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Tracing the invisible along the surface-subsurface continuum: bisphenols and microplastic pollution in karst system waters

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ABSTRACT

Bisphenols are emerging organic contaminants often released into the environment, especially in aquatic systems. Specifically, Bisphenol A (BPA) is generally used in synthetic polymers and thermal paper production, and has been found in different environments and organisms, affecting ecosystems and human life. However, bisphenol contamination in karst systems remains poorly studied, although karst groundwaters are widely exploited for drinking use. This study presents a preliminary screening of BPA, bisphenol AP (BPAP) and C (BPC) in a karst system, from surface watercourses to cave groundwaters, using the novel AptaStick system for bisphenol family. Results confirmed BPA pollution in the karst system, with highest values inside a cave. BPAP and BPC were not detected in water samples. Potential pollution sources are likely linked to diffuse anthropogenic inputs within the catchment area, combined with event-driven transport dynamics. As a potential source of BPA contamination, microplastic (MP) pollution was concurrently investigated during high-flow conditions, finding from 0 to 7.7. MPs/L. These data expand previous assessments conducted under low-flow conditions in the same karst system. Natural and regenerated microfibres (MFs) were analyzed too, finding from 0 to 136 items/L. This work wants to be a first evaluation of both bisphenol and MP pollution in karst systems, stressing the importance of monitoring pollutants in these highly vulnerable environments. Moreover, it provides a first evaluations of how pollutant distribution may vary within the different hydrological compartments of a karst system, not only between surface and groundwater environments, but also among flowing, percolation, and stagnant waters within the subterranean network. This approach contributes to a better understanding of the spatial heterogeneity and transport dynamics of emerging contaminants in karst environments, supporting the development of appropriate conservation measures for habitats and species protection, and suitable drinking water resources management.

1. Introduction

Emerging organic contaminants (EOCs) are substances potentially harmful to the environment, such as pesticides, pharmaceuticals, microplastics (MPs) and bisphenols (García et al., 2020; Reberski et al., 2022). Many EOCs can accumulate in soil and water, including aquifers, especially through leaching, which involves their movement into deeper soil layers and eventually into groundwater sources (Narevski et al., 2021; Oppeltová et al., 2024; Pérez-Lucas et al., 2018; Reberski et al., 2022, lósarczyk and Jakóbczyk-Karpierz, 2026; Yamamoto and Yasuhara, 1999). The accumulation of EOCs in aquifers poses serious environmental and health risks, as these water sources are used for drinking water worldwide (Reberski et al., 2022). The transportation of EOCs to aquifers is determined by aquifer characteristics and subsurface conditions, such as hydrogeological factors (e.g., hydraulic conductivity, permeability, and recharge rates), groundwater residence time (e.g., rapid flow in karst systems versus long residence times in confined aquifers), and environmental conditions (e.g., pH, redox conditions, temperature, and organic matter content) (Ligavha-Mbelengwa et al., 2025; Oppeltová et al., 2024). Chemical, biological and physical

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processes may control EOCs migration into aquifers too (Stoppiello et al., 2020). Karst systems exhibit unique transport dynamics, sometimes rapid infiltration through sinks and conduit-dominated flow, which facilitate the swift transport of contaminants over long distances.

Bisphenol A (BPA) is one of the most highly produced chemicals worldwide used in the production of synthetic polymers, such as polycarbonate plastics and epoxy resins, and thermal paper (Hoekstra and Simoneau, 2013; Huang et al., 2012; Michałowicz, 2014). As a result, it is ubiquitously present in a wide range of consumer products, including paper products, electronic devices, toys, and water pipes, but also in medical equipment and dental products (Huang et al., 2012; Michałowicz, 2014). Thermal paper is produced in large quantities, due to its widespread use in register receipts, books, fax papers, and labels. Following recycling, it is also employed in the manufacture of newspapers, tickets, brochures, mailing envelopes, kitchen rolls, toilet paper, and food cartons (Michałowicz, 2014 and references therein). Furthermore, BPA is extensively utilized in the production of polyacrylates, and polyesters, and is incorporated into food contact materials, such as packaging, beverage bottles and lacquered coatings for metal cans, causing chronic human exposure through ingestion of contaminated food and drinks (Arnold et al., 2013; Cooper et al., 2011; Makris et al., 2013; Michałowicz, 2014; Niu et al., 2012). These materials, upon degradation, can represent significant sources of BPA in the environment too (Michałowicz, 2014). Therefore, among bisphenols, BPA is currently the most investigated. However, while BPA toxicity is well-documented, mainly due to its endocrine-disrupting potential, oxidative stress induction, mutagenic potential, as well as hypomethylation activity, among other evidences in organisms (Clayton et al., 2011; Keri et al., 2007; Richter et al., 2007), its transport behavior in the environment remains a critical knowledge gap.

