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1 **Preliminary investigations of microplastic pollution in karst systems, from surface watercourses to cave**
2 **waters**

3

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10

11 **Abstract**

12 Microplastic pollution in different environments has increasingly been documented in detail in recent

13 times, but it is still poorly studied in caves and karst aquifers. To deepen the knowledge of microplastic

14 pollution, for karst environment protection and conservation purposes, we collected and investigated

15 different water samples from a karst area of Italy, considering connected surface and cave waters.

16 Microplastics were extracted from water samples by filtration and subjected to organic matter removal

17 with 15% hydrogen peroxide solution. Microplastics on filters were counted and characterised (size, colour,

18 shape) via visual identification under a microscope, with and without UV light, exploiting fluorescence given

19 by fluorescent whitening additives (FWAs) contained in plastic materials. Finally, spectroscopic analyses

20 were carried out on 10% of the microplastics observed on each filter. The concentration of microplastics in

21 cave waters varied from 12 to 54 items/L, with a mean value of 28 items/L. In the surface water of a

22 tributary, it was of 23 items/L, and in the downstream, it was 29 items/L. Fibres represented the most

23 abundant shape (95.1%) in the karst system waters, and most microplastics (82.9%) were smaller than 1

24 mm. The majority of the microplastics were fluorescent under UV light (84.8%), and most fluorescent

25 particles were transparent (46%). However, black microplastics (68%) were more common among the non-

26 fluorescent ones. Polyethylene (51.7%) was the main type of microplastics found in the karst system

27 waters. Our results show the presence of microplastics in karst systems and provide useful information for
28 future research. Karst aquifers are open systems, subjected to possible contamination by surface
29 pollutants. Microplastics in karst systems can be consumed by animals, damage ecosystems and
30 contaminate water resources; surface karst areas and underground environments should therefore be
31 monitored and protected, especially regarding the management of water resources. To further understand
32 the sources and transport of microplastics within a karst system, analyses on a greater range of surface and
33 subterranean waters throughout the world are required.

34

35 **Keywords:** microplastics, show caves, aquifers, water pollution, karst areas, groundwaters

36

37 **1. Introduction**

38 Microplastics (MPs) are defined as plastic particles smaller than 5 mm and can be produced intentionally
39 (primary MPs), mainly for cosmetics or body care products, or via the degradation and/or fragmentation of
40 plastic products due to physical, chemical and biological processes (Corami et al., 2020; Henry and Klepp,
41 2018; Prata et al., 2019). Microplastic contamination has been found in marine and terrestrial
42 environments worldwide (e.g. Boyle and Örmeci, 2020; Sharma and Chatterjee, 2017; Wong et al., 2020)
43 from populated areas (e.g. Jahan et al., 2019; Zhou et al., 2019) to remote ones (e.g. Ambrosini et al., 2019;
44 Cabrera et al., 2020; Cincinelli et al., 2017; Van Cauwenberghe et al., 2013; Zhang et al., 2021) and can be
45 extremely mobile. Microplastics are consumed by several organisms (Assas et al., 2020; Devereux et al.,
46 2021; Henry and Klepp, 2018; Romeo et al., 2015; Wright et al., 2013), resulting in direct or indirect physical
47 harm, and can be sources and vectors for other contaminants, such as persistent organic pollutants (POPs),
48 heavy metals, pesticides and antibiotics (Li et al., 2018; Li et al., 2019; Rochman et al., 2013; Selvam et al.,
49 2021; Zhou et al., 2019). Assessing MP presence and distribution in natural environments, using accurate
50 methods for quantification, understanding their transport pathways and evaluating their impacts on
51 organisms and habitats are necessary steps to be addressed for conservation purposes.

52 However, research on MPs is generally focused on the marine environment and surface ecosystems, and
53 only few studies have examined MP pollution in caves and underground systems (e.g. Balestra and
54 Bellopede, 2022b; Christman, 2019; Vardar and Vidal Rodriguez, 2021). Regarding freshwater resources,
55 research is in its early stages. The potential contamination of groundwater is often only mentioned, and a
56 limited number of studies focused on groundwater MP pollution (e.g. Khant and Kim, 2022; Mintenig et al.,
57 2019; Panno et al., 2019; Samandra et al., 2022; Selvam et al., 2021; Viaroli et al., 2022). Moreover, MP
58 studies suffer from methodological discrepancies, providing heterogeneous analysis results, and the
59 invisibility of the subterranean karst system structure makes impedes the study of such systems, even if
60 they preserve a precious asset such as groundwater.

61 Sources of MPs to groundwater include wastewater, litter, atmospheric deposition and soil and surface
62 waters pollution. Atmospheric transport favours the distribution of MPs over long distances in relation to
63 wind direction and intensity, rain/snow precipitation intensity and duration (Allen et al., 2019). In the
64 hydrogeological cycle, atmospheric water contribution is the main aquifer recharge. The presence of MPs in
65 precipitation was detected in cities and remote areas (Allen et al., 2019; Liu et al., 2019), highlighting the
66 omnipresence of this contaminant. Therefore, MP contamination in soil is strongly related to precipitation,
67 facilitating MP infiltration (Zhou et al., 2021). When MPs are deposited on the ground, some can be
68 transported vertically into the subsoil, travelling over long distances throughout the rock fractures and
69 accumulating in the groundwater system (Chia et al., 2021; Wanner, 2021). Previous studies highlighted the
70 downward movement of microparticles through soil pores and cracks for smaller MPs (Fahrenfeld et al.,
71 2019; McGechan, 2002; Viaroli et al., 2022), and vertical and horizontal migration in soil matrices could be
72 also be related to bioturbation (Lwanga et al., 2017). The hyporheic zone, the zone between surface and
73 groundwater, is an important accumulation zone for MPs of small dimensions (Frei et al., 2019). The
74 hydrogeological setting of the basin and seasonal trends must be taken into account, as well as the flow
75 regime of streams and riverbeds. Therefore, hydrogeological information and aquifer types are decisive in
76 choosing the sampling approach and detection method.

77 Karst aquifers are groundwater environments usually made up of carbonate rock, constituting about
78 25% of the global drinking water sources (Panno et al., 2019). The hydrodynamic regime of these aquifers is
79 affected by the geology and the rock cluster fracturing, the karstification degree and the local
80 meteorological conditions (Braun, 1984; Chauve et al., 1990; Hottelet et al., 1993; Moindrot et al., 1988).
81 Considering the drainage system, three basic conceptual aquifer models have been described: systems with
82 dominant drainage (absent or reduced phreatic zone, high permeability), systems with interconnected
83 drainage (extensive phreatic zone, high permeability) and systems with dispersive circulation (highly
84 extensive phreatic zone, middle–low permeability) (Balestra et al., 2022a; Ford and Williams, 2013; Ford
85 and Williams, 1989; Vigna, 2007; Vigna and Banzato, 2015; White, 1969). Various intermediate situations
86 exist, which can be described using these models considering hydro-chemical data and spring hydrography
87 (Banzato et al., 2011). Besides being important drinking water resources, karst ecosystems and waters are
88 particular habitats for troglobitic and endemic species (e.g. Balestra et al., 2022b; Barzaghi et al., 2021),
89 which may be vulnerable to pollution (Manenti et al., 2018; Sket, 1999), and karst caves have an
90 exceptional scientific value and are a precious archive that must be protected from damage, allowing
91 paleo-environmental and paleo-climatic reconstructions and preserving palaeontological and
92 archaeological finds (Balestra et al., 2021; Cigna and Forti, 2013). The open nature of karst aquifers makes
93 them vulnerable to contamination by surface pollutants, which can be transported through the rock
94 fractures (White, 1988) or can directly access the karst systems through some caves, especially tourist ones,
95 increasing the pollution risk (Balestra and Bellopede, 2022b; Balestra et al., 2021). Fractured and karst
96 aquifers are subjected to greater pollution risk as MPs are prone to circulate throughout the discontinuities,
97 even if mechanical dispersion may attenuate the contaminant concentration in some zones (Viaroli et al.,
98 2022).

