addition, in many of the analyzed water intakes there is the presence of PE pipes, such as VM1, VM5 or VM6, or sheaths made of synthetic materials, such as in the aqueduct water intakes. Therefore, possible sources of pollution could be linked to the water intake itself.

Once in groundwaters, MPs can be directly or indirectly ingested by organisms too. Research spanning a wide range of taxa has documented direct and indirect ingestion of MPs within aquatic environments (Devereux et al., 2021; Jemec et al., 2016; Pennati et al., 2022), including subterranean species (Kokalj et al., 2025; Sforzi et al., 2024). In addition, MPs present in groundwater may alter boundary zones between subterranean and surface environments, particularly sensitive transition areas like springs.

Comprehensive studies are required to identify and characterize MP sources, dispersal routes, and accumulation processes within karst terrains, as well as to evaluate their ecological implications for aquatic biota. Long-term observation programs that include a broader range of both groundwater and surface-water sites will be vital for assessing the extent and persistence of MP contamination. Finally, the potential repercussions of MP pollution for groundwater safety - encompassing ecological functioning, biodiversity maintenance, ecosystem service provision, and human health - should be systematically explored.

7.6 Conclusions

These results improve knowledge on MP and MF pollution in groundwaters, drawing attention on water quality, and the potential risks that could affect to subterranean species and human health. This first investigation in the Italian deep waters documents the presence of MPs and MFs even in aquifers with high depth, drawing attention on the potential exposition of species and humans. Possible sources of pollution could be linked to nearby human activities, especially agricultural ones, and water intakes, providing useful references for further research.

Surface and underground environments are closely connected, therefore, greater efforts should be made to establish more comprehensive measure of protection, especially for groundwater resources. MP pollution monitoring, but also MF, in groundwaters must become a priority for the water resources management and ecosystems protection, implementing analyses on a larger number of aquifers, but also surface and subterranean habitats strictly linked to them.

Chapter 8

General considerations and conclusion

Origin of the Chapter

Part of this chapter is adapted from the following peer-reviewed articles, result of this thesis:

- "Microplastic pollution in show cave sediments: first evidence and detection technique" authored by Balestra and Bellopede (2022b), published in Environmental Pollution, Volume 292, Part A, 1 January 2022, 118261, DOI: **https://doi.org/10.1016/j.envpol.2021.118261.**

  The author of this dissertation was responsible for conceptualisation, methodology, validation, formal analysis, investigation, resources, data curation, writing – original draft, writing – review and editing, visualisation.

- "Automated method for routine microplastic detection and quantification" authored by Giardino et al. (2023), published in Science of the Total Environment, Volume 859, 2023, 160036, DOI: **http://dx.doi.org/10.1016/j.scitotenv.2022.160036.**

  The author of this dissertation was responsible for conceptualization, methodology, validation, formal analysis, investigation, resources, data curation, writing – original draft, writing – review and editing, visualization.

- "Preliminary investigations of microplastic pollution in karst systems, from surface watercourses to cave waters" authored by Balestra et al. (2023), published in Journal of Contaminant Hydrology, Volume 252, January 2023, 104117, DOI: **https://doi.org/10.1016/j.jconhyd.2022.104117.**

  The author of this dissertation was responsible for conceptualisation, methodology, validation, formal analysis, investigation, resources, data curation, writing – original draft, writing – review and editing, visualisation.

- "Microplastics in caves: a new threat in the most famous geo-heritage in the world. Analysis and comparison of Italian show caves deposits" authored by Balestra and Bellopede (2023), published in Journal of Environmental Management, Volume 342, 15 September 2023, 118189, DOI: **https://doi.org/10.1016/j.jenvman.2023.118189.**

  The author of this dissertation was responsible for term, conceptualisation, methodology, software, validation, formal analysis, investigation, resources, data curation, writing – original draft, writing – review and editing, visualisation.

- "Microplastic pollution calls for urgent investigations in stygobiont habitats: a case study from Classical Karst" authored by Balestra et al. (2024b), published in Journal of Environmental Management, Volume 356, April 2024, 120672, DOI: **https://doi.org/10.1016/j.jenvman.2024.120672**.