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

MPs pollute natural environments worldwide raising concerns for ecosystems and human health (e.g. Di Lorenzo et al., 2023; Ha and Yeo, 2018; Kannan and Vimalkumar, 2021; Sharma and Chatterjee, 2017). This pollutant can be taken up by organisms either directly or indirectly, exert toxic effects and constituting a serious ecological concern (Assas et al., 2020; Devereux et al., 2021; Jahan et al., 2019; Romeo et al., 2015). MPs may also contain or adsorb other contaminants, such as pesticides, persistent organic pollutants (POPs), bisphenol A (BPA), chemicals, heavy metals, and antibiotics, thereby amplifying their associated environmental risks (Cheng et al., 2023; Rochman et al., 2013; Selvam et al., 2021; Wanner, 2021; Zhou et al., 2019). Consequently, MPs are widely studied, particularly in marine environments. However, they remain poorly investigated in other settings, such as karst and hypogeal ecosystems. Crucially, MPs can act as transport vectors, adsorbing organic pollutants like bisphenols and thereby amplifying their environmental persistence and mobility (Sheng et al., 2021). In karst systems in which the hydrodynamic response is rapid, high-flow events can mobilize MPs from the surface into the subterranean continuum (Yang et al., 2025). The co-occurrence of MPs and bisphenols in such systems is a critical but under-researched topic.

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

Groundwaters are fundamental resources and important ecosystems. Unfortunately, groundwaters are under increasing pressure from contamination due to anthropogenic activities (Balestra, 2026; Balestra et al., 2026; Reberski et al., 2022 and references therein). Generally, groundwaters are less vulnerable to contamination by EOCs than surface waters, however, once in groundwaters, these substances are persistent and difficult to treat (Reberski et al., 2022; White et al., 2019). BPA is one of the most relevant detected compounds in European groundwaters, with a detection frequency of 40%, and a reported maximum concentration reaching 100 μg/L (Lapworth et al., 2015; Loos et al., 2010).

Little studies on EOCs were done in karst, but only from a small number of countries, especially from Europe and USA, with few or no studies in many other parts of the world (Oppeltová et al., 2024; Reberski et al., 2022). However, they provide key insights into patterns of EOCs in karst aquifers, useful for a wide range of applications and conservation measures.

Research on BPA contamination in karst systems is still at an early stage, despite the widespread use of karst groundwater for drinking purposes (Reberski et al., 2022; Wilkinson et al., 2022). Karst aquifers are particularly susceptible to pollution, due to direct infiltration pathways, such as stream sinks, shafts, and caves (Ford and Williams, 2007; Goldscheider and Hötzl, 1999), which facilitate the rapid transport of pollutants, even reaching rapidly long distances (Worthington and Ford, 2009).

To the authors' knowledge, there is only one study which analyze BPA pollution in waters of boreholes, streams, wells and caves of the same karst area and possible sources (Oppeltová et al., 2024) and one in surface water and groundwaters of the same karst area (Ślósarczyk and Jakóbczyk-Karpierz, 2026). No one on less common bisphenols, such as Bisphenol C (BPC) or AP (BPAP). Studies on bisphenol pollution in caves, as well as surface and underground waters of the same karst system are lacking. Karst groundwater resources are highly vulnerable to pollution. Given their essential ecological functions and their role as a source of drinking water, it is crucial to implement environmental investigations to monitor environmental health and identify possible sources of pollution.

In this study we performed a preliminary screening using the innovative AptaStick system (Ochoa-Esteso et al., 2026) for bisphenol family, including BPA, BPAP, BPC in the karst system of Bossea, Piedmont, NW Italy, analyzing surface watercourses, a subterranean river, cave groundwaters and waters from a conduit overflow, providing a reference for further research. Specifically, the AptaStick operates as an active solid-phase extraction platform, and it consists of a 3D-printed stir-stick architecture functionalized with target-specific DNA aptamers that actively capture pollutants from the water matrix under controlled magnetic stirring. Unlike conventional solid-phase extraction methods that often suffer from clogging and poor selectivity in highly turbid karst matrices, the AptaStick avoid these problems through DNA-aptamer functionalization and an innovative stirring system. Additionally, the characteristics of the system and the area will be analyzed, as well as the surface anthropogenic activities to hypothesize the possible sources of pollution.

