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The problem of anthropogenic microfibres in karst systems: Assessment of water and submerged sediments

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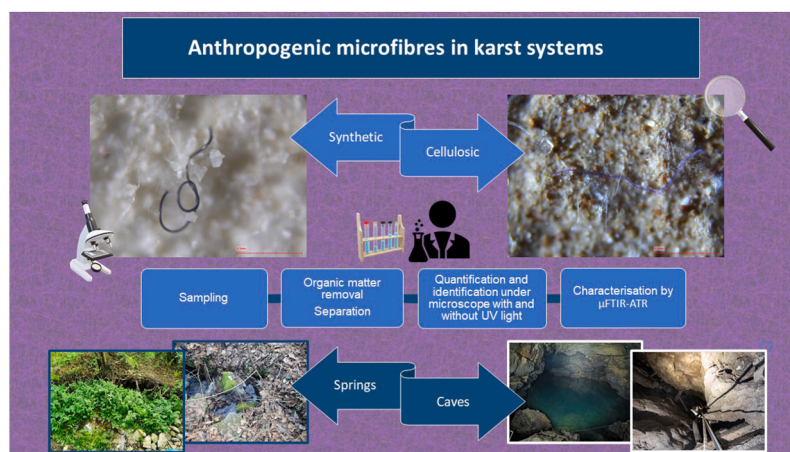
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HIGHLIGHTS

- Microfibres pollute karst surface and subterranean aquatic environments.
- An accumulation of microfibres in submerged sediment occurs.
- Microfibre amount increase with the decrease in the size considered.
- Natural and regenerated microfibres are more abundant than synthetic ones.
- Monitoring aquatic environments is fundamental for their conservation.

GRAPHICAL ABSTRACT



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ABSTRACT

A new worrying micropollutant threatens natural environments: the microfibres (MFs). Natural, regenerated and synthetic MFs have been detected in different environments, as well as in organisms. While synthetic MFs are generally detected in microplastic analyses, natural and regenerated MFs are not taken into account, or are wrongly considered plastics. They are generally considered biodegradable even if their degradation processes in ecosystems are poorly known. Their potential faster degradation could release toxic compounds, and their characteristics could lead to a long-term accumulation in the environment. Understanding their dangerousness and the possible impact they could have on ecosystems is fundamental for environment conservation. We collected and investigated water and submerged sediment samples in different caves and springs of the Classical Karst Region (NE Italy), rich in protected habitats and species. MFs were analysed via microscopy and spectroscopy. MFs were found in all samples, highlighting pollution in surface and subterranean habitats of the karst system. MF concentration was higher in submerged sediments respect to waters, highlighting an accumulation of

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MFs over time. Big microfibrils were less abundant, and MF amount increased with the decrease in the considered size. More than 80% of fibres were fluorescent under UV light. Fluorescent MFs were especially transparent, while non-fluorescent ones were mainly black and blue. Most MFs were cellulosic, and synthetic MFs represent only 15–22%, highlighting a significant gap between the MF composition detected in natural environments and the global production of synthetic textiles in recent times. Synthetic MFs were more abundant in waters. Our results improve the knowledge on micropollutants in karst environments, laying the foundations for future research. MF pollution monitoring in karst areas must become a priority for species protection, habitat conservation, and waters management, improving analyses on a larger number of aquatic environments, taking into account the ecological connections between surface and subterranean habitats.

1. Introduction

Microplastics (MPs) represent a worldwide concern; however, while marine environments have been extensively studied over time (e.g. Cutroneo et al., 2020; Leistenschneider et al., 2021; Tsang et al., 2017), research in atmosphere and terrestrial environments is at the initial stage, and different environments remain not well known, such as subterranean habitats (Balestra et al., 2023; Re, 2019; Viaroli et al., 2022). Plastic properties, such as resistance, high durability, and low weight, make these materials dangerous pollutants for natural environments. Small dimension (5mm–1µm) of MPs increases their dangerousness and make them easily transported even across long distances through different matrices (Allen et al., 2019; Liu et al., 2019; Lusher et al., 2015). MPs can be assimilated directly or indirectly by organisms, and can be toxic, representing an ecological emergency (Assas et al., 2020; Devereux et al., 2021; Jahan et al., 2019; Romeo et al., 2015). They may contain other pollutants and/or bind to other pollutants too, increasing environmental risks (Koelmans et al., 2013; Li et al., 2018; Rochman et al., 2013; Selvam et al., 2021; Wanner, 2021).

However, recent studies highlighted concern for other pollutants that impact natural environments and often are wrongly considered as MPs: the microfibrils (MFs) (Athey and Erdle, 2022; Dris et al., 2016; Finnegan et al., 2022; Hasenmueller et al., 2023; Stanton et al., 2019; Suaria et al., 2020a). MFs includes fibres <5 mm in length of any composition, derived from textile production during all their life cycle, cigarette filters, personal care products such as face masks and wet wipes, and other manufactured fibrous materials (Athey and Erdle, 2022). Textile MFs in natural environments are currently more studied than the other sources; however, recent research suggest that MFs from other sources can have presence into the environments that is comparable to the one of textile products (Belzagui et al., 2021; Ó Briain et al., 2020).

Anthropogenic fibres are commonly divided in three categories: natural, man-made cellulosic or regenerated, and synthetic (Finnegan et al., 2022; Stanton et al., 2019). Natural fibres derive from the processing of plant (cellulosic) and animal (proteinaceous) fibres, such as cotton or wool. Man-made cellulosic (MMC) fibres are regenerated, reconstituted from the dissolved plant material cellulose, such as rayon/viscose, and acetate. Synthetic fibres are made of plastic polymers, therefore, under 5 mm, they could be considered MPs.

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, MMC 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).

