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Research article

Microplastics in caves: A new threat in the most famous geo-heritage in the world. Analysis and comparison of Italian show caves deposits

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ABSTRACT

Microplastic pollution represent a worldwide concern, however, in karst areas is still largely unknown, especially in underground environments. Caves are the most important geological heritage worldwide, rich in speleothems, unique ecosystems custodians of important drinking water reserves, and a significant economic resource. Thank to their relatively stable environmental conditions, they can preserve information for a long time such as paleontological/archaeological remains, however, these characteristics make caves vulnerable environments too, easily damaged by climate variations and pollution. To increase the current knowledge of microplastic pollution, the deposits of different Italian show caves were investigated, improving the method for microplastic separation. Microplastic were identified and characterised using MUPL automated software, observed with and without UV light under a microscope, and verified under μFTIR-ATR, highlighting the importance of combine different methods. Microplastics were present in sediments of all examined caves, and were always greater along the tourist route (an average of 4300 items/kg) than the speleological areas (an average of 2570 items/kg). Microplastics less than 1 mm dominated the samples and the amount increased with the decrease in the size considered. Fibre-shaped dominated the samples and 74% particles was fluorescent under UV light. Analysed sediment samples contained especially polyesters and polyolefins. Our results highlight the presence of microplastic pollution in show caves, giving useful information to assess risks posed by microplastics in show caves and emphasizing the importance of pollutants monitoring in underground environments to define strategies for the conservation and management of caves and natural resources.

1. Introduction

In scientific literature microplastics (MPs) are generally defined as polymers with a dimension between 5 mm and 1 μm, even if there is no general consensus on the value of their maximum size and the ISO/TR 21960 define MPs as particles smaller than 1 mm (International Organization for Standardization and European Committee for Standardization, 2020). Microplastics are directly produced with a small dimension (primary production) or originated from biological, chemical or physical activities on bigger plastics (secondary production). Microplastics pollution represents an ecological emergence and is a worldwide concern: they can be consumed or assimilated by organisms (Assas et al., 2020; Devereux et al., 2021; Romeo et al., 2015), are easily to transport (Allen et al., 2019; Liu et al., 2019), and can be sources and vectors for other pollutants, such as chemicals (Rochman et al., 2013), pesticides (Wanner, 2021), persistent organic pollutants (POPs) (Koelmans et al., 2013), heavy metal (Li et al., 2019; Selvam et al., 2021), or antibiotics (Li et al., 2018). Microplastic have been recorded in different environments, especially marine ones (e.g. De Lucia et al., 2018; Liu et al., 2022; Phuong et al., 2018; Tsang et al., 2017). Recently, terrestrial environments have been monitored more (e.g. Bertoldi et al., 2021; Boyle and Örmeci, 2020; Wong et al., 2020), witnessing the presence of MPs even in remote areas (e.g. Ambrosini et al., 2019; Neelavannan et al., 2022; Zhang et al., 2021). However, MP pollution in several environments is little investigated, such as underground environment (e.g. Balestra and Bellopede, 2022; Balestra et al., 2023; Panno et al., 2019; Romano et al., 2023; Valentić et al., 2022). The potential MP contamination for subterranean environments is often only mentioned or limited to

Abbreviations: MP, Microplastic; MPs, Microplastics; DIATI, Department of Environment, Land and Infrastructure Engineering; ARPA, Agenzia Regionale per la Protezione Ambientale; INRIM, Istituto Nazionale di Ricerca Metrologica; FWAs, Fluorescent Whitening Agents; OMR, organic matter removal; FTIR, Fourier transform infrared spectroscopy.

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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 (Balestra et al., 2023).