Being MPs a possible source of BPA pollution, in parallel to bisphenols, MP pollution was analyzed. This system was previously analyzed by some of the authors to understand MP pollution in sediments and waters, highlighting MP pollution from surface watercourses to cave waters and springs, including also highly specialized crustaceans that live in these waters (Balestra and Bellopede, 2022, 2023; Balestra et al., 2023; Sforzi et al., 2024). Furthermore, pollution was investigated during a high-flow/recharge period, whereas previous monitoring activities were conducted under low-flow conditions (Balestra et al., 2023), in order to evaluate the influence of different hydrological

conditions on MP occurrence and distribution within the karst system. To the best of our knowledge, this is the first study assessing the co-occurrence of MPs and BPA, BPAP and BPC within a karst system, considering both surface and subterranean compartments of the same hydrological continuum. Therefore, the study provides novel baseline information on the simultaneous presence of these emerging contaminants in highly vulnerable subterranean environments.

To further expand the current knowledge, natural and regenerated microfibres (MFs) were analyzed too. Recent studies highlighted concern for this pollutant that impact natural environments, especially regarding natural and regenerated MFs (Athey and Erdle, 2022; Balestra and Bellopede, 2025; Balestra et al., 2024a; Dris et al., 2016; Finnegan et al., 2022; Hasenmueller et al., 2023; Liu et al., 2022; Miller et al., 2017; Okyere et al., 2025; Stanton et al., 2019; Suaria et al., 2020), highlighting the need of monitoring also these materials.

Therefore, the aims of this study were, for the first time: i) to evaluate the occurrence of BPA, BPAP, and BPC in karst systems using the innovative AptaStick system (Ochoa-Esteso et al., 2026); ii) to investigate the co-occurrence of MPs and BPA, BPAP and BPC in karst systems; iii) to assess MP pollution during high-flow conditions in the Bossea karst system; and iv) to evaluate MF pollution through the entire karst system network, from surface watercourses to cave groundwaters.

Moreover, this study provides an evaluation of how pollutant distribution varies within different hydrological conditions and compartments of a karst system, examining the interplay not only between surface and groundwater environments, but also among flowing, percolation, and stagnant waters within the subterranean network. By evaluating the co-occurrence of these contaminants in the immediate aftermath of a high-flow recharge event, this study offers clear insights into residual distribution patterns and the influence of recharge dynamics on the subterranean migration of these contaminants. These findings contribute to a better understanding of the spatial heterogeneity and transport dynamics of emerging contaminants in karst environments, highlighting the importance of integrated monitoring strategies. Furthermore, the study provides preliminary baseline data on these emerging contaminants in karst and subterranean environments, encouraging further research in supporting the development of appropriate conservation measures for these highly vulnerable environments and for a sustainable management of groundwater resources useable for drinking water supply.

2. Materials and method

2.1. Study area

The recharge area of the Bossea karst system, Piedmont, northwestern Italy (Fig. 1), is characterized by a belt of carbonate lithologies, laterally confined by low-permeability units, including quartzites and metavolcanic rocks (Balestra et al., 2023 and references therein). The Bossea Cave (Fig.s 1 and 2), located in the Frabosa Soprana (CN)