MFs have been detected in different environments (Stanton et al., 2019; Suaria et al., 2020a, 2020b), in atmosphere (Finnegan et al., 2022), and even in food and drinks (Cox et al., 2019; Kosuth et al., 2018), as well as in human (Pauly et al., 1998) and animal organs (Le Guen et al., 2020; Remy et al., 2015; Zhao et al., 2016). Adverse effects on animal health have been observed under laboratory conditions (Jemec et al., 2016; Watts et al., 2015). MFs are often the most prevalent morphology found in MP pollution surveys worldwide, although synthetic MFs are generally just a fraction of the total number of MFs (Athey and Erdle, 2022; Finnegan et al., 2022; Hasenmueller et al., 2023; Suaria et al., 2020a). However, synthetic MFs pose the highest risk for ecotoxicity compared to other morphologies (Bucci and Rochman, 2022; Gray and Weinstein, 2017).

Natural and MMC fibres have been likely included in MPs monitoring by hundreds of studies in the past, increasing MP concentrations in both environments and organisms (Wesch et al., 2016). Not-synthetic MFs have been often considered MPs because of their colours, although without chemical analysis, and MMC fibres have been considered MPs by different researchers because they are extruded and processed industrially (Obbard et al., 2014; Woodall et al., 2014). However, MMC fibres are an available source of carbon for microorganisms (Zambrano et al., 2019), and their biodegradation processes seems to be similar to cotton fibres (Park et al., 2004), therefore, they should not be considered MPs.

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., 2021; Lusher et al., 2013).

Recently, growing efforts were done to study micropollutants in natural environments, however, several ecosystems, such as karst systems, remain poorly studied, despite their high pollution risk. Karst systems and other underground environments have a high environmental value, but are often subjected to contamination by surface pollution (Chiarini et al., 2022). Karst systems are characterized by the

presence of dissolution feature in soluble carbonate rock, such as caves, conduits, and sinkholes, which enhance the surface and subterranean environment connections. These environments host aquifers supplying drinking water (Moldovan et al., 2020), and support fragile ecosystems and species, such as troglobiont (i.e. specialist of underground environments) (Culver and Pipan, 2019; Mammola, 2019). Consequently, these areas often are within protected areas, national and regional parks. Despite the ecological and economic significance of these environments, assessments of microplastic pollution in karst systems are rare (e.g. Balestra et al., 2024; Balestra et al., 2023; Panno et al., 2019; Shu et al., 2023; Valentić et al., 2022) and, to our knowledge, only in one American karst system research hints at natural and regenerated materials

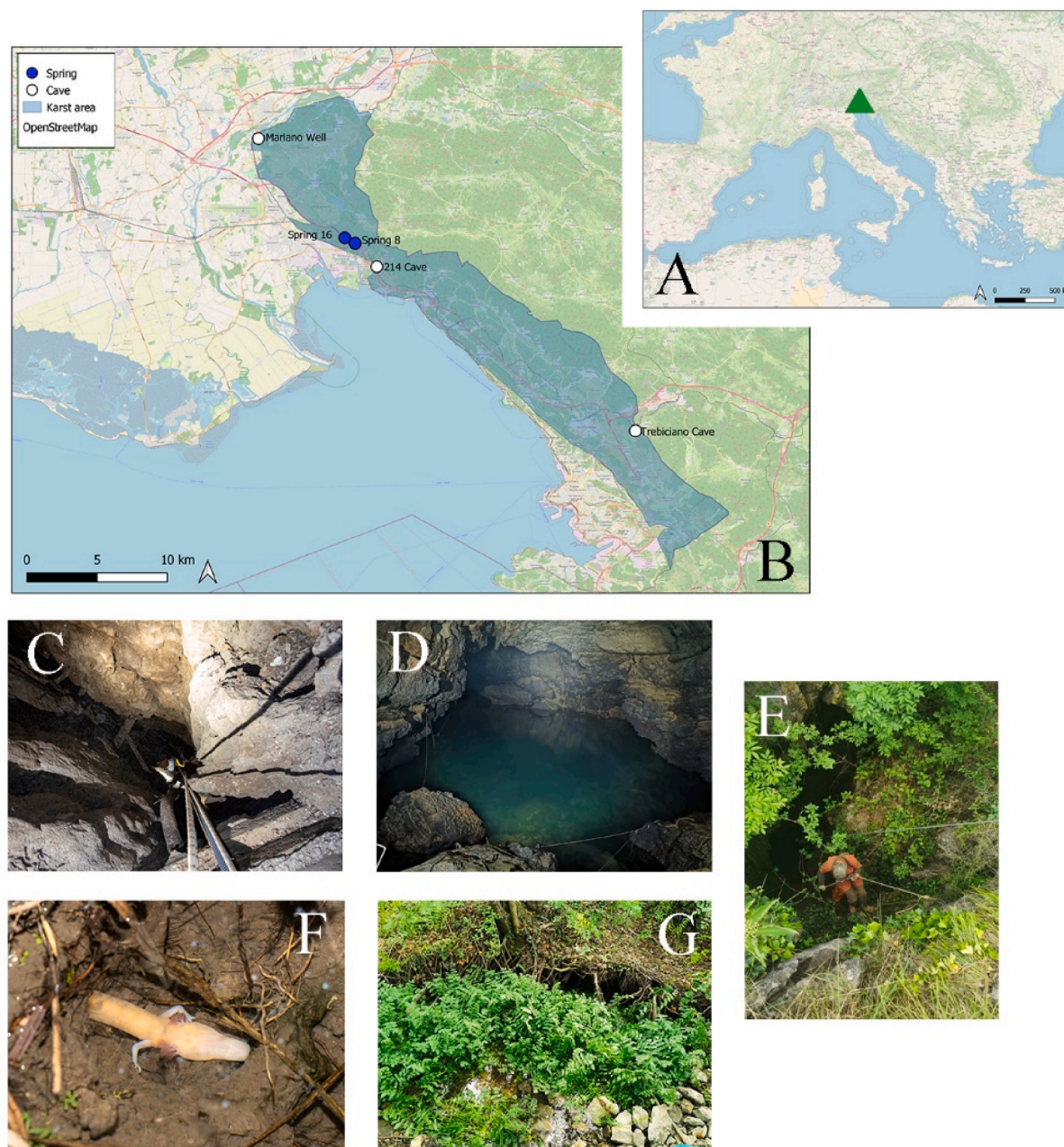


Fig. 1. Study area and sampling points in the Italian sector of the Classical Karst, Friuli-Venezia-Giulia Region, Italy. A: Study area (Maps created with QGIS Desktop 3.12.1 with GRASS 7.8.2 using OpenStreetMap map, modified - openstreetmap.org/copyright); B: Karst area in light blue, springs in blue 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). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

pollution (Baraza and Hasenmueller, 2023; Hasenmueller et al., 2023).