  The author of this dissertation was responsible for conceptualisation, methodology, validation, formal analysis, investigation, resources, data curation, writing – original draft, writing – review and editing, visualisation, project administration.

- "The problem of anthropogenic microfibres in karst systems: Assessment of water and submerged sediments" authored by Balestra et al. (2024a), published in Chemosphere, Volume 363, September 2024, 142811, DOI: **https://doi.org/10.1016/j.chemosphere.2024.142811.**

  The author of this dissertation was responsible for term, conceptualisation, methodology, validation, formal analysis, investigation, resources, data curation, writing – original draft, writing – review and editing, visualisation, supervision, project administration.

- "(Micro-)plastics in saturated and unsaturated groundwater bodies: first evidence of presence in groundwater fauna and habitats" authored by Sforzi et al. (2024), published in Sustainability, Volume 16(6), 2024, 2532, DOI: **https://doi.org/10.3390/su16062532.**

  The author of this dissertation was responsible for validation, sample collection, data curation, writing - original draft preparation, writing - review and editing.

- "Explorations in the dark continent: did microplastics and microfibres get here before us?" authored by Balestra and Bellopede (2025), published in Science of The Total Environment, Volume 977, May 2025, 179328, DOI: **https://doi.org/10.1016/j.scitotenv.2025.179328**.

  The author of this dissertation was responsible for conceptualisation, methodology, validation, formal analysis, investigation, resources, data curation, writing – original draft, writing – review and editing, visualisation, project administration.

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8.1 Methodological considerations

Research on MPs is challenging for several reasons, including external contamination, the selection of appropriate methodologies, and issues related to sampling and analysis (Boyle and Örmeci, 2020; Eerkes-Medrano et al., 2015; Henry and Klepp, 2018; Hidalgo-Ruz et al., 2012; Munno et al., 2023; Song et al., 2015; Xu et al., 2019). MF research is in its early stage, making it even more complex than MP one. Moreover, the methodologies developed for detecting and characterizing MFs were initially designed for MPs, therefore, analysis on natural and regenerated materials could be challenging (Athey and Erdle, 2022), from OMR to microscopic and spectroscopic analysis. The main critical issues found in this work are reported below.

8.1.1 Speleology

Conducting research in underground and dark environments presents significant logistical and technical challenges. Progression through unexplored subsurface systems is often difficult, and extensive sampling is generally discouraged in such fragile ecosystems. Material collection and transport are further limited by the presence of narrow passages, vertical shafts and unstable substrates. Despite these obstacles, underground habitats remain among the least studied components of the Earth's biosphere - the so-called “dark continent” -, and each new dataset contributes significantly to understanding their ecological dynamics and potential vulnerabilities. Expanding sampling efforts within subsurface environments, even during exploration activities, is essential to advance knowledge of these critical systems.

Although the sampling in subterranean environments was carried out according to the chosen methodology, it should be considered that contamination, albeit limited, may be occurred during sampling, because of the material of the speleological clothes. However, speleologists wore speleological suits made of polyamide (PA) and cordura, a type of rayon, of really limited colours and of a particular manufacturing, easily identifiable under microscopy and spectroscopy. Helmets have outer shell in acrylonitrile butadiene styrene (ABS) or polycarbonate (PC) and inner foam in expanded polystyrene (EPS) or expanded polypropylene (EPP). The harnesses are composed of PA and polyvinyl chloride (PVC). The speleological bags are made of PVC or thermoplastic polyurethane (TPU), and gloves are generally made of polyethylene (PE), polypropylene (PP), PA with nitrile coatings, a synthetic rubber. The ropes are made of PA or polyester. The boots are commonly boots for trekking, made of various synthetic materials. However, for sampling, nitrile gloves have always been used, the sample collection took place with glass containers directly or with metal spoons, and the operator always positioned himself next to the area to be analyzed, not above.

8.1.2 Contamination control

High levels of AMPs were detected in the chemicals and solutions tested in these research, especially natural and regenerated MFs, highlighting the importance of pre-cleaning and filtering all products before laboratory analysis. Increased awareness is needed regarding the extent to which these materials can contaminate samples, both for scientists and producers. Immediate action is necessary to improve the quality of their products and reduce potential pollution.