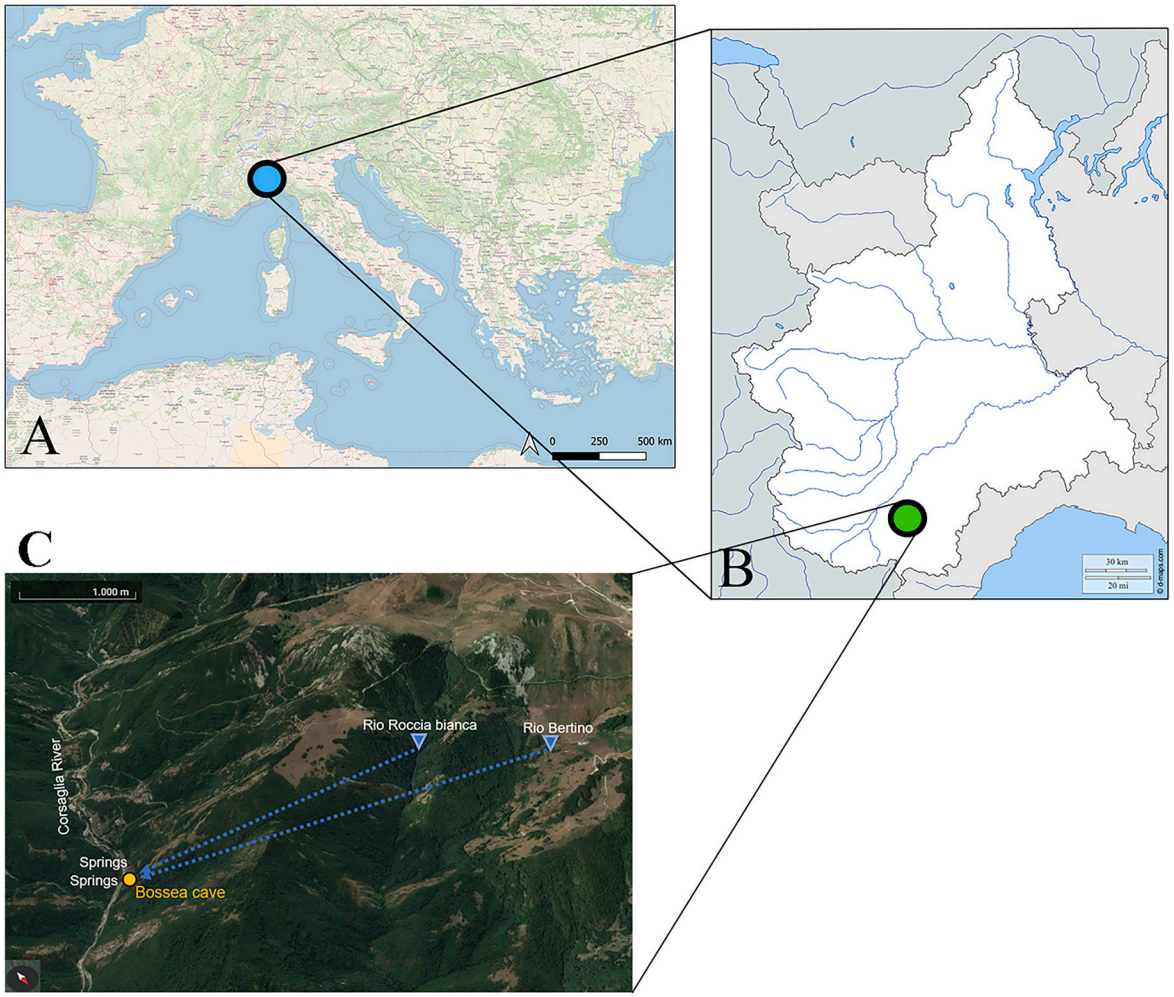


Fig. 1. Study site. A and B: Location of the sampling area (map A created with QGIS Desktop 3.12.1 with GRASS 7.8.2 using OpenStreetMap map, modified - openstreetmap.org/copyright, map B from https://d-maps.com/carte.php?num_car=8256&lang=en, modified); C: Turquois triangles for the main surface water supply (Rio Roccia bianca and Rio Bertino), orange circle for Bossea cave and springs (Map from https://earth.google.com [accessed: 07/06/2022], modified).