99 Currently, to the authors' knowledge, there is only one study on MP pollution in waters of karst areas,
100 monitoring springs and wells under low-flow conditions (Panno et al., 2019). Studies on cave waters, as well
101 as MP pollution in surface and underground waters of the same karst area, are lacking. The vulnerability of
102 groundwater resources to pollution, anthropogenic pressure and climate change, as well as their important

103 role in natural ecosystems and as a fundamental drinking water reserve, require actions targeted at
104 underground environment protection and sustainable resources management (Balestra et al., 2021; Re,
105 2019). Therefore, investigations are required for understanding the system dynamics and monitoring the
106 state of the environmental health, detecting the possible pollution and its sources. The aims of this study,
107 which is the first of its kind, are therefore i) to preliminarily investigate MP pollution in karst systems, from
108 surface watercourses to cave waters, to provide a reference for further research, and ii) to discuss the
109 abundance, morphological characteristic and types of MPs in the karst system.

110

111 **2. Materials & Method**

112 *2.1. Study area*

113 The Bossea karst system feeding area (Piedmont, NW Italy) (Fig. 1) is characterised by a belt of
114 carbonate rocks laterally confined by poorly permeable rocks (quartzites and meta-volcanics) (Fig. 2).
115 Bossea Cave (Frabosa Soprana (CN), Piedmont, Italy) represents the final part of the karst system
116 developing in the Maudagna-Corsaglia watershed, between the Prato Nevoso basin and the Corsaglia River
117 (Fig. 2). The land cover is mostly composed of broadleaf forests of larches, alternated with mountain
118 meadows and pastures, although in a part of it, there are several ski slopes which attract many tourists in
119 winter (Fig. 2A).

120 The recharge of this aquifer (Fig. 2B) is mainly linked to primary and/or secondary inputs: the primary
121 inputs (or authigenic) are by rain or snow melt, directly recharging the aquifer, whereas the secondary
122 inputs (or allogenic) of concern are the surface runoff waters continuously recharging the aquifer through
123 sinkholes in the sub-riverbed (Vigna, 2020). Rio Roccia bianca is the main secondary supply contributing to
124 the Bossea karst system recharge (Fig. 2). A series of tests were previously carried out in the two main
125 absorbent valleys (Rio Roccia bianca and Rio Bertino) (Vigna, 2020), using two different dyes (Tinopal and
126 fluorescein), to examine their arrivals in Bossea Cave. This aquifer has a water circulation along karst
127 conduits and fractures, with an extensive and well-developed saturated zone, and can therefore be

128 described as a system with interconnected drainage, with a medium-high permeability (Banzato et al.,
129 2011).

130 Bossea Cave is the first show cave of Italy, open to the public since 1874. The cavity has a single
131 entrance at 836 m a.s.l. and an ascending structure (Fig. 3), with a 2,800-m development in the tectonic
132 contact between the Middle Triassic Dolomie di San Pietro dei Monti formation (dolostone and carbonate
133 rocks) and Permotriassic Metavolcanics (Antonellini et al., 2019). Different underground karst laboratories
134 to study and monitor hydrogeology, subterranean meteorology, radon activity and subterranean biology
135 are located in the cave (touristic and non-touristic areas), managed by Struttura Operativa Bossea C.A.I.,
136 Biologia Sotterranea Piemonte – Gruppo di Ricerca and the Department of Environment, Land and
137 Infrastructure Engineering (DIATI) of the Politecnico di Torino, working together with ARPA Piemonte.
138 Automatic detectors were installed in different parts of the cave to continuously (hourly) monitor water
139 quantitative and qualitative data such as flow rate (Q), electrical conductivity (EC) and temperature (T). The
140 flow rate is calculated through overflow vents or calibrated flow sections, depending on the water levels.
141 Bossea Cave offers an ideal situation to study the groundwater circulation in a carbonate rock mass: a main
142 water collector, called Mora River, and several water supplies with different flow rates, related to rock
143 fractures, are present (Fig. 3B). Continuous monitoring data carried out in Bossea Cave by the DIATI -
144 Politecnico di Torino team - revealed that the collector has a flow rate ranging from 50 to 1,200 L/s,
145 developing for about 1.5 km in the cave and directly flowing in the Corsaglia River, with a set of springs
146 (Banzato et al., 2011; Fiorucci et al., 2015; Fiorucci and Vigna, 2015; Peano et al., 2011; Vigna et al., 2017).
147 The discontinuities are poorly or not karstified, characterised by low flow circulation ranging from 0.01 to
148 2.5 L/s (pools of water or drips), such as Polla delle anatre, one of the most important monitoring points in
149 the Bossea Cave, tracked for over 40 years, with a highly constant flow rate (0.5 to 2.5 L/s) (Balestra et al.,
150 2022a; Vigna et al., 2017). Moreover, secondary inputs continuously recharging the aquifer were previously
151 monitored, proving their arrivals in Bossea Cave and highlighting the surface and underground environment
152 connections. Finally, as the hydrodynamic response to rainfall events is characterised by a rather rapid

153 increase in flow (Banzato et al., 2011), the movement of pollutants from the surface to the underground
154 environments could be rapid, making this type of karst systems more subject to MP pollution.

155

156 *2.2. Field sampling*

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

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

167 One surface water sample was taken from Rio Roccia bianca, upstream the Bossea Cave (Fig. 2), four
168 groundwater samples were collected from different areas of the Bossea Cave (Figs. 2–3, Table 1), and one
169 surface water sample was taken from the Corsaglia River, where the water of the collector emerges (Fig. 2).
170 In the Bossea Cave, two samples were collected nearer to the tourist path (Uovo and Sala frane) and two in
171 different non-tourist areas (Sifone and Polla delle anatre) (Fig. 3, Table 1) as reference samples of
172 unpolluted waters because these areas are not frequented by tourists or in close contact with human
173 activities (Fig. 3, Table 1). In the tourist section, the collector water flows from the innermost areas towards
174 the entrance of the cave, where the Sala frane sampling area is located. In this zone, the collector water
175 flows quickly before leaving the cave. In certain areas of the cave, landslides and speleothems convey a
176 small amount of this water along secondary paths, feeding dams of various sizes and slowing the flow, such
177 as in the Uovo sampling area (Fig. 3, Table 1). Here, water flows between different gours, a particular type
178 of speleothem (cave formation) in the form of a stone dam (Hill and Forti, 1997), adjacent to the tourist