In this work, we quantified and characterized MFs in water and sediment samples from aquatic surface (springs) and subterranean (caves) environments of the Italian sector of the Classical Karst Region. This research provides a baseline for future work related to the anthropogenic microfibres pollution in karst environments, which are under-represented in literature respect to MPs, and useful for mitigation and management plans. The aims of this study are, for the first time: i) to understand if human-sourced MFs are present in the Classical Karst Region; ii) to highlight the extent to which anthropogenic MFs dominate karst environments in both cave and spring environments, and in water and submerged sediments, and iii) to characterize detected MFs by size, shape, colour, fluorescence and typology to facilitate a more critical future consideration of their environmental impact. In detail, we want to investigate the following questions: a) is there an accumulation of MFs in submerged sediments or concentration are higher in waters? b) does the MF amount increase with the decrease in the size considered? c) are synthetic fibres less abundant than natural and regenerated ones?

2. Materials and method

2.1. Study area

This study was carried out in the Italian sector of the Classical Karst Region (CKR) - Kras (Fig. 1), a karst plateau stretching from NE Italy and SW Slovenia, for a total 15–20 km wide and 40 km long area in the SW-NW direction (Visintin and Cucchi, 2010). The CKR was the first studied karst areas, from which the “karst” term derives (Gunn, 2004). This area is intensely karstified, mainly composed by Cretaceous to Eocene carbonate rocks (Comeno/Komen Unit), and is famous for its caves (Cucchi et al., 1987; Placer, 1981). More than 80 sinkholes and 3500 caves are reported for the Italian sector (Zini et al., 2010). In this sector, the major aquifers are connected with the Vipacco, Isonzo, and Timavo rivers, and with local subterranean systems. The Timavo River flows almost entirely in underground cavities and conduits for more than 70 km (Zini et al., 2010). Despite frequent changes of direction in the preferential flows, under flood periods the waters of this river take up only two days en route (Zini et al., 2010).

This karst area is characterized by important habitats, prioritized according to the European Union classification. These habitats are featured by the presence of dissolution morphologies in soluble carbonate rock, which improve connections between surface and subterranean environments, such as caves, karst lakes, and springs. A lot of these habitats host different stygobionts, such as obligate groundwater specialized species, including the endemic crustaceans *Troglocaris planinensis* and *Monolista racovitzai*, and the salamander *Proteus anguinus*, the only troglobiont vertebrate present in Europe (Balestra et al., 2024; Manenti et al., 2024; Stoch, 2017).

Thanks to the important underground water reserves and their high quality, this region has been heavily exploited in the past, and the hydrology of the karst system was irreversibly modified (Fornasir, 1929; Gemit, 2004; Marocco and Melis, 2009). Economic development and urbanization over time made the water sources extremely threatened (Zini et al., 2010). Although different protected areas were established in this region for conservation purposes, karst ecosystems are open and close to urban and industrial areas, threatening species and habitats.

2.2. Sampling sites

Water and submerged sediment samples were collected from two surface (springs) and three subterranean (caves) aquatic environments, selected based on their geographical and biological relevance (Fig. 1). Samples were collected at the beginning of the lean period, in May 2022, using bulk samples (Hidalgo-Ruz et al., 2012), placed into pre-cleaned glass jars. Water samples were directly collected using 1 L glass jars, while submerged sediment samples were collected with a metal spoon,

cleaned with ethanol every time.

Working in extreme and fragile environments such as not touristic caves is not easy. These sensitive environments should not be damaged, and large volumes of samples are difficult to collect. The transport of samples can be difficult too, due to narrow passages, wells, rope passages, or crossing in unstable areas. However, these environments are rarely studied, consequently, any new information is crucial to better understand these ecosystem and possible threats, formulating appropriate subsequent conservation measures. Following the precautionary principle and assessing the environmental characteristics of the studied sites, we limited the amount of collected samples. Cave samples were wrapped with anti-impact material to transport them safely in the speleological bags. The amount of the collected samples depended on water and sediment pool availability, and on the difficulty of sampling: water samples had to be able to be sampled without moving the sediment on the bottom, then analysed separately.

Surface sites (Spring 8 and Spring 16) are permanent small springs flowing from the ground (Fig. 1G and H). Preliminary investigations pointed out these springs as emitters of a complex karst system connecting several subterranean sites with surface habitats of the Doberdò and Pietrarossa Lakes, in the homonymous Regional Nature Reserve (Cucchi et al., 2008). This protected area is adjacent to a very busy highway connecting Italy with Eastern Europe, and local roads.

Subterranean environments samples were collected in small puddles and still waters in caves with vertical entrances, reachable using caving equipment.

The pit 214 Cave (Pozzo presso S. Giovanni di Duino, 214/226VG) is located north of Trieste, in Duino Aurisina municipality, where the Timavo River waters rise after 40 km underground (Fig. 1F). The cave, with the entrance at about 50 m a.s.l., has a 47 m vertical drop which ends with a water basin on the bottom, due to a bank of gravel. Cave waters are strictly connected with the nearby resurgences of the Timavo River, however, water table is clearly marginal (Galli, 2012). The railway tracks are closed to the cave entrance, which is also surrounded by the highway and the provincial road.