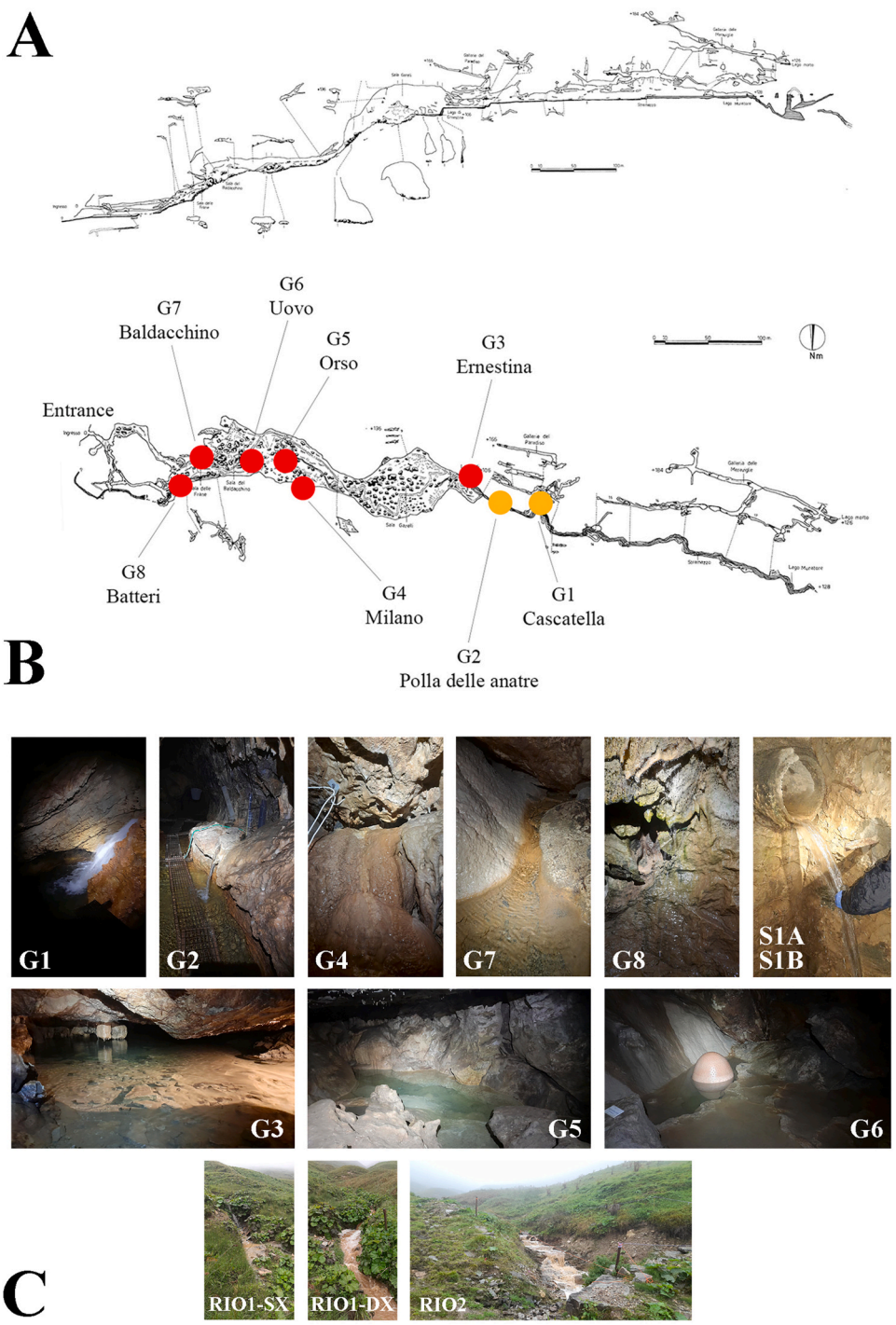


Fig. 2. Sampling points. A,B: Bossea cave surveys. Red circles for water sampling in the tourist area, orange circles for water sampling in the speleological area (Surveys – section (A) and plan (B) - from Elia and Calleris (1988), modified); C: photos of all sampling points (photo S1A-S1B by E. Lana, all the other photos by V. Balestra).

municipality, represents the terminal segment of the karst system developed within the Maudagna-Corsaglia watershed, spanning the area between the Prato Nevoso basin and the Corsaglia River. The prevailing land cover consists of broadleaf larch forests interspersed with alpine meadows and pastures; however, portions of the area are impacted by anthropogenic activity, notably ski infrastructure attracting seasonal tourism.

The aquifer recharge predominantly occurs through both direct (autogenic) and indirect (allogenic) mechanisms (Vigna, 2020). Autogenic recharge is derived from precipitation and snowmelt infiltrating directly into the karst system, whereas allogenic recharge involves surface runoff entering the subsurface through swallow holes located within the riverbed. The Rio Roccia Bianca constitutes the principal allogenic input contributing to the recharge of the Bossea karst system. Previous hydrogeological tracer tests with fluorescent dyes were conducted in the main losing streams of Rio Roccia Bianca and Rio Bertino (Fig. 1C) to trace hydrological connections with Bossea cave (Vigna, 2020). The aquifer exhibits groundwater flow along karst conduits and structural discontinuities, with a well-developed saturated zone. Accordingly, it can be classified as a karstic system with interconnected

drainage networks and medium to high permeability (Banzato et al., 2011).

Bossea cave (Fig. 2) holds historical significance as the oldest show cave in Italy, having been accessible to the public since 1874. The cave has a single entrance at an elevation of 836 m a.s.l. and features an upward-trending morphology, extending approximately 2800 m along a tectonic boundary between the Middle Triassic Dolomie di San Pietro dei Monti Formation (dolostones and carbonates) and the Permo-Triassic metavolcanic sequence (Antonellini et al., 2019). Several subterranean research laboratories are situated within both tourist and non-tourist areas of the cave, enabling investigations into hydrogeology, geochemistry, microclimate, radon, microplastics, and subterranean biology (Biamino et al., 2024; Lana and Balestra, 2020; Vigna et al., 2017b).