Contamination of not synthetic AMPs was found in all the blanks, highlighting the importance of control airborne and operator-related contamination during sampling and analysis (Scopetani et al., 2020; Shruti and Kutralam-Muniasamy, 2023).

8.1.3 Organic matter removal

OMR is an important step for different matrices, especially before density separation. However, certain chemicals used in the process may lead to the partial or complete degradation of non-synthetic AMPs (Athey and Erdle, 2022; Treilles et al., 2020). H_2O_2 digestion is the most commonly used method for MP and MF analysis (Athey and Erdle, 2022), but it can alter the mechanical properties and IR spectra of the samples, increasing the fragility of certain types of MFs, especially cellulosic. The brittleness could potentially lead to MF fragmentation, causing counting errors and potential overestimation (Treilles et al., 2020).

Groundwater and cave waters generally have a little amount of organic material, however, having no previous feedback or few information, it is advisable to do it anyway, perhaps with small amounts of H_2O_2.

Sediments and spring waters, especially in caves or areas subjected to the passage of water and, therefore, to a possible accumulation of materials over time, are often rich in organic matter (and pollutants), therefore, OMR is a necessary step.

8.1.4 Density separation

The density separation method used for sediment samples in this research may limit our ability to capture anthropogenic materials with densities greater than 1.2 kg/L. The specific density of some plastics and different cellulosic materials is higher than that of several plastics, such as polyester, polypropylene, and nylon/acrylics. However, the densities of materials in natural environments may differ from those of newly produced materials. Due to factors like increased porosity, degradation, biofouling, or organic matter adsorption, the density of these materials can either increase or decrease over time in natural environments (Kaiser et al., 2017). Indeed, we observed materials with reported densities greater than 1.2 kg/L, consistent with findings from surface water systems (Horton et al., 2017). However, it is important to consider that some materials with higher densities may remain in the sediments due to their inability to be separated by our method, underestimating pollution.

8.1.5 Microscopy and spectroscopy

Microscopy is one of the most commonly used methods for MP and MF characterization, especially due to its cost-effectiveness. However, visual analysis cannot provide information on the chemical composition of the particles, is a hardworking process, and is not recommended alone for small particles (European Commission, 2013; Hidalgo-Ruz et al., 2012; Huang et al., 2023; Song et al., 2015).

Upside, this method allows for a clearer observation of the surface morphology of AMPs, as well as important characteristics such as color. Preliminary microscopic screening can be helpful in distinguishing between synthetic materials and natural or regenerated ones.

Spectroscopic analyses are essential for determining the chemical composition of microparticles, helping to confirm or refute the nature of particles identified through microscopy. However, these methods are time-consuming too, require expensive equipment and specialized personnel. The surfaces of particles collected from natural environments are often coated with other materials, impurities, and/or contaminated with additional pollutants, making it challenging to match the obtained spectra with high confidence to spectra libraries (Song et al., 2015; Xu et al., 2019). Natural polymers exhibit lower signal intensities compared to synthetic ones and are more susceptible to interference from dyes. Additionally, the FTIR spectra of natural and regenerated polymers are nearly identical (Comnea-Stancu et al., 2017), and the presence of dyes, oxidation, and microbial degradation can also alter the absorption bands of cellulose in the FTIR spectra (Li et al., 2010; Remy et al., 2015; Zambrano et al., 2019). Therefore, distinguishing between them is extremely challenging. Misestimations in library matches could pose significant issues, which is why only high-quality matches (over 70%) of spectra were used in this research.

A combination of multiple methods is the most effective approach for identifying AMPs in natural environments (Song et al., 2015), as detected in this study.

8.2 Microplastics and microfibres in karst systems

In this research, unfortunately, MPs and other anthropogenic microparticles, especially MFs, were found in all the analyzed matrices, from water to sediments, and fauna. This data, although preliminary, is alarming, as these environments, being extreme, are currently less influenced by anthropic activities than superficial ones. However, MPs were not found in some unexplored caves and in different deep aquifers.

Although the data obtained so far in underground environments and karst systems are still few, these studies highlight some important information, and different observations can be made regarding the possible sources of pollution and transport of these materials (Table 45).