179 route, where an egg-shaped statue is placed (Fig. 3, Table 1). In Sifone, the water comes out from the rock,
180 from below, in a siphoning section, and accumulates in a lake (Fig. 3, Table 1). It is possible to access the
181 lake only by a rubber dinghy, in a speleological section of the cave. Polla delle anatre is a small pool of
182 water entering in the inner collector, located in a non-touristic cave zone, visited only by researchers and
183 speleologists (Fig. 3, Table 1). This water flows out of a fracture in the rock and is collected in a small tank
184 with a weir for flow monitoring. Polla delle anatre flow has the typical characteristics of an interconnected
185 drainage system, with a flow rate ranging from 0.5 to 2.5 L/s (Balestra et al., 2022a). Groundwater and
186 surface water samples were collected in September 2021 under low-flow conditions. Being a preliminary
187 investigation, water samples were collected into 1-L glass vessels and capped immediately to prevent
188 atmospheric contamination. All sampling materials were pre-cleaned with ethanol and distilled water to
189 avoid contamination. All samples were transported to the analytical laboratories of the Politecnico di
190 Torino in ice-filled coolers and kept refrigerated at 6°C until analysis.

191

192 *2.3. Laboratory analysis*

193 Microplastic samples were processed according to the previously published method for cave sediments
194 (Balestra and Bellopede, 2022b), adapted for the liquid matrix and improved to determine the particles
195 using the infrared (IR) spectroscopy. All researchers used nitrile gloves and cotton coats in all steps, and all
196 surfaces and materials used during sampling and laboratory analysis were cleaned with ethanol and
197 distilled water to avoid MP contamination. Plastic equipment was replaced with glass or metal utensils.

198 Each water sample was filtered through an 0.8- μm pore size silver filter (GVS Life Sciences, Membrane
199 Disk 47 mm) to allow a better analysis by IR spectroscopy. Filters were placed on glass petri dishes covered
200 with aluminium foil and dried in an oven at 40°C for 2 h. As previous studies have shown the importance of
201 organic matter removal (e.g. Balestra and Bellopede, 2022b), dried filters were subjected to this
202 fundamental step through the application of 2 mL of 15% hydrogen peroxide solution, left to react for 30
203 min at room temperature and dried again for 2 h at 50°C.

204 Fluorescent whitening agents (FWAs) are often used in the production of plastic (Qiu et al., 2015), and
205 MPs with FWAs can be easily detected under ultraviolet (UV) light (Ehlers et al., 2020; Klein and Fischer,
206 2019). Microplastics on filters were observed alongside with and without an UV flashlight (Alonefire SV10
207 365 nm UV flashlight 5W) under a Leitz ORTHOLUX II POL-MK microscope equipped with a DeltaPix Invenio
208 12EIII 12 Mpx Camera, with a 2.5x, 4x, 10x or higher magnification. As some particles are difficult to
209 distinguish from natural ones, especially fibres, comparisons with images of natural, artificial and synthetic
210 fibres under the microscope, taken in previous works (e.g. Houck, 2009; Khan et al., 2017; Zhang, 2014),
211 were made. The UV flashlight was positioned on a pedestal with an inclination of 45°. Visual identification
212 was performed to identify MPs in agreement with the strict selection criteria reported in previous works
213 (Crawford and Quinn, 2016; Hidalgo-Ruz et al., 2012; Noren, 2007): fibres must have the same diameter
214 along their entire length, particles must have unvarying colours, no cellular or organic structures must be
215 present, and red, transparent or white particles must be examined under a high-magnification or
216 fluorescence microscope. Particles smaller than 0.1 mm were cut off as suggested by the European
217 Commission (2013)) for visual identification under a microscope. Particles that could not be identified as
218 MPs were not take into consideration. Filters were observed by the same laboratory operator to ensure MP
219 quantification and identification and reduce human errors. The MPs were categorised in relation to shape,
220 size and colour, according to the standardised size and colour sorting system (SCS) proposed by Crawford
221 and Quinn (2016)).

222 Usually, an average from 1% to 10% of the sample is analysed to determine the chemical composition of
223 the MPs (International Organization for Standardization and European Committee for Standardization,
224 2020). Randomly, 10% of MPs of each filter were analysed using a micro-Fourier Transform Infrared
225 Spectroscopy (micro-FTIR) Shimadzu AIM-9000 microscope equipped with a Shimadzu IRTracer-100
226 spectrophotometer. Spectra were compared with the Shimadzu standard library.

227

228 **3. Results and Discussion**

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

237

238 *3.1. Microplastic abundance*

239 Microplastics were found in all water samples, including the two samples collected in the non-touristic
240 areas of the Bossea Cave. Microplastic abundance, shape, dimension and fluorescence are shown in Table
241 2. The concentration of MPs in cave water varied from 12 to 54 items/L, with a mean of 28 items/L. The MP
242 abundance in the surface water of Rio Roccia bianca was 23 items/L, whereas in the Corsaglia River, it was
243 29 items/L (Table 2). Because there is no standardised method for assessing MPs in water bodies,
244 comparisons are challenging since measurement methods and treatments can affect the results. However,
245 some assumptions on the amounts of MPs in karst environments can be made. Comparisons with other
246 show cave water MP abundances are not possible at the moment as this study is the first one dealing with
247 cave waters; however, a comparison with other groundwater and surface water samples from different
248 areas of the world is possible (Table 3). To the authors' knowledge, the only work on a karst area,
249 monitoring springs and wells from two karst aquifers in Illinois, USA, documented the presence of a
250 maximum MP concentration of 15.2 items/L (mean of 7.9 items/L) in springs and a maximum concentration
251 of 4.4 items/L (mean of 2.8 items/L) in wells, under low-flow conditions (Panno et al., 2019). Selvam et al.
252 (2021)) analysed 24 groundwater samples from wells and bore wells, collected in the post-monsoon season
253 in coastal south India, pointing out a median concentration of 4.2 items/L, with a maximum concentration
254 of 10.1 items/L. Other values are reported in Mintenig et al. (2019)) for NW Germany groundwater, with

255 5.6 items/L. Much higher values were found in an alluvial unconfined groundwater aquifer in Victoria,
256 Australia, with an average concentration of 38 ± 8 items/L (Samandra et al., 2022). Very low values (1
257 items/L) were found in chalk and sandstone aquifers of the UK (Johnson et al., 2020). Surface water
258 samples in the same studied aquifers were analysed by Selvam et al. (2021)), showing 7.8 items/L, with a
259 maximum of 19.9 items/L., whereas Mintenig et al. (2019)) reported 2.9 items/L and Samandra et al.
260 (2022)) 0.4 items/L. The microplastic abundances found in our study are high compared to those found in
261 subterranean and surface waters of Northwest Germany (Mintenig et al., 2019), Illinois, USA (Panno et al.,
262 2019) or South India (Selvam et al., 2021), whereas they are lower than those detected in groundwaters
263 examined in Victoria, Australia (Samandra et al., 2022). However, it should be considered that the size of
264 MPs taken into account, the sampling point, the flow rate, the season and the sources of MPs in the
265 catchment may impact the concentrations of MPs in waters.