Karst areas are characterised by carbonate rocks, representing the major Earth's cave systems, even if karst phenomena occur also in gypsum, halite and sometimes in quartzites (Wray and Sauro, 2017). 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, 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, 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, 2022; Chelius et al., 2009; Christman, 2019), creating favourable environments for lampenflora growth and bacteria activity (Bellopede et al., 2022; 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. Previously analysis of cave lint gave a synthetic fibre content between 30 and 75% (Christman, 2019; Jablonsky et al., 1993), while it was 85% from the MPs analysis in the cave sediments (Balestra and Bellopede, 2022).

Several methods have been developed to analyse and quantify MPs in natural environments, however, there is no established standard. Many existing methods require the use of specific and expensive instruments and specialised operators. However, monitoring concentrations of MPs in the environment is necessary to measure the relevance of this problem, the source and transport of pollutants and the impacts they could have on species, ecosystems and human health (Henry and Klepp, 2018; Prata et al., 2019). Assessing MP contamination in caves is crucial: 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 1200 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).

The aims of this study are: 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 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, we want 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?

2. 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, 2022) (Fig. 1), 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 1.

2.1. Toirano caves

Toirano caves (Fig. 1) are located in the Toirano municipality, Liguria, Italy, and develop in the Dolomie di San Pietro dei Monti formation. They are characterised 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, a 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).

2.2. Borgio Verezzi cave

Borgio Verezzi cave (Grotta di Valdemino) (Fig. 1) 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 33,000 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