Considering MP pollution, the main results obtained were shown in Table 46. Given the large number of case studies and the inherent heterogeneity of the investigated environments, it is not simple to harmonize the findings. Nevertheless, several general considerations can be drawn.

Firstly, it possible to observe that micropollutant amounts in sediments, submerged or not, are really high compared to waters, suggesting an accumulation in sediments over time, aligning with other data in the literature (e.g. Hasenmueller et al., 2023). This pattern is observed in both urbanized and pristine settings, indicating an accumulation process that is largely independent by direct or indirect local anthropogenic activity and instead reflects the intrinsic properties of karst systems, which are known to act as highly conservative environments.

Table 45. Summary of the main specificity of the examined karst systems and main possible sources of pollution.

Site	Environment	Region	Specificity	Possibile main sources of pollution
Bossea cave	show cave	Piedmont	Rural area, pastures and wooded areas	Tourists, Ski slopes, farming, garbage roads, air circulation, cavers, soil-water-atmosphere contamination
Rio Roccia Bianca	surface water	Piedmont	Pastures and wooded areas	Ski-slopes, farming, garbage, roads, soil-water-atmosphere contamination
Corsaglia river	spring	Piedmont	Rural area	Roads, urban activities, farming, agriculture, garbage, soil-water-atmosphere contamination
Borgio Verezzi cave	show cave	Liguria	Urban area	Tourists, urban activities, roads, cavers, garbage, air circulation, soil-water-atmosphere contamination
Toirano caves	show cave	Liguria	Rural area, wooden areas	Tourists, roads, garbage, cavers, air circulation, soil-water-atmosphere contamination
Torri di Slivia cave	show cave	Friuli-Venezia-Giulia	Rural area, wooden areas	Roads, railway, urban activities, agriculture, garbage, soil-water-atmosphere contamination
Spring 8 and 16	spring	Friuli-Venezia-Giulia	Rural area	Roads, garbage, soil-water-atmosphere contamination
214 Cave	cave	Friuli-Venezia-Giulia	Rural area	Roads, railway, garbage, soil-water-atmosphere contamination
Trebiciano Cave	cave	Friuli-Venezia-Giulia	Rural area	Cavers, garbage, roads, soil-water-atmosphere contamination
Mariano Well	cave	Friuli-Venezia-Giulia	Urban area	Roads, urban activities, garbage, soil-water-atmosphere contamination
SV2, SV4, SV5, SV6, SV8	unexplored cave	Abruzzo	Rural area, wooden areas	Quarry, garbage, soil-water-atmosphere contamination
VM1	deep aquifer	Piedmont	Rural area	Agricolture, roads, garbage, soil-water-atmosphere contamination
VM2	deep aquifer	Piedmont	Rural area	Agricolture, roads, garbage, soil-water-atmosphere contamination
VM3	deep aquifer	Piedmont	Rural area	Agricolture, roads, garbage, soil-water-atmosphere contamination
VM4	deep aquifer	Piedmont	Rural area	Agricolture, roads, garbage, soil-water-atmosphere contamination
VM5	deep aquifer	Piedmont	Rural area	Agricolture, roads, garbage, soil-water-atmosphere contamination
VM6	deep aquifer	Piedmont	Rural area	Agricolture, roads, garbage, soil-water-atmosphere contamination
VM8	deep aquifer	Piedmont	Rural area	Agricolture, roads, garbage, soil-water-atmosphere contamination
VM9	deep aquifer	Piedmont	Rural area	Agricolture, roads, garbage, soil-water-atmosphere contamination
VM10	deep aquifer	Piedmont	Rural area	Agricolture, roads, garbage, soil-water-atmosphere contamination
VM11	deep aquifer	Piedmont	Rural area	Agricolture, roads, garbage, soil-water-atmosphere contamination
VM12	deep aquifer	Piedmont	Rural area	Agricolture, roads, garbage, soil-water-atmosphere contamination
VM13	deep aquifer	Piedmont	Rural area	Agricolture, roads, garbage, soil-water-atmosphere contamination
VM16	deep aquifer	Piedmont	Rural area	Agricolture, roads, garbage, soil-water-atmosphere contamination
VM17	deep aquifer	Piedmont	Rural area	Agricolture, roads, garbage, soil-water-atmosphere contamination
VM18	deep aquifer	Piedmont	Rural area	Agricolture, roads, garbage, soil-water-atmosphere contamination
VM19	deep aquifer	Piedmont	Rural area	Agricolture, roads, garbage, soil-water-atmosphere contamination
VM20	deep aquifer	Piedmont	Rural area	Agricolture, roads, garbage, soil-water-atmosphere contamination
VM21	deep aquifer	Piedmont	Rural area	Agricolture, roads, garbage, soil-water-atmosphere contamination
VM22	deep aquifer	Piedmont	Rural area	Agricolture, roads, garbage, soil-water-atmosphere contamination
VM23	deep aquifer	Piedmont	Rural area	Agricolture, roads, garbage, soil-water-atmosphere contamination
VM24	deep aquifer	Piedmont	Rural area	Agricolture, roads, garbage, soil-water-atmosphere contamination
Maira 1	deep aquifer	Piedmont	Rural area	Agricolture, roads, garbage, soil-water-atmosphere contamination
Maira 2	deep aquifer	Piedmont	Rural area	Agricolture, roads, garbage, soil-water-atmosphere contamination
Cigliè 1	deep aquifer	Piedmont	Rural area	Agricolture, roads, garbage, soil-water-atmosphere contamination
Bastia 1	deep aquifer	Piedmont	Urban area	Urban activities, roads, garbage, agriculture, soil-water-atmosphere contamination
Bastia 6	deep aquifer	Piedmont	Rural area	Agricolture, roads, garbage, soil-water-atmosphere contamination
Bastia 7	deep aquifer	Piedmont	Rural area	Agriculture, roads, garbage, soil-water-atmosphere contamination
Bastia 8	deep aquifer	Piedmont	Rural area	Agriculture, roads, garbage, soil-water-atmosphere contamination