266 In non-tourist areas of the cave, the MP abundance in waters was similar to that in tourist areas, and a
267 high number of MPs in the karst system water was found in the inner area of the cave, where the water
268 comes out of a syphoning area. However, the Bossea Cave sediments collected in tourist areas contained
269 more than twice as many MPs as those collected in non-tourist areas, suggesting that pollution is related to
270 the passage of tourists. The microplastic presence in cave water, including water coming out of the rock in
271 the non-tourist sections (Sifone and Polla delle anatre), suggests pollution linked to hydrogeologic
272 connections from the surface to the underground aquifers. The high presence of fractures in the rocks of
273 this karst area could favour pollutant transport in the underground environment, as suggested in Panno et
274 al. (2019). It is reasonable to assume that the high degree of karstification in different zones, the presence
275 of a well-organised outflow network and the plenty water flows play a fundamental role in the transport of
276 MPs in this karst system. The contact between water and sediments could increase the MP concentrations
277 in the Bossea karst system water. Microplastic pollution in the soil of this karst area could be related to the
278 intense winter and summer activities in the zones above the karst system, and the area is rich in ski resorts
279 (Prato Nevoso country). The presence of waste in these areas is not unusual, possible sources include
280 plastic litter from anthropogenic activities and synthetic fibres from clothes. In these areas, surface waters

281 that feed Rio Roccia bianca and Rio Bertino streams were collected, the main secondary supplies
282 contributing to the Bossea karst system recharge (Vigna, 2020). A series of tests with dyes, previously
283 carried out in the two main absorbent valleys, indicated that the waters coming from Rio Roccia bianca and
284 Rio Bertino arrive in Bossea Cave in a relatively short time. During lean periods, the Rio Roccia Bianca
285 waters arrive in the Bossea Cave collector after 4 days (speed of about 644 m/day), whereas in flood
286 periods, they arrive after only 1 day (speed of about 2,460 m/day). Moreover, Rio Roccia bianca provides a
287 much higher quantity of water to the Bossea systems than Rio Bertino water. Although currently, there are
288 no studies studies on the atmospheric transport of pollutants in this area, soil pollution could be related to
289 or aggravated by the atmospheric transport of particles depositing on the ground thanks to precipitation
290 and wind as well as any other contamination between adjacent environments, linked to human activities
291 from the near countries of Fontane and Prato Nevoso. In underground environments, tourists are carriers
292 of alien particles such as lint, organic matter, dust and pollutants (Addesso et al., 2022; Balestra and
293 Bellopede, 2022b; Jablonsky et al., 1993; Liu et al., 2021; Puławska et al., 2021), and therefore, waters in
294 the tourist parts of the cave (Uovo and Sala frane) could be enriched with pollutants, such as MPs in cave
295 sediments (Balestra and Bellopede, 2022b), which could be transported into different parts of the cave and
296 outside, in the Corsaglia River. Our data do not show a definitive link to the suggested sources of pollution
297 but offer information about the hydrogeologic connections, showing opportunities for future research on
298 MP dynamics in subterranean waters in karst environments.

299

300 *3.2. Microplastic shape and size*

301 Microplastics were categorised according to the standardised size and colour sorting system (SCS)
302 described in Crawford and Quinn (2016)) (Table 2). Fibres represented the majority of the MPs present in
303 cave and surface waters (17.1% between 1 and 5 mm and 78% between 0.1 and 0.99 mm of length) (Figs.
304 4–5 and Table 2), followed by fragments (4.3% micro-fragments) (Fig. 5 and Table 2). Some microbeads
305 (0.6%) were also observed, whereas no foam and films were found in this karst system (Fig. 5 and Table 2).
306 As in previous research, fibres and fragments are the main shapes present in the groundwaters, albeit at

307 different concentrations, such as 94% fragments (Samandra et al., 2022) or 100% fibres (Panno et al.,
308 2019). In this work, fibre-shaped fragments dominated the samples. As more than 60% of the world textiles
309 are produced from synthetic fibres (Henry and Klepp, 2018), synthetic clothes are the main source of MP
310 pollution in this karst system. The fragment source could be linked to human activity on the surface or the
311 presence of degraded waste in soil or could have been produced during the electric system works in the
312 cave.

313 According to Crawford and Quinn (2016)), MPs are categorised into microplastics (1–5 mm) and mini-
314 microplastics (1 mm–1 µm). Using visual identification under a microscope facilitated the analysis of MP
315 particles from 5 to 0.1 mm. In this investigation, mini-MPs accounted for 82.9% of the total MPs found in
316 the water karst system and for 84% of the MPs found in cave water only (Fig. 5 and Table 2).

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

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

332

333 3.3. *Microplastic fluorescence and colour*

334 The highest MP abundance was fluorescent under UV light (84.8%) (Fig. 4, Table 2), with a percentage
335 similar to that found for MPs in cave sediments (87.7%) (Balestra and Bellopede, 2022a; Balestra and
336 Bellopede, 2022b). Exploiting fluorescent whitening agents (FWAs) in plastics, MPs can be easily found
337 using an inexpensive UV flashlight (Fig. 4); however, there are also several limitations (Qiu et al., 2015): not
338 all plastics contain FWAs (Fig. 4A-B, orange arrow), and different naturally fluorescent organic and inorganic
339 matter compounds could be misunderstood as MPs. Organic fluorescent particles may account for more
340 than half of the total fluorescent particles in the samples analysed in cave environment (Balestra and
341 Bellopede, 2022b), and therefore, organic matter removal is a fundamental step in MP determination.

342 Figures 6 shows the different colours of the collected MPs. Of the fluorescent particles (Fig. 6A), 46%
343 were transparent, followed by red (16.5%), blue (10.8%), beige (7.2%) and amber (4.3%) ones. The MPs
344 with other colours accounted for less than 4% of the total fluorescent ones. Non-fluorescent MPs (Fig. 6B)
345 were mainly black (68%) or blue (20%), followed by green, transparent and violet ones (4%). The major part
346 of the fluorescent particles in water are transparent, as reported in Balestra and Bellopede (2022b)) for
347 Bossea Cave sediments. The other main colours are similar, with the exception of red particles, which are
348 more abundant in water. The non-fluorescent MP particles were mainly black and blue, confirming the data
349 found for Bossea Cave sediments (Balestra and Bellopede, 2022b). However, the percentages were
350 different, as well as the colour of the less abundant MPs. These facts could be related to the presence of
351 particles with different densities, more easily transportable in water, with a different degree of
352 biodeterioration or a diverse pollution source. Transparent MPs were also prevalent in groundwaters
353 analysed in Selvam et al. (2021)), followed by white and blue ones, whereas in Panno et al. (2019)), 65% of
354 the analysed MPs were blue and/or clear, whereas the other common colours were red (15%) and grey
355 (13%).

356 The MP colour could provide an indication of the chemical pollutants; for example, yellow and black
357 MPs could indicate high levels of pollutants (Frias et al., 2010; Karapanagioti et al., 2011). In the Bossea
358 karst system, about 68% of non-fluorescent MPs were black, and future investigations should be carried

359 out to monitor possible environmental contaminations. Moreover, the colour of MPs can be correlated
360 with the consumption by organisms (Carpenter et al., 1972; Romeo et al., 2015; Shaw and Day, 1994), being
361 confused with trophic resources. In natural subterranean systems, there is no light, and therefore, the
362 colour of MPs does not play a particular role in hypogeal animal ingestion. However, the colour and
363 fluorescence of MP particles may be relevant for less cave-adapted and epigeal organisms in watercourses
364 and adjacent habitats.