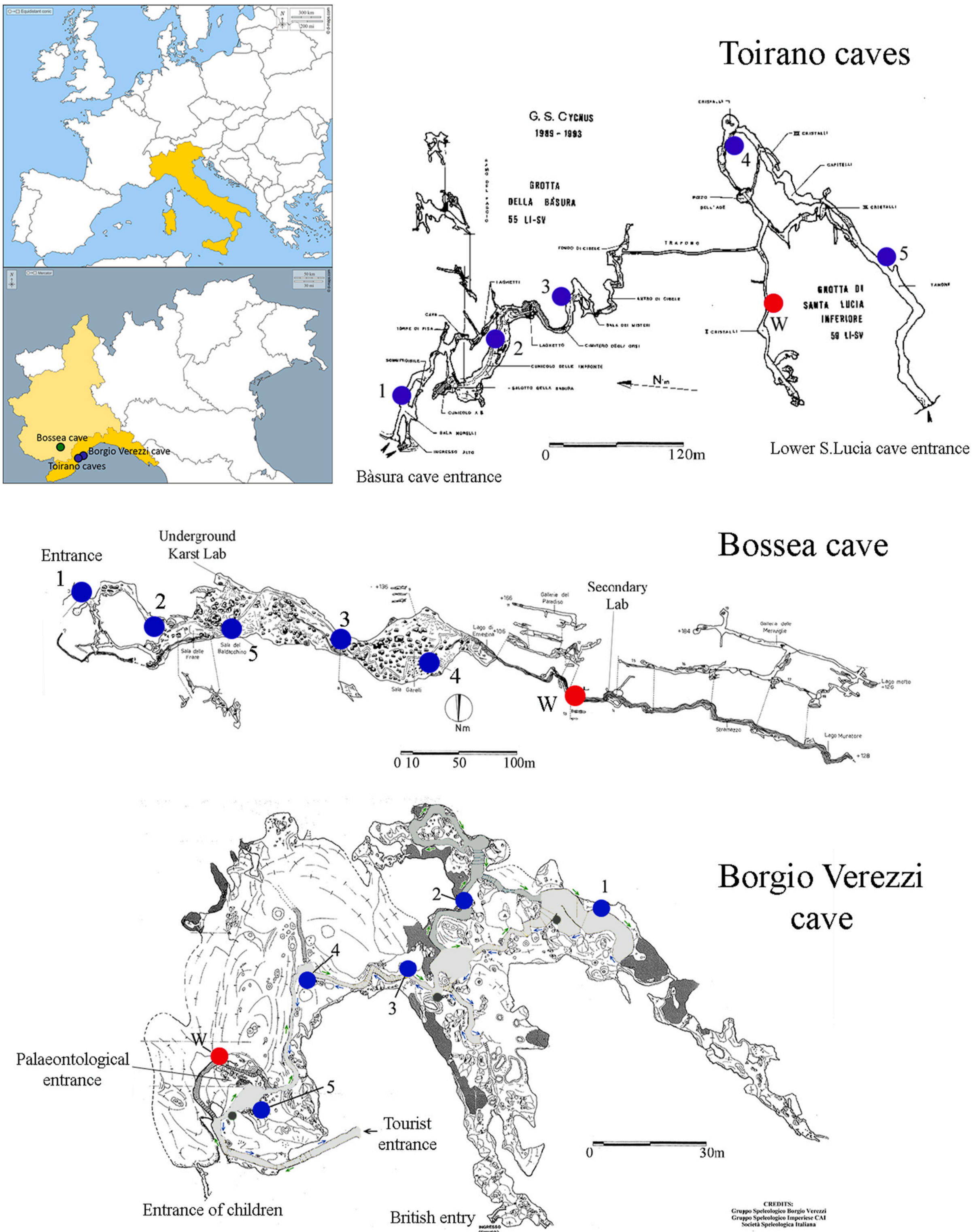

Fig. 1. Location of the examined show caves and sampling areas. Borgio Verezzi and Toirano caves are located in Liguria and Bossea cave in Piedmont, NW Italy. (Maps used for the plate and modified, retrieved from: https://d-maps.com/carte.php?num_car=2254&lang=it and https://d-maps.com/carte.php?num_car=24424&lang=it). Show caves sampling areas: 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. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
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 known 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)	40,000	33,000	18,000
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
Data collection	2021	2021	2020 (Balestra and Bellopede, 2022)

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).

2.3. Bossea cave

Bossea cave (Grotta di Bossea) (Fig. 1) 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 18,000 tourist/year. The cavity is crossed for about 1.5 km by a subterranean collector (Mora River), with a 50–1200 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. 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 first research on MP pollution in show cave deposits and water were realized in this cave (Balestra and Bellopede, 2022; Balestra et al., 2023), paving the way for future and fundamental research aiming karst systems conservation.

3. Materials and method

3.1. Field sampling and data collection

Field sampling method and the number of sampling areas depend on the monitored environment. Sediments containing MPs not easily identifiable visually or with low MP abundance can be collected using bulk sample (Hidalgo-Ruz et al., 2012). In all examined show caves, six sampling areas of 1 × 1 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. 1). For each sampling area, a minimum of 150 g of superficial sediments (upper 5 cm) were collected with a metal spoon, placed in pre-cleaned glass boxes, and stored in the fridge at 6 °C until laboratory analysis. Sediments samples were collected in 2020 in Bossea cave (Balestra and Bellopede, 2022) and in 2021 in Borgio Verezzi and Toirano caves. 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 a monitoring over time could be crucial.