Table 46. Summary of the main obtained results for microplastic pollution.

				Fluorescence [%]		Shape [%]					Size [%]	
Site	Environment	Matrix	MP abundance	Fluorescent MPs	Non fluorescent MPs	Fibre	Fragment	Foam	Film	Pellet/sphere	1-5 mm	>1 mm
Bossea cave	show cave	sediments	4390	87.7	12.3	94.2	5.5	0	0.3	0.1	27.3	72.7
Borgio Verezzi cave	show cave	sediments	4695	87.9	12.1	87.9	12.1	0	0	0	27.5	72.5
Toirano caves	show cave	sediments	3823	93.7	6.3	93.7	6.3	0	0	0	25.3	74.7
Spring 8	spring	sediments	775.6	62.5	37.5	75.0	22.5	0.0	0.0	2.5	12.5	87.5
214 Cave	cave	sediments	1153.3	80.7	19.3	78.9	14.0	0.0	3.5	3.5	14.0	86.0
Trebiciano Cave	cave	sediments	2064.4	59.2	40.8	35.7	32.7	0.0	0.0	31.6	12.2	87.8
Mariano Well	cave	sediments	797.8	82.9	17.1	80.5	17.1	0.0	0.0	2.4	17.1	82.9
SV2	unexplored cave	sediments	22.2	94.2	5.8	95.9	4.1	0.0	0.0	0.0	8.0	92.0
SV4	unexplored cave	sediments	0.0									
SV5	unexplored cave	sediments	29.6	95.8	4.2	98.4	1.4	0.0	0.0	0.2	8.6	91.4
SV6	unexplored cave	sediments	14.8	96.0	4.0	98.7	1.3	0.0	0.0	0.0	9.0	91.0
SV8	unexplored cave	sediments	0.0									
Torri di Slivia cave	show cave	dripping	6.6	90.9	9.1	81.6	17.1	0.0	1.3	0.0	10.7	89.3
Bossea cave	show cave	waters	28	73.2	26.8	93.8	5.4	0	0	0.8	18	82
Rio Roccia Bianca	surface water	waters	23	78.3	21.7	95.7	4.3	0	0	0	8.7	91.3
Corsaglia river	spring	waters	29	93.1	6.9	100	0	0	0	0	27.6	72.4
Spring 16	spring	waters	40.5	66.1	33.9	76.8	21.4	0.0	0.0	1.8	23.2	76.8
214 Cave	cave	waters	36.9	60.8	39.2	80.4	17.6	0.0	2.0	0.0	29.4	70.6
Trebiciano Cave	cave	waters	85.6	70.8	29.2	72.9	22.9	0.0	4.2	0.0	27.1	72.9
Mariano Well	cave	waters	72.3	64.2	35.8	45.3	45.3	1.1	1.1	7.4	10.5	89.5
VM1	deep aquifer	waters	4.1	100.0	0.0	33.3	66.7	0.0	0.0	0.0	0.0	100.0
VM2	deep aquifer	waters	5.7	50.0	50.0	50.0	50.0	0.0	0.0	0.0	50.0	50.0
VM3	deep aquifer	waters	0.0									
VM4	deep aquifer	waters	2.7	50.0	50.0	0.0	100.0	0.0	0.0	0.0	0.0	100.0
VM5	deep aquifer	waters	2.9	0.0	100.0	100.0	0.0	0.0	0.0	0.0	0.0	100.0
VM6	deep aquifer	waters	2.5	0.0	100.0	0.0	100.0	0.0	0.0	0.0	0.0	100.0
VM8	deep aquifer	waters	0.0									
VM9	deep aquifer	waters	3.1	100.0	0.0	0.0	100.0	0.0	0.0	0.0	0.0	100.0
VM10	deep aquifer	waters	0.0									