Trebiciano Cave (Grotta di Trebiciano, 3/17VG) is located in a wooden area of the Trieste municipality (Fig. 1E). The cave, with the entrance at about 342 m a.s.l., develops for about 1198 m, with a vertical drop of 329 m. The cave has a long series of wells of different depths that follow each other up to emerge on a huge pile of sand located inside a giant hall, in which the Timavo River flows. The water of the Timavo River disappears in the San Canziano Cave, Slovenia, and reappears only in Trebiciano and Luftloch caves before flowing into the springs located in S. Giovanni di Duino. An underground laboratory was built inside the cave to study these waters, and different works were done to preserve and enhance the route in the cavity over time, equipped in 1983 with fixed via ferrata ladders, contributing (voluntarily or not) to the pollution of the cave, even in deep areas.

Mariano Well (Pozzo primo di Castelveccchio, 8186/6611VG) is located in an urban area of the Sagrado municipality, which extends between the Isonzo River left bank and the karst plateau (Fig. 1D). The cave has a total vertical drop of about 40 m, with a water basin of 17 m. The cave entrance, at about 28 m a.s.l., opens into the cellars of a building, since this cave was used to collect water for drinking purposes in the past. The cave was then equipped with metal structures directly in contact with water, which degraded over time. The structures were subsequently removed due to their dangerousness, however, this structure and other different sources of pollution could have polluted this cavity, being in an urban area.

Samples were stored in a fridge at 6 °C until laboratory analysis. Unfortunately, water of Spring 8 and submerged sediment of Spring 16 samples were damaged during transport, consequently, they were not analysed in this study.

2.3. Laboratory analysis

2.3.1. Contamination control

To avoid contamination, plastic labware were replaced with glass and metal equipment, wherever possible. All working surfaces and labware were cleaned with milliQ water and absolute ethanol. After washing, labware was let dry covered with aluminum foil, to prevent possible air contamination. Laboratory analyses were carried out under a hood, covering all open glass jars and labware with an aluminum foil during all steps. Nitrile gloves and white cotton coats were used by researcher during laboratory analysis. Blank controls on ethanol absolute (VWR Chemicals), milliQ water, H₂O₂ 30% (Merck), and NaCl solution (Carlo Erba NaCl + milliQ water) were done to determine possible contaminations during laboratory analysis. For water samples, milliQ water, H₂O₂ 30% and absolute ethanol were analysed, however, milliQ water, H₂O₂ 30%, absolute ethanol, and NaCl solution were considered for submerged sediment samples. Four samples for each chemical, four for milliQ water, and eight for NaCl solution were tested, calculating the average for each one (see [Supplementary Tables 1 and 2](#)). Finally, all the different average blank types were added and then the sum of the blanks was subtracted from the sample total amounts. All blanks were analysed using the same methodology of the samples.

2.3.2. Water analysis

Analyses on water samples were done according to the method described in [Balestra et al. \(2023\)](#), adapted to the samples characteristics, and improved adding organic matter removal as pre-treatment, to obtain cleaner and homogenous filters. During pre-treatment, in order to digest organic materials, samples were poured in glass beakers with a 1:1 30% H₂O₂ solution, covered with aluminum foil, and left to react for a week at room temperature. Each sample was then filtered by a vacuum pump through a glass fibre filter (Phenomenex, Ø 47 mm, 1.2-µm pore size). Filters were placed on pre-cleaned glass petri dishes, covered with aluminum foil, and dried in the oven at 40 °C until completely dry.

2.3.3. Submerged sediment analysis

Submerged sediment samples were analysed following the method described in [Balestra and Bellopede \(2023\)](#). Sediments were placed in aluminum box covered with aluminum foil and dried into the oven at 40 °C to constant weight. During pre-treatment, in order to digest organic materials, dried sediments were poured in glass beakers with a 1:1 30% H₂O₂ solution, covered with aluminum foil, left to react for a week at room temperature, and dried again at 40 °C to constant weight. Each sample was divided in three sub-samples of 15 g by the coning and quartering method. Sub-samples were put into beakers with 150 ml NaCl solution ($\rho = 1.2$), mixed for 2 min with a magnetic mixer, and left to rest for 24 h. The supernatant (50 ml) was then aspired with a glass pipet and filtered through a glass microfibre filter (Phenomenex, Ø 47 mm, 1.2-µm pore size) by a vacuum pump. Filters were placed on pre-cleaned glass petri dishes, covered with an aluminum foil, and dried in the oven at 40 °C until completely dry.

2.4. Microfibre identification and characterization

A lot of plastic materials and anthropogenic fibres are mixed with chemicals that improve their properties, such as Fluorescent Whitening Agents (FWAs) ([Qiu et al., 2015](#)). These materials can be identified thanks to their fluorescence under an ultraviolet (UV) light (e.g. [Balestra and Bellopede, 2022, 2023](#); [Balestra et al., 2023](#); [Ehlers et al., 2020](#); [Giardino et al., 2023](#); [Klein and Fischer, 2019](#); [Qiu et al., 2015](#)). However, also natural matter, organic and inorganic, can be fluorescent under a UV light, and not all anthropogenic fibres are treated with whitening fluorescent additives, consequently, analysis under UV light alone could be insufficient. Organic matter removal is an indispensable step in MF detection. Preliminary screening investigations on filters can be very useful to understand which methodology is best used. A

combination of several methods is probably the best solution for the identification of MFs, as suggested for MP identification in natural environments ([Song et al., 2015](#)). In this work, MFs were detected by means of microscopic and spectroscopic techniques.