Although low volumes of samples could be not representative, overestimating or underestimating the abundance of pollutants, in extreme environments it is not always possible to collect large quantities of material for analysis, and sensitive areas must be damaged as little as possible. In cave environment, concreting of cave deposits and/or materials movement due to water flows can limit the volume of sediment available per site. However, monitoring pollution in these particular environments is fundamental for their conservation.

3.2. Laboratory analysis

Plastic equipment was avoided, using glass and metal materials only. During all steps, nitrile gloves and cotton coats were used by researches as well as all working surfaces and laboratory materials were cleaned with ethanol and milliQ water to avoid MP contamination. Sediment samples of Bossea cave were previously analysed using the method described in Balestra and Bellopede (2022); samples collected in Borgio Verezzi and Toirano caves were analysed according to this method, improved and tested for these sediments, rich in organic matter. A comparison between the different steps used is shown in Table 2. In relation to the remaining quantity of dry sediments, trying to use as much sediment as possible, three sub-samples of 10–20 g were selected for each sample via coning and quartering for analysis (Table 2). Major variations in the methodology concern the organic matter removal (OMR) step: Toirano caves samples were treated through the application of 0.5 ml of 30% H_2O_2 solution during post-treatment on filters, Borgio Verezzi cave sediments were pre-treated through the application of 1:1 30% H_2O_2 solution (Table 2).