365

366 *3.4. Microplastic characterisation*

367 Visual identification is one of the most inexpensive and commonly used methods for MP detection (e.g.
368 Alomar et al., 2016; Cannas et al., 2017; Guerranti et al., 2017; Hidalgo-Ruz et al., 2012). However, it does
369 not allow the determination of small particles (European Commission, 2013), is susceptible to operator
370 experience and errors (Crawford and Quinn, 2016; Prata et al., 2019) and does not allow conclusions about
371 polymer composition. In-depth analyses can be done with high-magnification microscopes, staining
372 techniques and via spectroscopy. However, over- or underestimation of MPs can occur for different
373 techniques (Hidalgo-Ruz et al., 2012; Song et al., 2015), and dyes have different limitations related to MP
374 particle shape, typology, hydrophobicity and superficial alteration (Erni-Cassola et al., 2017; Prata et al.,
375 2019; Shim et al., 2016; Tamminga et al., 2017). Reasonably, a set of several methodologies is the optimal
376 choice, and therefore, in this work, spectroscopy was selected to confirm the results obtained via visual
377 counting under the microscope.

378 The identification of MPs by spectroscopy is time-consuming, and generally, only an average from 1% to
379 10% of the sample is analysed (International Organization for Standardization and European Committee for
380 Standardization, 2020). Moreover, the spectra of samples are difficult to match with the library ones,
381 obtaining high percentages because of the contaminated surface of plastics found in natural environments
382 (Song et al., 2015). In this study, 10% of MPs in each filter was analysed by micro-FTIR, taking random MP
383 particles (Fig. 7). Polyethylene (51.4%) and polyvinyl alcohol (31.4%) were the main types of MPs found in
384 the karst system waters, followed by polyester (8.6%), ethylene vinyl alcohol (2.9%), polyvinyl chloride

385 (2.9%) and acrylic adhesive (2.9%). Considering cave groundwaters only, polyethylene (56.5%) and polyvinyl
386 alcohol (30.4%) were the main types of MPs. Polyethylene, polyester and polyvinyl chloride were also
387 found in other analysed groundwaters (Mintenig et al., 2019; Panno et al., 2019; Samandra et al., 2022;
388 Selvam et al., 2021). Other different MPs were identified in previous works, such as polyamide (nylon)
389 (Mintenig et al., 2019; Samandra et al., 2022; Selvam et al., 2021), epoxy resin (Mintenig et al., 2019),
390 polypropylene, polystyrene, polycarbonate, polyethylene terephthalate and polymethylmethacrylate
391 (Samandra et al., 2022). The MP composition is useful to understand the likely sources of pollution. Many
392 of the plastics found are used in the production of textiles, corroborating earlier assumptions on the origin
393 of microplastics found in the waters of the karst system.

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

399

400 **4. Conclusions**

401 This preliminary investigation documents the presence of microplastics in the examined karst system,
402 from surface watercourses to cave waters. The concentration of MPs in the karst system water varied from
403 12 to 54 items/L. Based on the analysis of karst waters, microplastics less than 1 mm dominated the
404 samples (82.9%), and fibre was the main shape present (95.1%). The detection of MP contamination is
405 essential to define the issues related to pollution and establish strategies for karst areas and water
406 conservation. This work suggests that karst morphology and associated aquifers could allow the movement
407 of microplastics into underground water flow systems, providing useful references for further research.
408 Future studies are needed to understand microplastic dynamics and transport in the karst system, to
409 determine pollution sources and to understand the potential effects on organisms and ecosystems. As karst
410 aquifers are open systems susceptible to contamination by surface pollutants, the superficial areas should

411 also be monitored. Greater efforts should be made to protect karst areas and underground resources,
412 implementing new strategies to monitor and valuate MP pollution and providing education to all
413 stakeholders to find adequate solutions, following the environmental sustainability principles, especially for
414 the management of water resources.

415

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427

428 **CRedit authorship contribution statement**

429 Valentina Balestra: Conceptualisation, methodology, validation, formal analysis, investigation, resources,
430 data curation, writing – original draft, writing – review and editing, visualisation. Bartolomeo Vigna:
431 Investigation, writing – review and editing, visualisation. Sean de Costanzo: Investigation. Rossana
432 Bellopede: Conceptualisation, methodology, validation, resources, writing – review and editing,
433 visualisation, project administration, funding acquisition.

434

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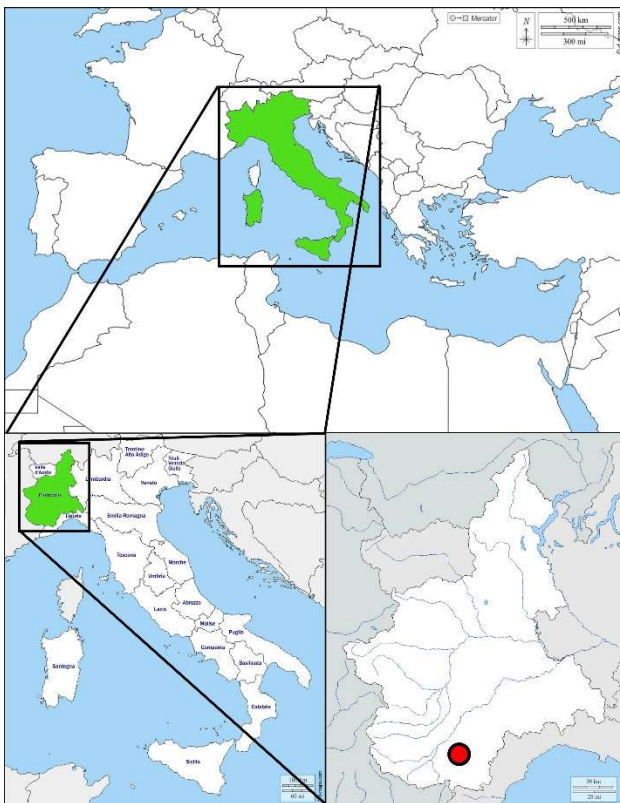
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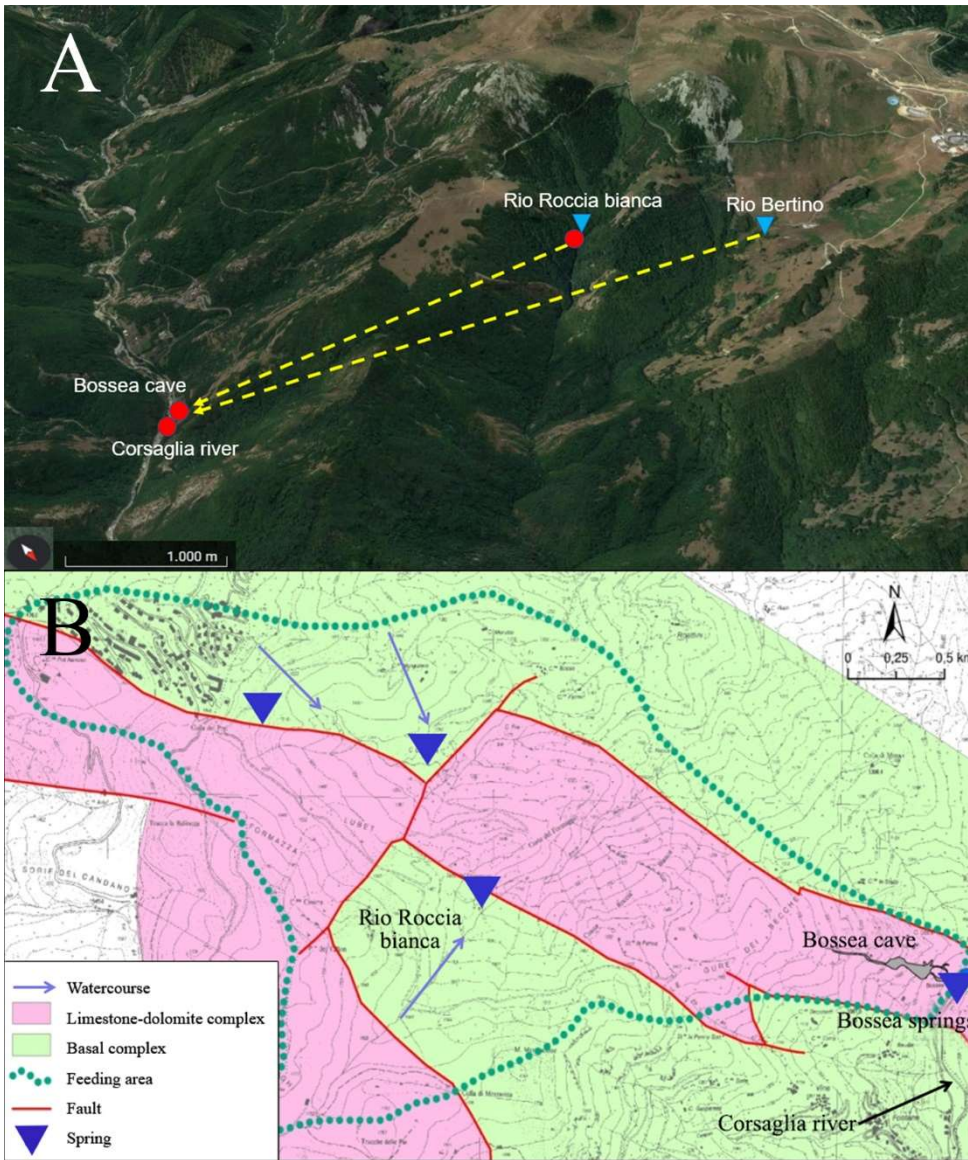
649