MFs on filters were observed at the microscope (Leitz ORTHOLUX II POL-MK, equipped with a DeltaPix Invenio 12EIII 12 Mpx Camera) with and without a UV flashlight (Alonefire SV10 365 nm UV flashlight 5 W). MFs were counted at 2.5 × magnification, enlarged to 10 × or higher magnifications for MF identification and characterization ([Balestra and Bellopede, 2022, 2023](#); [Balestra et al., 2023, 2024](#)), performed in agreement with the strict selection criteria reported in previous works ([Crawford and Quinn, 2016](#); [Hidalgo-Ruz et al., 2012](#); [Noren, 2007](#)), with the help of x-sectional and longitudinal microscopic images of natural, MMC and synthetic fibres (e.g. [Khan et al., 2017](#)). Being the accuracy in visually identifying small particles less reliable than with big ones ([Hidalgo-Ruz et al., 2012](#); [Song et al., 2015](#)), particles <0.1 mm were not considered, as well as MFs not clearly identifiable as of anthropogenic origin ([European Commission, 2013](#)). Observed microfibres were divided in synthetic (MPs - plastic polymers), natural and man-made (natural materials worked through chemical processes) and unknown (degraded anthropogenic material not clearly identifiable) MFs.

An average from 1% to 10% of detected particle is usually analysed to determine the chemical composition of MPs ([International Organization for Standardization and European Committee for Standardization, 2020](#)). In this work, randomly 10% of MFs for each filter was identified using a micro-Fourier Transform Infrared Spectroscopy (µFTIR) Shimadzu AIM-9000 microscope equipped with a Shimadzu IRTracer-100 spectrophotometer, and operated in attenuated total reflection (ATR) mode (Shimadzu ATR with a germanium prism). MFs were handpicked from the glass filters and transferred with a inline needles on a silver filter (GVS Life Sciences, Membrane Disk 47 mm, 0.8 µm pore size) placed onto a slide. Spectral range of analysis was set between 4000 and 700 cm⁻¹, with 40 scans per item. MF spectra were improved applying atmosphere corrections. Automatic comparison with the Shimadzu Lab Solution Library ATR Polymer 2 were done together with visual analysis of the characteristic bands in the reference spectrum. To reduce errors, only spectra with a match degree ≥75% were taken into account (e.g. [Fossi et al., 2017](#); [Suaria et al., 2020a](#)).

3. Results

3.1. Microfibre abundance

Procedural blank indicated a possible contamination linked to pre-existing material in the chemical products used during laboratory activities ([Supplementary Tables 1 and 2](#)). Analysed MFs of our blank were mainly comprised of cellulose ([Supplementary Tables 1 and 2](#)). Reported data in this chapter were corrected with the removal of the blank. Uncorrected data were reported in [Supplementary Fig. 1](#) and [Supplementary Tables 3, 4 and 5](#).

MFs were found in all water and submerged sediment samples, highlighting MF pollution in surface and subterranean habitats of the karst system ([Tables 1 and 2, Fig. 2](#)). A mean concentration of 163.5 MF/L was found in waters, and of 4776.7 MF/kg in sediments. An accumulation of MFs in sediments was highlighted ([Fig. 2](#)). Most of the analysed MFs (>60%) were cellulotics ([Fig. 2](#)). Synthetic MFs were more abundant in waters, and were only 22.2% in waters and 14.7% in submerged sediments (corrected values) ([Fig. 2](#)). Degraded (Unknown) MFs were mainly present in submerged sediments ([Fig. 2](#)).

3.2. 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. 3](#)). The highest

Table 1

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

Filter Water	Examined amount [ml]	TOT [MFs/L]	Natural and MMC [MFs/L]	Synthetic [MFs/L]	Unknown [MFs/L]
Spring 16	1100	110.4	59.5	28.1	22.7
214 Cave	1080	149.1	97.1	27.0	25.0
Trebiciano Cave	500	192.0	121.0	65.0	6.0
Mariano Well	1150	202.7	143.4	25.4	33.9
TOT		654.2	421.1	145.4	87.6
%		100.0	64.4	22.2	13.4
Mean		163.5	105.3	36.4	21.9

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. 3).

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.

3.3. Microfibre fluorescence and colour

Most MFs were fluorescent under UV light: a mean of 81.9% in waters and 93.0% in submerged sediments (Figs. 4A,B and 5). 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 (Fig. 4A and 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 (Figs. 4C,D and 5). Other fluorescence colours were found with values less than 1%. Only in Trebiciano Cave percentages were slightly different in water samples (Fig. 4C).

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% (Figs. 4E,F and 5). 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% (Figs. 4G,H and 5).

3.4. Microfibre characterisation by μ FTIR-ATR

A half of the total analysed fibres did not exceed a match of 75%, respectively 56.3% for water and 43.8% for submerged sediments samples (Fig. 6).

μ FTIR-ATR characterization revealed that 28.9% of the analysed fibres in waters and 47.9% in submerged sediments were anthropogenic cellulotics (natural and MMC). Except a pair of ramie fibres, all analysed 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 MMC fibres, such as Cupro/Bemberg, Tencel/Lyocel and cellulose acetate. Different methyl cellulose and hydroxypropyl 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

Table 2

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]	TOT [MFs/15g]	Natural and MMC [MFs/15g]	Synthetic [MFs/15g]	Unknown [MFs/15g]
Spring 8	15	4537.8	68.1	34.8	8.6	24.7
214 Cave	15	8982.2	134.7	91.8	13.6	29.3
Trebiciano Cave	15	2648.9	39.7	20.8	10.3	8.7
Mariano Well	15	2937.8	44.1	26.5	9.6	8.0
TOT			286.6	173.9	42.1	70.7
%			100	60.7	14.7	24.7
Mean		4776.7	71.7	43.5	10.5	17.7