VM11	deep aquifer	waters	4.3	33.3	66.7	0.0	100.0	0.0	0.0	0.0	0.0	100.0
VM12	deep aquifer	waters	0.0									
VM13	deep aquifer	waters	0.0									
VM16	deep aquifer	waters	0.0									
VM17	deep aquifer	waters	2.9	100.0	0.0	100.0	0.0	0.0	0.0	0.0	100.0	0.0
VM18	deep aquifer	waters	4.5	66.7	33.3	100.0	0.0	0.0	0.0	0.0	66.7	33.3
VM19	deep aquifer	waters	2.7	100.0	0.0	0.0	100.0	0.0	0.0	0.0	0.0	100.0
VM20	deep aquifer	waters	2.8	100.0	0.0	0.0	100.0	0.0	0.0	0.0	0.0	100.0
VM21	deep aquifer	waters	2.8	50.0	50.0	0.0	100.0	0.0	0.0	0.0	0.0	100.0
VM22	deep aquifer	waters	0.0									
VM23	deep aquifer	waters	6.3	75.0	25.0	0.0	100.0	0.0	0.0	0.0	0.0	100.0
VM24	deep aquifer	waters	0.0									
Maira 1	deep aquifer	waters	0.0									
Maira 2	deep aquifer	waters	3.8	100.0	0.0	100.0	0.0	0.0	0.0	0.0	100.0	0.0
Cigliè 1	deep aquifer	waters	0.0									
Bastia 1	deep aquifer	waters	0.0									
Bastia 6	deep aquifer	waters	0.0									
Bastia 7	deep aquifer	waters	3.3	100.0	0.0	0.0	100.0	0.0	0.0	0.0	0.0	100.0
Bastia 8	deep aquifer	waters	0.0									
Bossea cave	cave	waters	1.7	66.7	33.3	0.0	100.0	0.0	0.0	0.0	0.0	100.0
Rio Roccia Bianca	surface water	waters	3.0	66.7	33.3	100.0	0.0	0.0	0.0	0.0	0.0	100.0
Overflow	spring	waters	2.0	0.0	100.0	33.3	66.7	0.0	0.0	0.0	0.0	100.0
Max				100.0	100.0	100.0	100.0	1.1	4.2	31.6	100.0	100.0
Min				0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Mean				72.1	27.9	58.4	39.9	0.0	0.3	1.4	17.6	82.4
SEDIMENTS												
Max				96.2	40.8	98.7	32.7	0.0	3.5	31.6	27.5	92.4
Min				12.3	3.8	17.2	1.3	0.0	0.0	0.0	7.3	7.3
Mean				81.4	11.0	80.6	8.2	0.0	0.5	4.6	11.7	79.8
WATERS												
Max				100.0	100.0	100.0	100.0	1.1	4.2	7.4	100.0	100.0
Min				0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Mean				67.5	32.5	48.6	50.7	0.0	0.3	0.4	18.1	81.9

The major part of the analyzed microparticles was fluorescent under UV light (mean 72.1% for MPs), with especially blue fluorescence. This data confirmed that using UV light in searching MPs and other anthropogenic microparticles such as MFs could be very helpful. Considering only sediment samples, the percentages of microparticles fluorescent under UV light found increase (81.4% for MPs), while in water samples decrease (67.5% for MPs).