650 **Figures**



651

652 *Fig. 1. Location of the sampling area. Bossea Cave is located in SW Piedmont (Italy). (Maps used for the plate and modified,*
653 *retrieved from: https://d-maps.com/carte.php?num_car=3126&lang=en, https://d-maps.com/carte.php?num_car=4828&lang=it*
654 *and https://d-maps.com/carte.php?num_car=8256&lang=en).*



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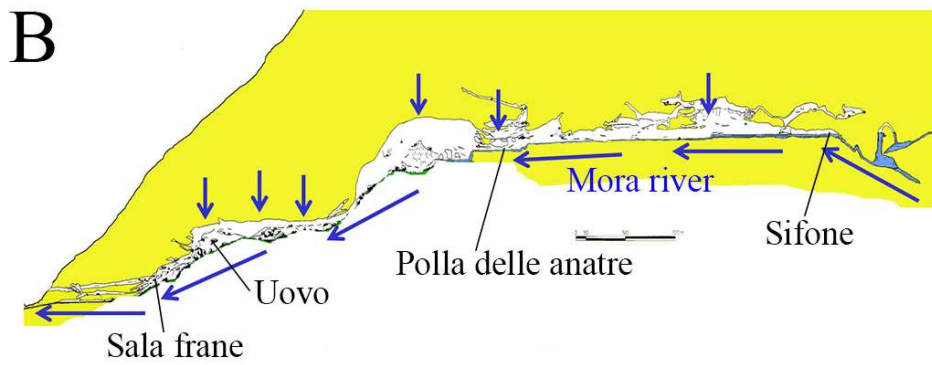
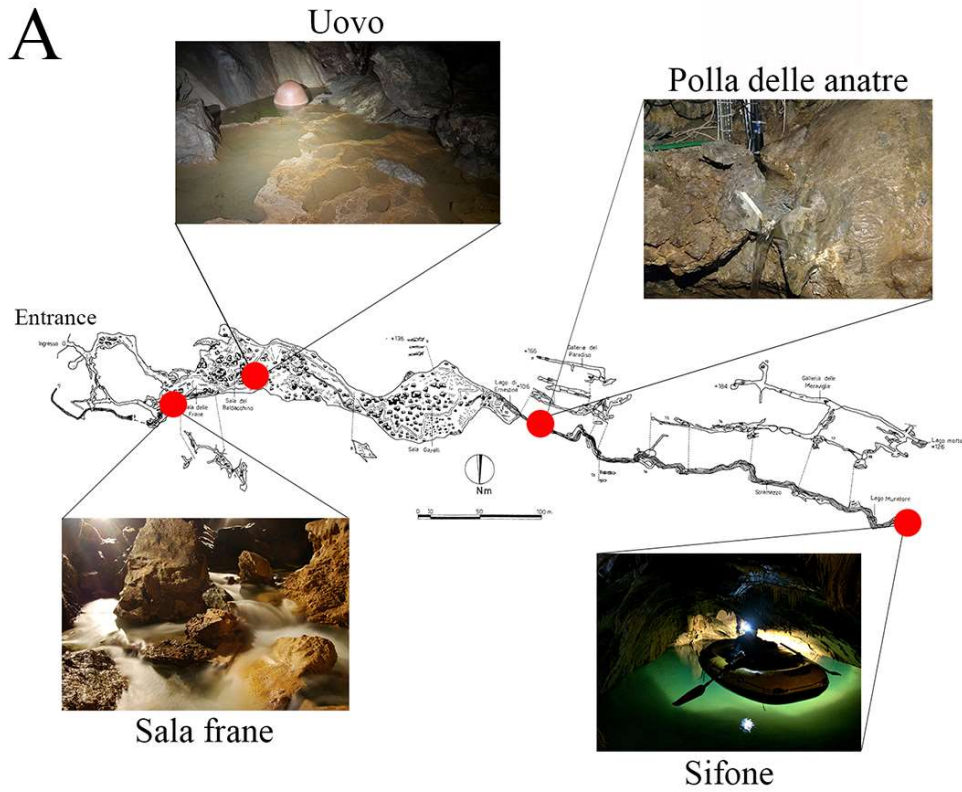
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Fig. 2. Bossea karst system. A: Turquoise triangles for the main surface water supply (Rio Roccia bianca and Rio Bertino), red circle for sampling areas, yellow arrow for the results of the staining tests carried out previously: the waters of the secondary supply contributing the Bossea karst system recharge (Rio Roccia bianca e Rio Bertino) trough sinkholes in the sub-riverbed, ending up in the collector of the Bossea Cave. Map from <https://earth.google.com> [accessed: 07/06/2022], modified; B: Bossea karst system feeding area. Map from Vigna (2020)), modified.