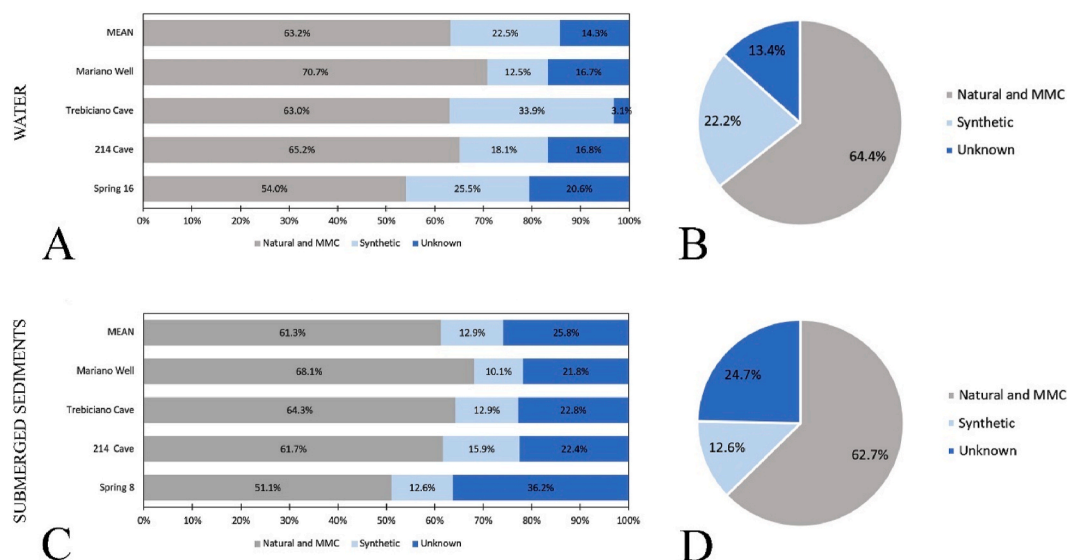


Fig. 2. Abundance of microfibres in water and submerged sediment samples in surface and subterranean aquatic environments of the Classical Karst Region. A, C: Water samples abundances by sampling area; B, D: Total amount of microfibres in water.

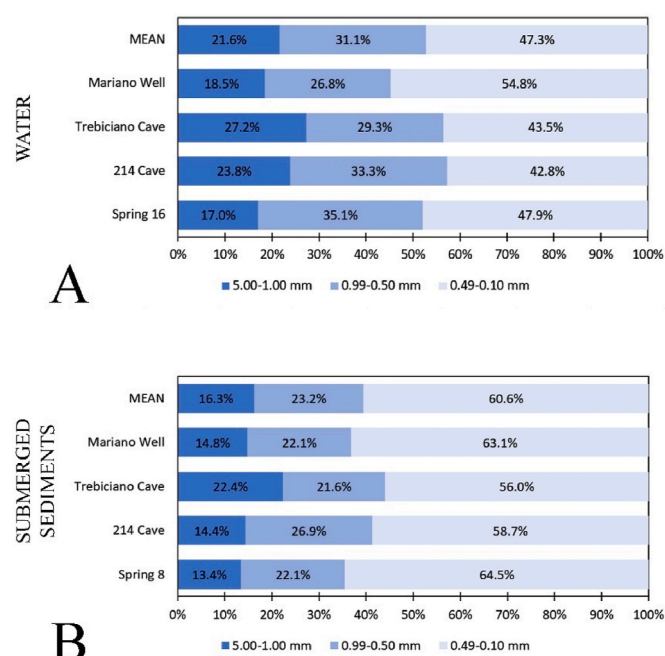


Fig. 3. Size of analysed microfibres. A: Size of microfibres found in waters samples; B: Size of microfibres found in submerged sediment samples.

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.

4. Discussion

4.1. Anthropogenic microfibres pollute karst systems

In this study, 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 analysing 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 (e.g. Balestra and Bellopede, 2022, 2023; Balestra et al., 2024; Balestra et al., 2023; Valentić et al., 2022), generally in show caves and nearby environments; to our knowledge anthropogenic materials (5 mm - 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 MMC 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 can be done only with part of the data reported in Hasenmueller et al. (2023) and Baraza and Hasenmueller (2023), which analysed 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 \pm 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 MMC and only 1% was plastic, while in sediments, 31% of particle was MMC 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 our 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 than 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.

4.2. Microfibre typology and possible sources

Except a pair of probable ramie fibres, all natural fibres analysed 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 MMC MFs comprised Cupro/Bemberg, Tencel/Lyocel and cellulose acetate MFs, commonly used in textile production, methyl cellulose and hydroxypropyl 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,