Blank controls with 100 ml milliQ water, H_2O_2 and NaCl solutions were done to determine possible contamination during laboratory analysis. The blank samples did not contain MPs.

3.3. Microplastic identification and characterisation

Microplastics containing Fluorescent Whitening Agents (FWAs) can be easily detected under an ultraviolet (UV) light (e.g. Balestra et al., 2023; Ehlers et al., 2020; Klein and Fischer, 2019; Qiu et al., 2015). However, a lot of inorganic and organic materials are fluorescent under a UV light and not all plastics contain these additives (Balestra and Bellopede, 2022), therefore, an initial screening analysis was conducted to observe primary MP characteristics.

Comparisons between spectroscopic and microscopic techniques highlighted overestimation or underestimation of MPs (e.g. Hidalgo-Ruz et al., 2012; Song et al., 2015), therefore, a combination of several methods is probably the optimal choice to identify MP particles, as

Table 2
Comparison between the different steps for sampling preparation and analysis.

Step	Bossea cave method (Balestra and Bellopede, 2022)	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 microfiber filter (Whatman, Ø 47 mm)	1.2-μm pore size glass microfiber filter (Whatman, Ø 47 mm)	1.2-μm pore size glass microfiber 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	–

suggested in Song et al. (2015). Moreover, the identification of MPs using spectroscopy is time-consuming and particles surface of samples of natural environments are often contaminated, therefore, the spectra of MPs are difficult to match with high percentages with spectra libraries (Song et al., 2015). However, this analysis is useful to confirm microscopic analysis, and the polymer composition can help to understand the likely sources of pollution. In this work, MPs were detected by means of automated counting software, visual identification under microscope with and without UV light, and spectroscopic techniques.

Particles on filters of Bossea cave were observed with and without a UV flashlight (Alonefire SV10 365 nm UV flashlight 5 W) under a Leitz ORTHOLUX II POL-MK microscope equipped with a DeltaPix Invenio 12EIII 12 Mpx Camera, with 2.5×, 4×, 10× or higher magnifications (Balestra and Bellopede, 2022). The accuracy in visually identifying very small particles is less reliable than with larger ones (Hidalgo-Ruz et al., 2012; Song et al., 2015), therefore, MPs were analysed up to 0.1 mm, as suggested in European Commission (2013). 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).

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. The MUPL setting parameters used are reported in Supplementary Material 1 (Tables 1 and 2). 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 and 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, a portion of MPs found in the Toirano and Borgio Verezzi caves was 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. Generally, an average from 1% to 10% particles of the total is analysed to determine the MPs chemical composition (International Organization for Standardization and European Committee for Standardization, 2020). Randomly 12% of the total MPs of Borgio Verezzi and Toirano caves were analysed. 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. Samples were measured in a spectral range of 4000 to 700 cm^{-1} with 45 scansions. Atmosphere corrections were made on obtained spectra. Spectra were compared with the Shimadzu Lab Solution Library ATR Polymer 2, followed by a visual analysis comparison of characteristic bands in the reference spectrum, and accepted only with a match degree ≥80%, as suggested in Fossi et al. (2017).

4. Results and discussion

4.1. 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.

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 (Table 2). The use of 30% H_2O_2 solution is not recommended as post-treatment on filter (Table 2), because it makes the surface of the filter less uniform and bubble, making more difficult the characterisation 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 characterisation 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. Using the OMR as pre-treatment (Table 2), pouring a volume of 1:1 30% of hydrogen peroxide solution directly on sediments, 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. As it is possible to observe in the Supplementary Material 1(Tables 1 and 2), 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 characterisation too. In addition, filters with a cleaner background were better also for visual identification under microscope.