Although in this work no correlations have been made between the color/fluorescence of the microparticles found and their possible ingestion by organisms, color and fluorescence remain important characteristics to be reported, when possible. Information on this topic is currently scarce, especially in non-marine environments, consequently, it becomes essential to delve deeper into this topic in the future. At the moment, it is not possible to make any kind of supposition with the data of this study, as the colors of the particles studied have been the most varied. However, it is possible to observe that non-fluorescent particles have generally dark colours (black, blue, brown…), probably because whiteners are not used in these kinds of plastics.

Regarding the shape, major part of the analyzed MPs were fibres (58.4%) and fragments (39.9%). Considering only sediments, 80.6% were fibres, while only 8.2% were fragments. Instead, in water samples 48.6% were fibres and 50.7% fragments. MP shape could suggest their origin, especially when combined with the polymer type. For example, microspheres found in underground water in an urban context suggest an origin linked to sewage system leaks, as these beads are often used in personal care products.

Taking into the account the size, only 17.6% of the analyzed MP had dimensions between 1-5 mm (big MPs), highlighting an increase of MP amounts, considering small sizes.

There are no particular similarities in typology and morphological characteristics of the analyzed pollutants between one site and another, highlighting the complexity of monitoring these environments, extremely varied and dependent on environmental and climatic characteristics, and direct and indirect anthropogenic surface activities.

Show caves consistently exhibit substantially higher levels of contamination than speleological ones, regardless of their geographical setting. This pattern is most likely attributable to the direct and sustained anthropogenic pressure exerted within the subterranean environment by continuous visitor presence. In contrast, contamination observed in speleological underground environments and at karst springs appears to be primarily controlled by surface-derived activities and soil-water-atmosphere contamination, resulting in highly site-specific and spatially variable pollution signatures. Considering unexplored hypogeal environments and deep aquifers, contamination levels are fortunately still markedly lower, although they are not entirely absent.

8.3 Possible sources and transport

In karst systems, the occurrence of MPs and other AMPs primarily reflects both direct and indirect human pressures and is modulated by aquifer flow dynamics, lithological and structural characteristics, the intensity of karst development, site-specific climatic conditions, and atmospheric transport processes.

In caves, point-source inputs arise from recreational activities in tourist caves, as well as from the presence of cavers and scientific personnel in speleological caves, in addition to litter. Conversely, indirect contamination of subterranean environments can originate from a wide range of surface-based activities, including waste dumping, landfills, effluent releases, agricultural practices, transportation networks, industrial operations, and the contamination of soils and surface waters, as well as from atmospheric fallout deposited at the land surface (e.g. Allen et al., 2019; Corradini et al., 2019; Kim et al., 2021b; Liu et al., 2019a; Liu et al., 2019b; Oppeltová et al., 2024; Zhou et al., 2021).

The karst areas studied in this thesis showed marked heterogeneity in terms of land use and environmental context (Table 45). Some of these sites are located in urbanized contexts, while others are immersed in agricultural landscapes or wooded areas. The caves themselves include tourist caves, caves frequented exclusively for speleological purposes, and systems that had not been explored previously. With the exception of show caves, which are likely to be subject to substantial contamination due to the daily pressure of visitors, the direct contributions of speleologists and scientific activities in caves can be considered of secondary importance. Instead, it is reasonable to infer that the main sources of pollution affecting underground environments arise from contamination of soils and surface water, as well as from atmospheric transport and deposition processes. In line with this interpretation, the present study also documented the presence MPs and MFs in percolation waters emerging directly from the rocks. Many of the investigated surface areas were also located in proximity to railway lines and major road infrastructures or are affected by the presence of improperly managed waste.