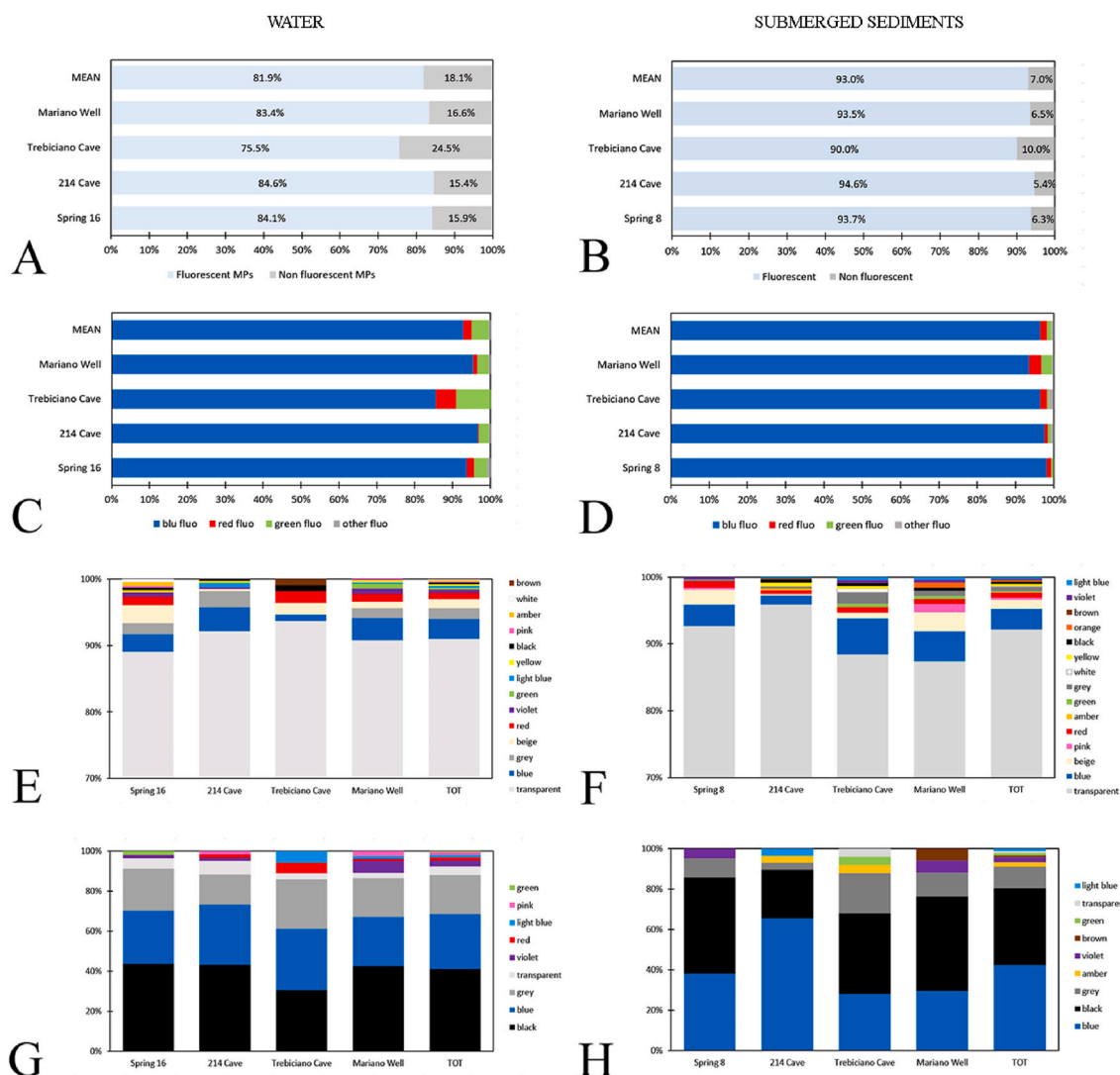


Fig. 4. Fluorescence and colours of analysed microfibres. A: Percentages of fluorescent microfibres in waters; B: Percentages of fluorescent microfibres in submerged sediments; C: Fluorescence colour of microfibres in waters; D: Fluorescence colour of microfibres in submerged sediments; E: Colours of 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. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

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 foodstuff; 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 our 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 (Saravanja 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 point, 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

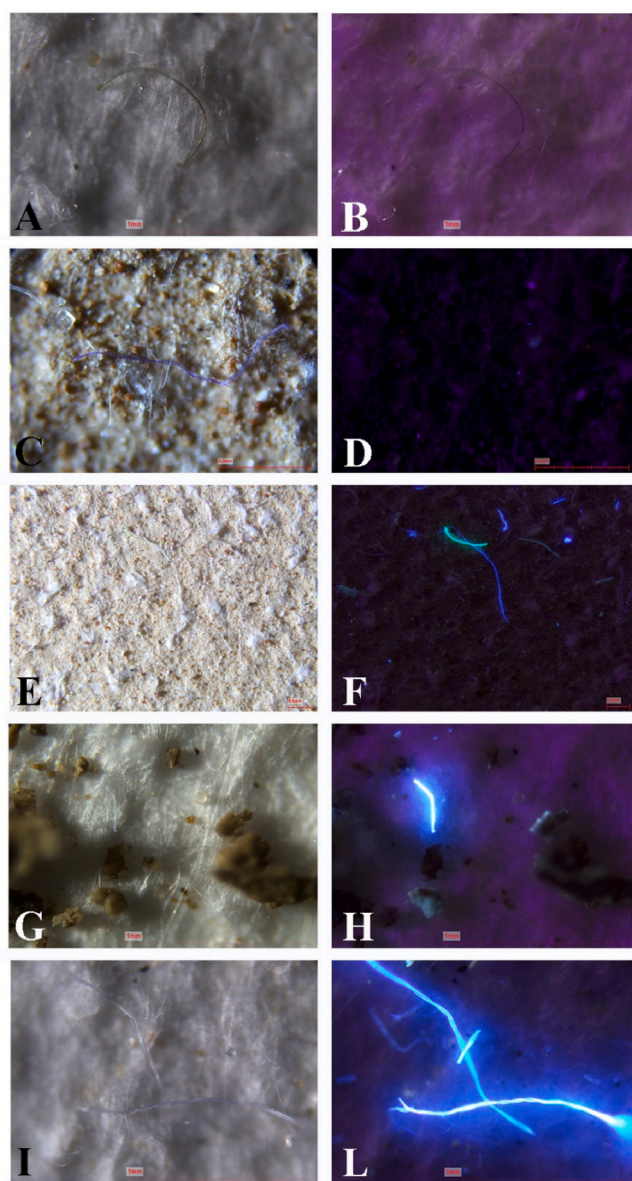


Fig. 5. Images of microfibres of anthropogenic origin found in the Italian Classical Karst aquatic environments under microscope, with and without UV light. A, B: green synthetic fibre without fluorescence; C, D: violet cellulosic (cotton) fibre without fluorescence; E, F: yellow synthetic fibre with 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). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

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., 2019). 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 contribute 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.

4.3. Microfibre fluorescence and colour

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., 2021; Remy et al., 2015; Zhang et al., 2023), 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.

4.4. Methodological considerations

Research on MFs is challenging, and the analysis of natural and re-generated 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).