4.2. Investigation and morphological characterisation of microplastics

4.2.1. Microplastic abundance and size

The use of MUPL software (Giardino et al., 2022, 2023) allowed us to detect fluorescent MP particles between 5.0 and 0.4 mm, their size and shape. According to Crawford and Quinn (2016), MPs are categorized 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 (Figs. 2 and 3).

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 (Figs. 2A and 3A). Figs. 2C and 3C 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 (Figs. 2D and 3A). Figs. 2F and 3C 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. The comparing between the average MP abundance values between 5 and 0.4 mm, their size and shape in the three examined show caves are shown in Fig. 3. 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. 3A). 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. 3A). 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 MP/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. 3A).

Fig. 3C 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<1 mm accounted for more than 70% in sediments of all caves (Fig. 3C), highlighting MP amount increase with decreasing in considered size.

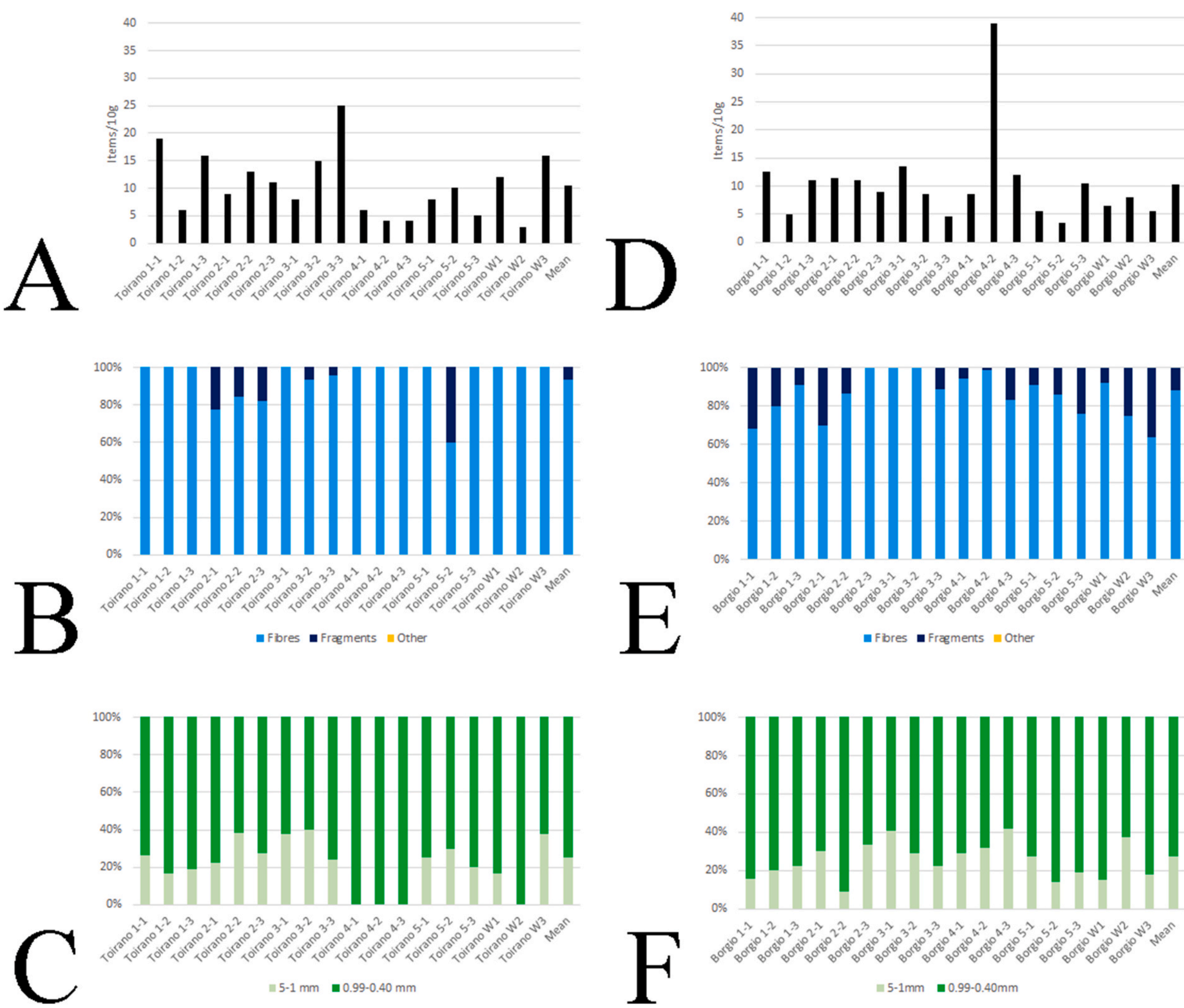

Fig. 2. Toirano and Borgio Verezzi caves samples abundance, shape and size percentages detected by MUPL software (fluorescent particles from 5 to 0.4 mm). Toirano caves samples abundance (A), shape (B) and size (C) percentages. Borgio Verezzi cave samples abundance (D), shape (E) and size (F) percentages.