Bossea Cave provides an optimal natural laboratory for examining groundwater flow within fractured carbonate rock masses. It features a principal conduit, the Mora River, which receives inputs from multiple secondary sources associated with structural discontinuities and variable discharge rates. Continuous monitoring by the DIATI – Politecnico di Torino research team has documented discharge variations in the Mora River ranging from 50 to 1200 L/s over a 1.5 km reach, ultimately discharging into the Corsaglia River via a series of springs (Banzato et al., 2011; Fiorucci et al., 2015; Fiorucci and Vigna, 2015; Peano et al., 2011; Vigna et al., 2017a). In contrast, weakly developed karst features and fractures exhibit significantly lower flow rates (0.01–2.5 L/s), forming pools or drip points, such as Polla delle Anatre (Fig. 2). This site, monitored for over four decades, consistently displays low but stable discharge (0.5–2.5 L/s) (Balestra et al., 2022; Vigna et al., 2017a). Furthermore, allogenic recharge inputs have been repeatedly validated through tracer studies, demonstrating the connectivity between surface and subterranean environments. The system exhibits a rapid hydrological response to precipitation events (Banzato et al., 2011) indicating a high vulnerability to surface-derived contaminants, including MP pollution (Balestra et al., 2023). Microplastics and microfibres pollutants were found also in sediments (Balestra and Bellopede, 2022, 2023) and stygofauna (Sforzi et al., 2024) of this system, highlighting high contamination levels, especially inside the touristic area of the cave, probably due to the tourist fluxes.

2.2. Contamination control

All materials and working surfaces were pre-cleaned with ethanol and milliQ water. Nitrile gloves were used by the operators during all procedures. White cotton coats were dressed by the operators during all laboratory activities. Plastic materials were replaced with glass and metal ones, wherever possible, and all open glass jars and equipment were covered with aluminum foils.

For MP and MF analysis, milliQ water, 30% H_2O_2 (Merck), and ethanol absolute (VWR Chemicals) were filtered twice through a 1.2 μm pore size glass fiber filter (47 mm) (Enrico Bruno srl) before use, avoiding possible contaminations. Laboratory analyses were conducted within a fume hood. Contamination control on air was carried out using one open pre-cleaned Petri dish during the laboratory analysis. Once the analysis was completed, the petri was washed with bi-filtered MilliQ water and filtered using the same procedure as the other samples. The blank correction method applied for airbone contamination in this study assesses the results of unknown samples by subtracting the blank contribution (Shruti and Kutralam-Muniasamy, 2023).

2.3. Field sampling

Sampling was done by Biologia Sotterranea Piemonte – Gruppo di Ricerca researchers. Sampling points were set in surface watercourses, subterranean river, cave groundwaters, and overflow conduit from the cave of the same karst system (Fig. 2, Table 1), trying to use the same sampling areas monitored in previous years (Balestra et al., 2023).

Table 1
Water sample characteristics in the Bossea karst system.

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

(continued on next page)

Table 1 (*continued*)

Sample ID	Sample name	Sampling site type	Water characteristics	ml
			the Bossea karst system recharge. Secondary left flow	
RIO1-DX	Rio Roccia Bianca DX	Surface watercourse	Surface watercourse upstream the Bossea cave. Main secondary supply contributing to the Bossea karst system recharge. Secondary right flow	500
RIO2	Rio Roccia Bianca (RIO1-DX + RIO1-SX)	Surface watercourse	Surface watercourse upstream the Bossea cave. Main secondary supply contributing to the Bossea karst system recharge. Main watercourse	540

However, due to heavy rains and the resulting rise in water levels, it was not possible to reach them all. Therefore, new sampling points were added. Sampling points and sample characteristics are listed in Table 1. A total of 13 samples for bisphenols were collected simultaneously to other 13 samples for MP and other anthropogenic microparticle (AMP) analysis. Three sampling points were established in surface watercourses, eight inside Bossea cave, and two in the overflow conduit.

Water bulk samples (Hidalgo-Ruz et al., 2012) were collected wearing nitrile gloves by directly immersing pre-cleaned (filtered ethanol and Milli-Q water) glass jars into the water (for river, flowing water, and lakes), immediately after opening. For waters directly coming from the rocks, the jars were immediately placed under the outflow to allow direct filling, in order to minimize external contamination. Once filled, the containers were closed immediately with the cap covered by aluminum foil; therefore, the probability of external contamination can be considered negligible. For each sampling area, a quantity between 200 and 550 mL of water was collected. Samples were stored in the fridge at 6 °C until laboratory analysis.