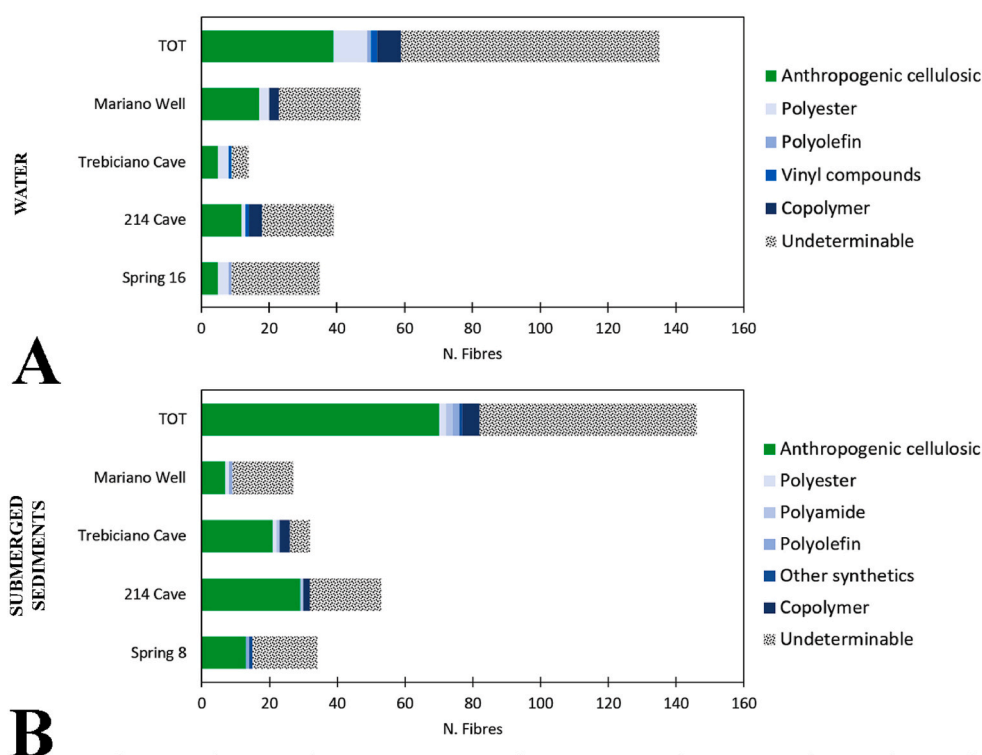


Fig. 6. Microfibre typologies. Green for natural and regenerated microfibrs (e.g. cotton), blue shades for synthetic microfibrs, texture for undeterminable microfibrs (degraded microfibrs and microfibrs with FTIR library spectrum match <75%). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

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 both 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).

The specific density of different cellulosic material is higher than the one of several synthetic polymers, such as polyester, polypropylene, and nylon/acrylics. Our density separation method for sediment samples could limit our ability to capture anthropogenic materials with densities >1.2 kg/L, however, the densities of newly produced materials are not necessarily the same as those in natural environments. Due to enhanced porosity, degradation, biofouling, organic matter adsorption, or degradation, their density can increase or decrease in natural environments (Kaiser et al., 2017). In fact, we observed different materials with reported densities >1.2 kg/L, in accordance with observations from surface water systems (Horton et al., 2017). However, it should be taken into account that some materials have higher density, therefore can remain into the sediments.

Spectroscopic analysis of natural and MMC MFs can be more challenging compared to MPs. Spectroscopic analysis 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, it is not always possible efficiently analyze microparticles collected in natural environments:

surfaces of these materials are often covered by impurities and micro-organisms, oxidated and/or contaminated by other pollutants, making spectra inaccurate and difficult to match with reference libraries (Song et al., 2015). Natural polymers have lower signal intensities compared to synthetic ones, and are more prone to dye interference. Natural and MMC 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. Library misestimations could be problematic, consequently, only high-quality match (>75%) of spectra was employed in this research. The high percentage of undeterminable MFs could be linked to the above-mentioned problems.

Visual analysis by microscopy is widely used for MPs, however, it is a hardworking method, and cannot provide information on the chemical composition of materials (Huang et al., 2023). Moreover, there is a limit of analysis on too small pollutants (European Commission, 2013; Hidalgo-Ruz et al., 2012; Song et al., 2015). However, it allows to better see MF surface morphology and some important characteristics such as color (Fig. 5). A preliminary screening step under microscope can be useful to distinguish between synthetic materials and natural and regenerated ones (Fig. 5). A combination of microscopy and spectroscopy methods is probably the optimal choice to identify MFs in natural matrices.

5. Conclusion

Most studies on microfibre pollution in natural environment focused on synthetic fibres only, neglecting a major component of anthropogenic microfibre pollution: cellulosic fibres. Our results improve knowledge on microfibre pollution in karst systems, showing the co-occurrence of natural, regenerated and synthetic fibres. Microfibrs are frequent in karst systems, from caves to springs, and are distributed in both water

and sediment reservoirs, with potential impacts on habitat, species and water quality. Microfibre abundances are particularly high in submerged sediments, suggesting a major role of sediments for the storage of microparticle pollution in subterranean and surface environments.

Microfibres monitoring is fundamental to understand the health status of the karst environment, and possible threats. Including natural and regenerated microfibres in future studies will help to understand the sources and effects of microfibres in karst systems. More restrictive management guidelines, taking into account the ecological connections between surface and subterranean karst environments, are essential for the conservation of these ecosystems, especially in karst areas nearby important connecting and transport routes. 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). Implementing analyses on a larger number of habitats and karst areas will help to understand the extend of the problem. We also encourage political and economic changes toward a sustainable production and life cycle of fibrous materials, and environmental education at all levels that can make people and future generations aware of more sustainable behaviors.

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CRediT authorship contribution statement

Valentina Balestra: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Matteo Galbiati:** Investigation. **Stefano Lapadula:** Investigation. **Benedetta Barzaghi:** Resources, Investigation. **Raoul Manenti:** Writing – review & editing, Resources, Investigation, Funding acquisition. **Gentile Francesco Ficetola:** Writing – review & editing, Resources, Funding acquisition. **Rossana Bellopede:** Writing – review & editing, Validation, Resources, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.chemosphere.2024.142811>.

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