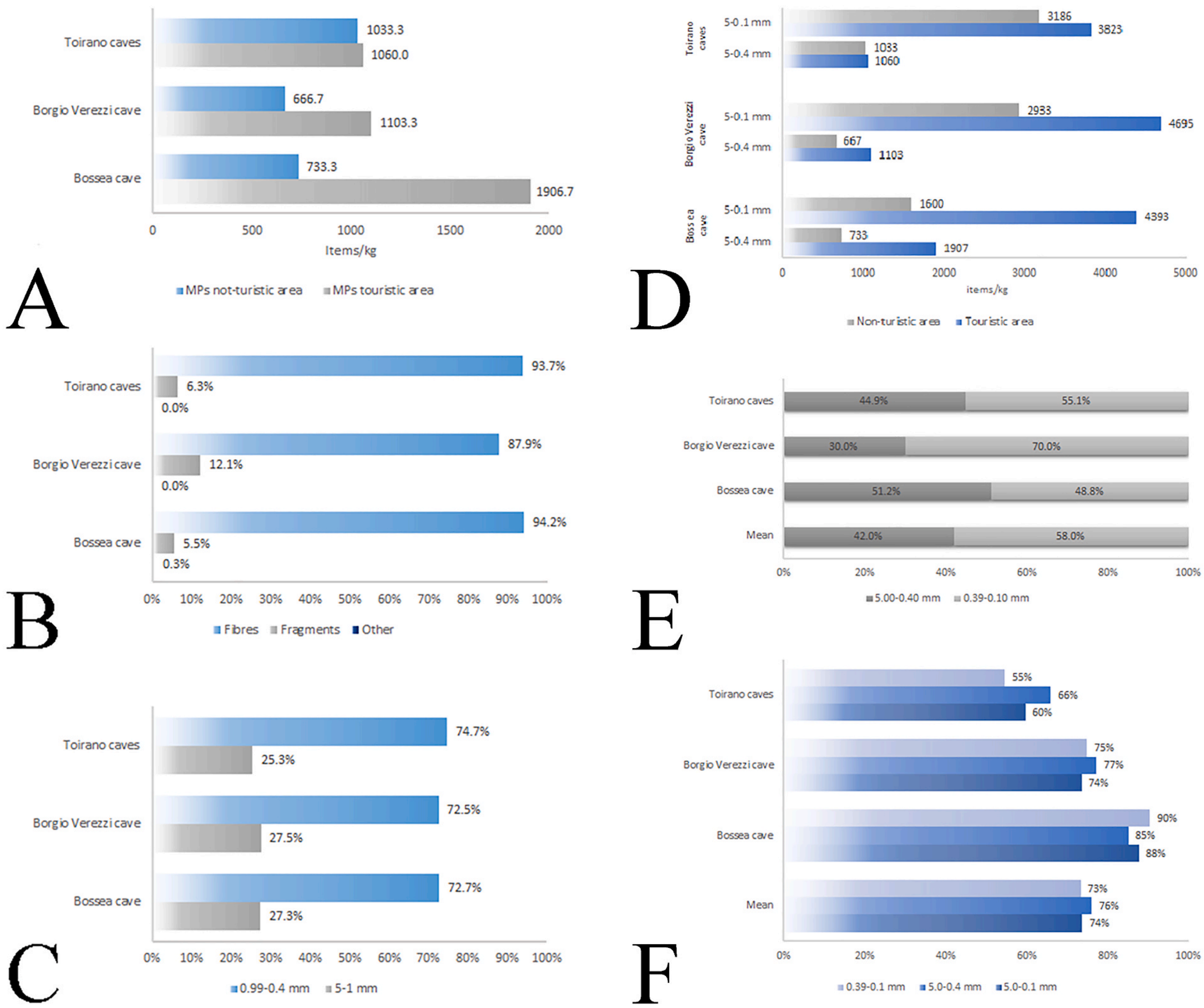

Fig. 3. Comparison between the microplastic abundance values and their morphological characteristics average percentages of the three examined show caves. A: Microplastic abundance (5–0.4 mm fluorescent particles only); B: Microplastic shape (5–0.4 mm fluorescent particles only); C: Microplastic size (5–0.4 mm fluorescent particles only); D: Microplastic abundance considering different size range; E: Microplastic size (visual counting under microscope for 5–0.1 mm particles); F: Fluorescent microplastics percentages considering different size ranges (visual counting under microscope for 5–0.1 mm particles).

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, 2022). 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. 3D, E, F.

Microplastic abundance between 5-0.4 mm and 5–0.1 mm in the three examined show caves are shown in Fig. 3D, highlighting the MP amount increase with decreasing in considered size, as shown in Fig. 3C. 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. 3D). 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. 3D). 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 a 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 are 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, comparisons with other show caves are 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 60,000 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.

Fig. 3E 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 characterize 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. Future research will be carried out to understand the sources of MPs pollution in these caves and to monitor smaller particles.

4.2.2. *Microplastic shape*

In Toirano caves fibre shape dominated all cave sediments samples (93.7%), while a 6.3% of particles were fragments (Figs. 2B and 3B). In Borgio Verezzi cave fibre shape dominated all cave sediment samples (87.9%), while a 12.1% of particles were fragments (Figs. 2E and 3B). Other shapes were not be detected by the MUPL software in both caves.

Comparing the data of the three examined caves (Fig. 3B), 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 characterisation. 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.

4.2.3. *Microplastic fluorescence*

Fig. 3F underline 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 account for each cave (Fig. 3F): about 60% in Toirano caves, 74% in Borgio Verezzi cave, and 88% in Bossea cave, with a percentage mean value of about 74%.

4.2.4. *Characterisation of microplastic by μFTIR-ATR*

In Borgio Verezzi cave sediments were found PA (polyamide), polyester, PE (polyethylene), PET (polyethylene terephthalate), PVAc (polyvinyl acetate), PVFM (polyvinyl formal), PAM (polyacrylamide), EVOH (ethylene vinyl alcohol) and copolymer (Fig. 4). Sediment samples of Toirano caves contained polyester, PET, PAM, PP (polypropylene), EVA (ethylene vinyl acetate) and copolymer (Fig. 4). Most of the analysed particles were identified by the FTIR library as plastic materials, however, it was established to validate only spectra with match $\geq$80% as suggested by Fossi et al. (2017). The most common identified particles are polyesters such as PET and polyolefins such as PE and PP. Some μFTIR-ATR spectra were shown in Supplementary Material 2.