Samples were collected on 18-09-2024 during a very rainy day, after a sunny period, with about 5 °C near Rio Roccia Bianca watercourse. Unfortunately, sample RIO 2 was damaged during shipment by post to Spain and G6 for MP and MF analysis was broken in the lab.

2.4. Bisphenol analyses

Bisphenols analysis were conducted in the Department of Analytical Chemistry of the University of Valencia. All details regarding analytical standards (purity and suppliers), chemical reagents, and solvents used during the extraction and chromatographic procedures are thoroughly described in Ochoa-Esteso et al. (2026).

2.4.1. Preparation of the AptaStick

The AptaStick device was prepared following a previously reported protocol (Ochoa-Esteso et al., 2026). Briefly, a BPA-specific aptamer, also effective against other bisphenols and modified at its 5′ end with an amine group and a C6 spacer arm (5′–NH_2–$(CH_2)_6$/CCG GTG GGT GGT CAG GTG GGA TAG CGT TCC GCG TAT GGC CCA GCG CAT CAC) was immobilized onto the surface of the star-shape AptaStick 3D-printed device through a multistep surface derivatization strategy. The terminal extraction zone of the device was first chemically activated by alkaline treatment (2 M NaOH, 60 °C, 30 min), followed by acid washing to convert surface groups into their corresponding carboxylic acid form. Subsequent activation with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and N-hydroxysuccinimide enabled the formation of reactive intermediates, which were further modified using hexamethylenediamine to introduce primary amine functionalities. These were reacted with glutaraldehyde to generate aldehyde groups for covalent attachment of the aptamer. Prior to immobilization, the aptamer solution (initially 100 μmol L^{-1}) was thermally activated (95 °C, 10 min; 4 °C, 10 min) and diluted to 2.5 μmol L^{-1}. The extraction star-shape tip of the device was then immersed in the aptamer solution under agitation (30 min, 25 °C). Finally, the formed imine bonds were reduced using sodium cyanoborohydride (0.2 M, 30 min, 4 °C) to improve the stability of the immobilized structure. The resulting functionalized device was referred to as AptaStick.

2.4.2. Extraction procedure

The extraction procedure consisted of three sequential steps: loading, washing, and elution. For the loading step, 3 mL of sample (standard solution or blank) prepared in 50 mM phosphate-buffered saline (PBS, pH 7.4) were placed in a vial, and the AptaStick was immersed ensuring complete coverage of the extraction zone. The system was stirred at 300 rpm for 15 min at room temperature. In the case of real samples, appropriate dilution with concentrated PBS was performed to maintain consistent buffer conditions. After extraction, the device was rinsed with ultrapure water to remove weakly retained matrix components. Elution of the retained analytes was carried out by immersing the device in 0.5 mL of a solution containing 50 mM adenosin triphosphate (ATP) in 10 mM PBS–ACN (1:1, v/v), followed by stirring at 300 rpm for 15 min at room temperature. The eluate was directly injected into the chromatographic system. Based on our previous publication, the addition of ATP is crucial as it induces a conformational change in the DNA aptamer structure, forcing the rapid release of the target bisphenols into the elution medium (Ochoa-Esteso et al., 2026).

2.4.3. Chromatographic analysis

Chromatographic analysis was performed using a C18 column (150 × 4.60 mm, 2.6 μm) operating under isocratic conditions with a mobile phase consisting of water and acetonitrile (60:40, v/v), both containing 50 mM formic acid, at a flow rate of 1.0 mL min^{-1}. Detection was carried out by fluorescence, with excitation and emission wavelengths set at 205 and 300 nm, respectively, and an injection volume of 50 μL.

The method was applied to the determination of bisphenol analogues in karstic cave water samples. The limit of detection (LOD) of the method was 0.094, 0.134 and 0.142 μg/L for BPA, BPAP and BPC, respectively. Quantification was performed using external standard calibration curves, which exhibited excellent linearity within the concentration range of 2.5 to 2000 μg/L (with a determination coefficient of $r^2 > 0.996$) for all analyzed bisphenols. Due to the absence of detectable levels of BPAP and BPC in the analyzed samples, recovery studies were only performed for BPA. The obtained recoveries ranged from 70.4% to 110%, depending on the sample matrix (G1: 89.0%, G2: 100%, G3: 99.0%, G5: 70.4%, G6: 110%, G7: 104%, G8: 86.4%, S1A: 88.4%, S1B: 82.3%, RIO1-DX: 71.4%, RIO1-SX: 88.4%). These recovery values were used to correct the measured concentrations in real samples.