After entering the soil matrix, these materials may undergo progressive fragmentation and are subsequently mobilized through percolation within soil pores and rock discontinuities, facilitating their downward transfer into deeper horizons and their eventual accumulation in subsurface compartments and groundwater systems (Chia et al., 2021; Fahrenfeld et al., 2019; Frei et al., 2019; Lwanga et al., 2017; McGechan, 2002; Pérez-Lucas et al., 2018; Viaroli et al., 2022; Wanner, 2021). In addition, a combination of biological, chemical, and physical mechanisms governs the transport and fate of pollutants in underground environments and aquatic systems (Stoppiello et al., 2020).

8.4 General conclusion

MP and MF monitoring in karst systems and aquifers is a key step to understand the health status of the environment and to establish management and conservation strategies. Future studies are needed to better understand MP and MF sources and transport in karst areas, verifying the potential effects on ecosystems and organisms. Potentially negative consequences from MP and MF pollution for subterranean water safety at all levels, such as ecological functionality, biodiversity distribution, ecosystem services and human health should be investigated too.

While research on MPs advanced in all fields, knowledge about natural and regenerated MF threats is too limited at the moment. Therefore, greater efforts must be made in the environmental and health fields regarding these pollutants.

Long-term monitoring, considering seasonal variations, spatial distribution, and more subterranean and surficial sites, will help understanding the impact of MP and MF pollution on habitats and species, including humans.

Improving MP monitoring in karst systems, including pollutants linked to MPs, could provide important information on the health of the ecosystems and the possible damage that these pollutants could cause even to tiny organisms.

Working in extreme and dark environments is not easy, and the difficulties of progression in unknown environments can be really hard. However, underground environments are rarely studied, and any new information is crucial for a better understanding of the ecosystems themselves, and possible threats. Greater effort in sampling subterranean environments is essential, enhancing our knowledge on these very important systems.

The presence of MPs within groundwater systems gives rise to multiple environmental challenges, as these long-lived microparticles exhibit reactive physicochemical characteristics and function as vectors for additional contaminants. Groundwater is commonly perceived as a safer and more reliable source of fresh water than surface water bodies. However, the infiltration of MPs into aquifers, as well as other anthropogenic microparticles like MFs, alters this perception by introducing multiple toxicological and ecological concerns (Lapworth et al., 2023). As the prevalence of MP contamination in groundwater systems becomes progressively more evident, an integrated, multi-level framework including preventive strategies, advanced remediation technologies and sustainable resource management is imperative to mitigate its long-term repercussions on the environment and public health.

Surface and underground environments in karst areas are closely connected, therefore, greater efforts should be made to establish more comprehensive measure of protection. A combination of awareness, willingness and new ideas will allow us to curtail the threat that pollution poses to wildlife and other species, including humans.

Promoting political and social changes is crucial to support genuine plastic reduction initiatives and encourage manufacturers to adopt more sustainable, eco-friendly materials for all products. Promoting sustainable alternatives, improving wastewater treatment efficiency, and regulating the production of products with MPs are practices that must now be implemented. Where monitoring shows an evident pollution, removal technologies can be applied, using filtration technologies, adsorption and coagulation methods, or biodegradation approaches (Sumam et al., 2025).

Education at all levels, grounded in the principles of environmental sustainability, will be key to the management and conservation of water resources, protected habitats, and species.

Abbreviations

ABS: acrylonitrile-butadiene-styrene
AI: artificial intelligence
AMP: anthropogenic microparticle
ATR: attenuated total reflection
BSP: Biologia Sotterranea Piemonte – Gruppo di Ricerca
EPP: expanded polypropylene
EPS: expanded polystyrene
EVOH: ethylene vinyl alcohol
FWAs: fluorescent whitening agents
IR: infrared
MP: microplastic
MF: microfibre
MUPL: MicroplasticLab software
OMR: organic matter removal
PA: polyamide (nylon)
PAC: polyacetylene
PAM: polyacrylamide
PC: polycarbonate
PE: polyethylene
PES: polyester
PET: polyethylene terephthalate
PP: polypropylene
PS: polystyrene
PVA: polyvinyl alcohol
PVC: polyvinyl chloride
PU: polyurethane
TPU: thermoplastic polyurethane
μFTIR: micro Fourier Transformed Infrared

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