The 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.

At the moment, comparisons with other show caves are 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

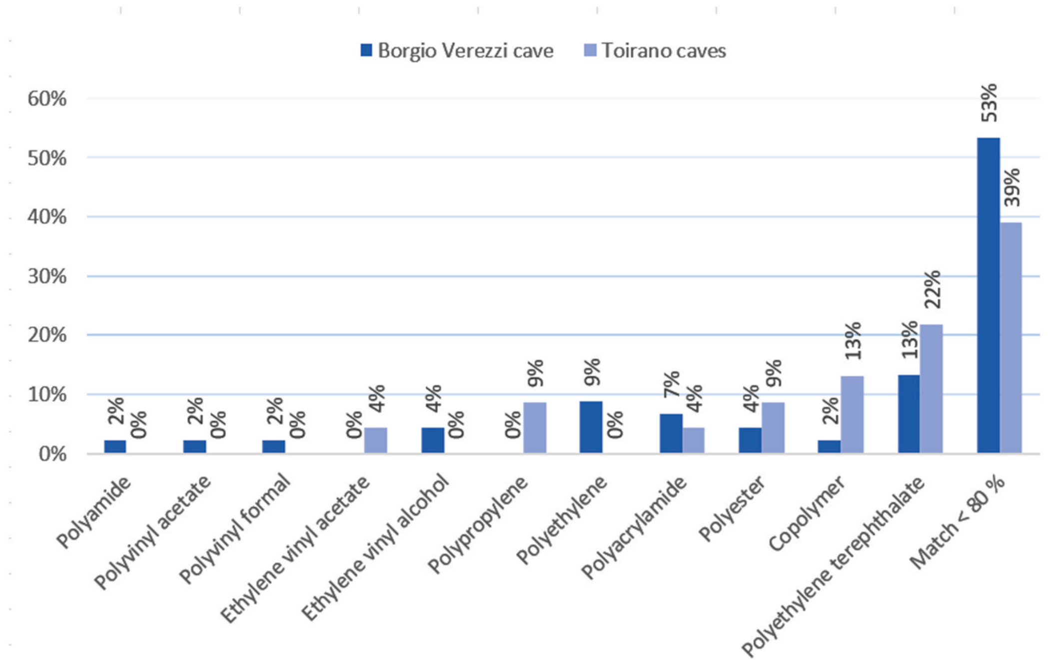

Fig. 4. Plastic typology found on 12% of the total microplastics of Borgio Verezzi and Toirano caves.

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).

5. Conclusion

This study documented the presence of microplastics in sediments of all examined show caves, filling a gap in the study of microplastic pollution. Improving organic matter removal technique to separate MPs in cave sediments helped to get better filter for particles identification and characterisation. Visual identification under microscope and MUPL automated software were used to obtain as much information as possible and compare data, underlining the importance of combine 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 not-fluorescent MPs and detect smaller particles, which significantly increase as the size decreases. Microplastics were present in touristic and non-touristic areas of all caves, with higher amount along the tourist paths. Fibre-shaped, microplastics less than 1 mm, and polyesters and polyolefins dominated the samples, advising that synthetic clothes are the main source of microplastic 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. Microplastic monitoring is crucial in karst environments to establish the current degree of pollution and define strategies for the protection and management of this geological heritages and their resources, even providing education and implementing new strategies for a sustainable development.

Credit author statement

Valentina Balestra: term, conceptualisation, methodology, software, validation, formal analysis, investigation, resources, data curation, writing – original draft, writing – review and editing, visualisation. Rossana Bellopede: conceptualisation, methodology, resources, writing – review and editing, visualisation, project administration, funding acquisition.

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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.jenvman.2023.118189.

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