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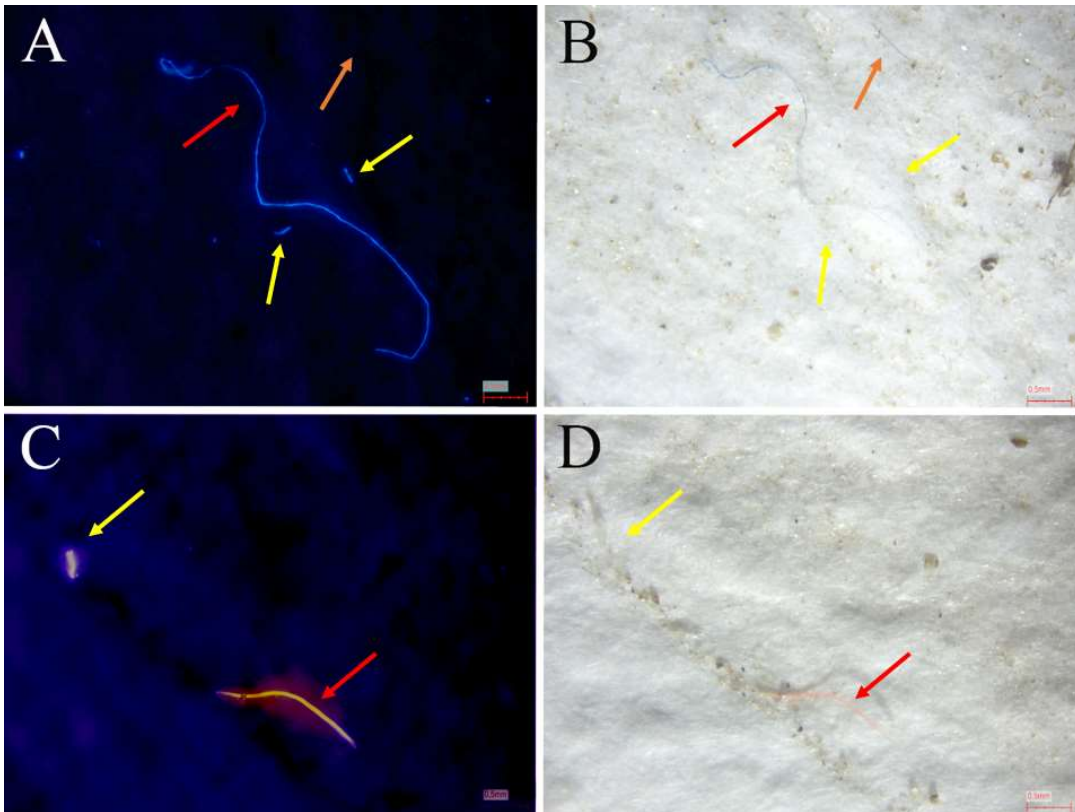
662 *Fig. 1. Bossea karst system. A: Bossea Cave sampling areas. Red circles for water sampling. Survey (plan) by Elia and Callaris (1988)),*

663 *modified. Photos by Valentina Balestra and Bartolomeo Vigna; B: Bossea Cave displacement of water with sampling areas. Blue*

664 *arrows for primary and secondary water intakes. Survey (section) by Elia and Callaris (1988), modified).*

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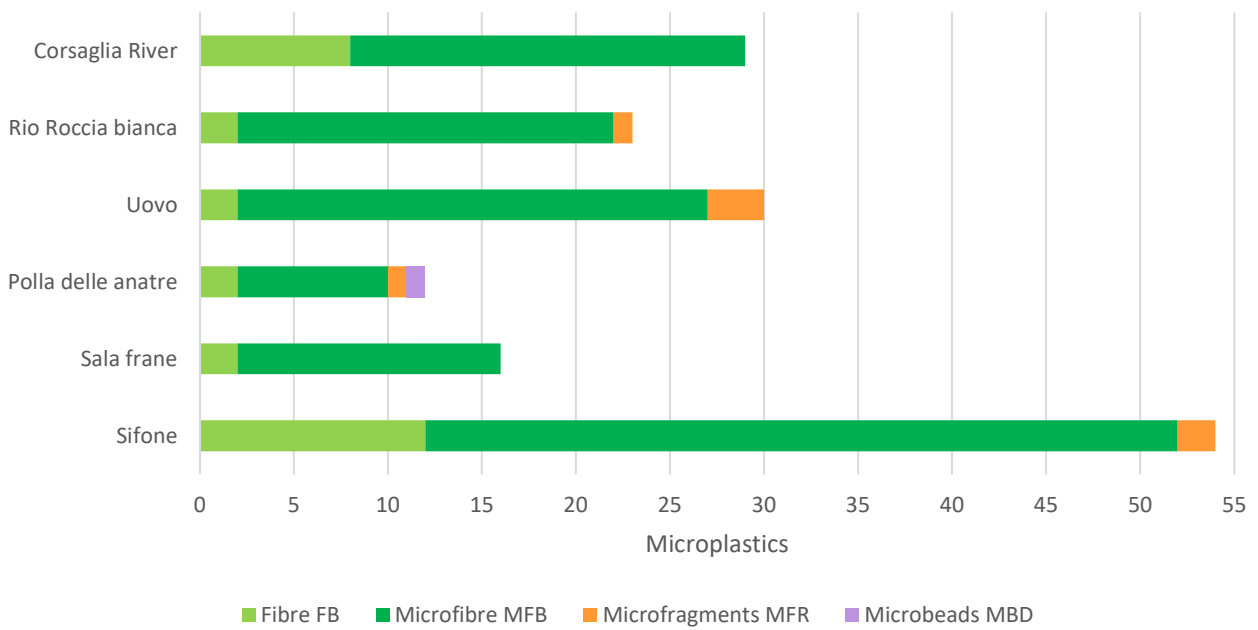
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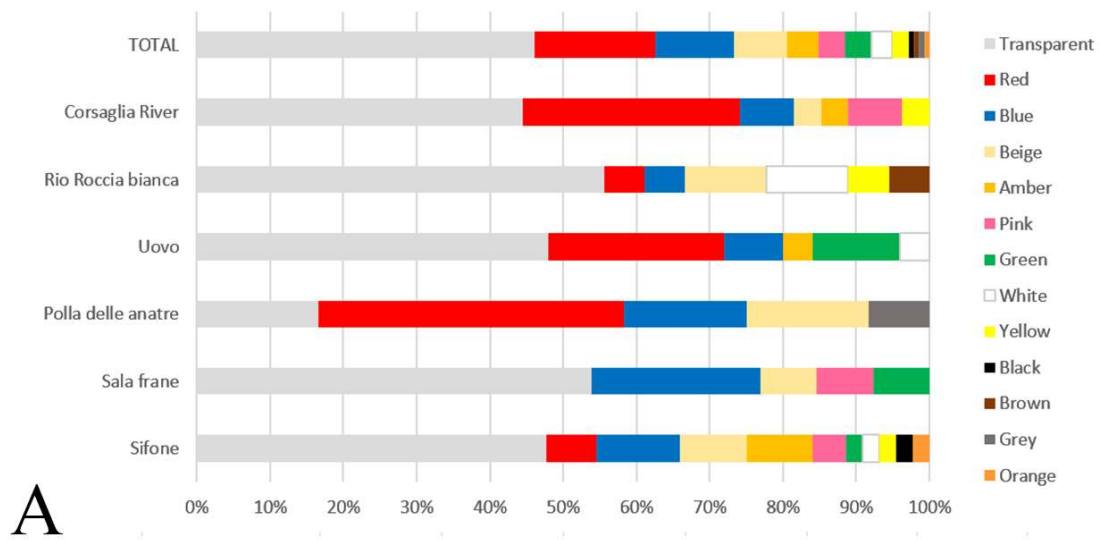
668 *Fig. 2. Microplastic particles on filters observed under a microscope, with and without a UV flashlight. Yellow arrows for transparent*
 669 *microplastics, fluorescent under UV light, orange arrows for coloured microplastic, non-fluorescent under UV light, and red arrows*
 670 *for coloured microplastics, fluorescent under UV light. A–B: microplastic fibres; C–D: microplastic fibre and fragment.*

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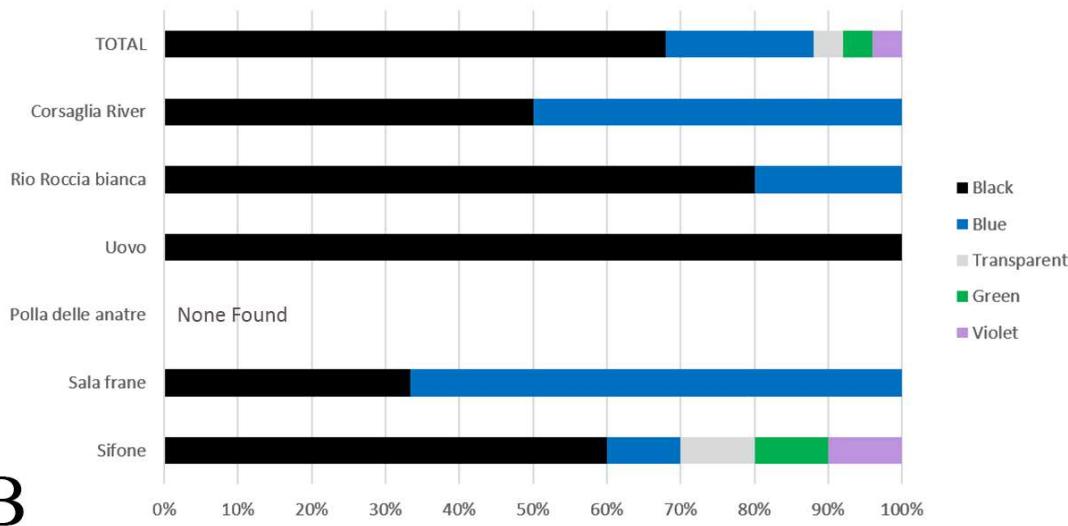


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673 *Fig. 3. Microplastic shape and size for the examined karst system waters.*



A



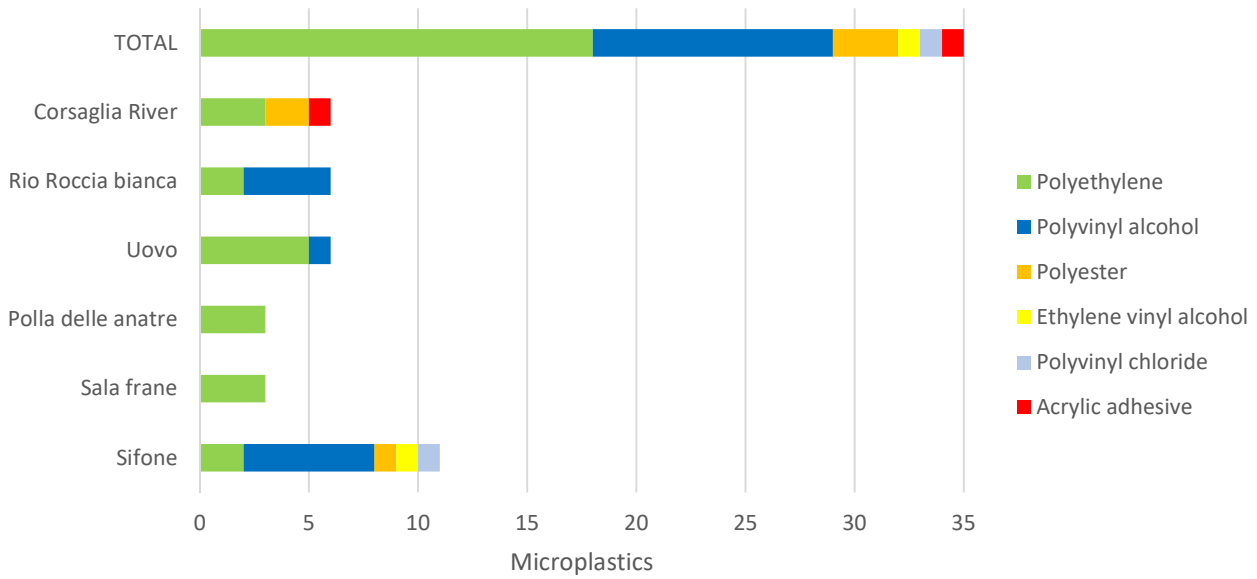
B

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676 Fig. 6. Percentages of colours of microplastic particles on filters from water sampled in different Bossea karst system areas. Uovo,

677 Polla delle anatre, Sala frane and Sifone were sampled in Bossea Cave. A: Percentages of colours of fluorescent microplastics. B:

678 Percentages of colours of non-fluorescent microplastics.



679

680 *Fig. 7. Identification of 10% of the MP particles of each filter by micro-FTIR spectroscopy.*

681

682 Tables

683 *Table 1. Bossea Cave sampling area characteristics.*

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

684

685 *Table 2. Abundance [items/L], shape, size and fluorescence of MPs in Bossea system waters. Shape and size were defined using the*
 686 *SCS Method (Crawford and Quinn, 2016).*

Filter	MP	Fluorescent MPs	Non fluorescent MPs	Fibre	Microfibre	Fragments	Microfragments	Film	Microfilm	Pellet	Microbeads	Foam	Microfoam
				FB	MFB	FR	MFR	FI	MFI	PT	MBD	FM	MFM
Sifone	54	44	10	12	40	0	2	0	0	0	0	0	0
Polla delle anatre	12	12	0	2	8	0	1	0	0	0	1	0	0
Uovo	30	25	5	2	25	0	3	0	0	0	0	0	0
Sala frane	16	13	3	2	14	0	0	0	0	0	0	0	0
Cave total	112	82	30	18	87	0	6	0	0	0	1	0	0

%	100	73.2	26.8	16.1	77.7	0.0	5.4	0.0	0.0	0.0	0.9	0.0	0.0
Rio Roccia bianca	23	18	5	2	20	0	1	0	0	0	0	0	0
Corsaglia river	29	27	2	8	21	0	0	0	0	0	0	0	0
Karst system total	164	127	37	28	128	0	7	0	0	0	1	0	0
%	100	84.8	15.2	17.1	78.0	0.0	4.3	0.0	0.0	0.0	0.6	0.0	0.0

687

688 *Table 3. MP pollution comparison of groundwater and surface water samples from different areas of the world.*

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

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