2.5. Microplastic and microfibre analysis

MP and MF analysis were done in the Department of Environment, Land and Infrastructure Engineering of the Politecnico di Torino.

In this study, MPs were defined as synthetic particles with a 5 mm – 1 μm dimension, resulting from the fragmentation of bigger plastic (secondary production) or intentionally produced with these dimensions (primary production). MFs were defined as any natural or artificial fibrous materials with length ranging from 5 mm to 1 μm, a diameter less than 50 μm, and length to diameter ratio greater than 100, originated from primary or secondary production. According to the available equipment, it was established to analyze MPs and MFs from 5 mm to 50 μm.

2.5.1. Laboratory analysis

Water samples were pre-treated by adding a 30% hydrogen peroxide

(H_2O_2) solution at a volume equal to 50% of the total sample volume, covered with aluminum foil and left to react for seven days under natural conditions. Each water sample was subsequently vacuum-filtered through a 1.2 μm pore-size silver membrane filter (GVS Life Sciences, 47 mm membrane disk). Finally, the filters were transferred to glass Petri dishes, covered with aluminum foil, and dried in an oven at 40 °C until completely dry.

2.5.2. Microscopic and spectroscopic analysis

Particles that could not be clearly identified as MPs or MFs were excluded from the analysis. The observed microparticles were characterized according to the Standardised size and colour sorting system (SCS) (Crawford and Quinn, 2016), with the help of x-sectional and longitudinal microscopic images of natural, regenerated and synthetic fibres (Carr, 2017; Khan et al., 2017). Observed particles were divided into synthetics, natural and regenerated, and unknown (N.D. - degraded anthropogenic material not clearly identifiable).

Particles on filters were observed under a Leitz ORTHOLUX II POL-MK microscope equipped with a DeltaPix Invenio 12EIII 12 Mpx Camera. Microparticles were counted at 2.5× magnification, enlarging to 10× or higher magnifications for identification and characterization. Each filter was observed with and without a UV flashlight (Alonefire SV10 365 nm UV flashlight 5 W).

Particles were subsequently analyzed using a micro-Fourier transform infrared (μFTIR) spectroscope (Shimadzu AIM-9000 microscope coupled with a Shimadzu IRTracer-100 spectrophotometer) equipped with an ATR with a germanium crystal. Particles retained on filters were analyzed over a spectral range of 4000–700 cm^{-1}, using 40 scans per spectrum. Atmospheric corrections were applied to the collected spectra, which were then compared with the Shimadzu LabSolutions Library ATR Polymer 2. This was followed by a visual inspection of characteristic absorption bands against reference spectra. Only spectra with a match quality ≥70% were accepted.

2.6. Correlations

The software PAST, Version 5 (Hammer et al., 2001) was used to perform statistical analyses. The Spearman correlation was used to assess whether the presence of BPA could be directly associated with that of MPs.

3. Results

3.1. Bisphenols

The results confirmed that the karst system is polluted by BPA (Table 2), in surface and subterranean environments, with high values in the main part of the sampling points, especially inside the cave. The sampling points affected by BPA pollution within the cave are located in both tourist and non-tourist sections, and the highest concentrations were also observed across both areas. Contaminants were detected both in underground river waters and in waters coming from fractures in the cave walls and ceilings at different locations within the cave system.

BPAP and BPC were not detected in the analyzed water samples.

Table 2
Detected bisphenols in the Bossea karst system. LOD: 0.094, 0.134 and 0.142 μg/L for BPA, BPAP and BPC, respectively.

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

3.2. Microplastics and microfibres

Airborne contamination control highlighted a mean of 13 natural and regenerated MFs for each sampling. No MPs were found. The following results show the values after the value correction.

Analyses on AMPs highlighted pollution in the entire system (Table 3, Fig. 3), with totally different values between the sampling points, ranging from 0 to 136 items/L. AMPs resulted in 90.7% natural and regenerated materials; only 3.6% were synthetic.

MPs were found in only five sampling points, ranging from 2 to 7.7 MP/L (Table 3). Two sampling points resulted polluted in surface watercourses (n = 3), two in cave (n = 3), and one in the overflow (n = 3). Characteristic of MP found in the water sample of the Bossea karst system were reported in Table 4 and showed in Fig. 3. MPs found resulted in small dimensions, with abundances increasing as particle size decreased (Table 4). In surface watercourses MPs had bigger dimensions respect to underground ones, and were especially fibres, while in subterranean environments fragments (55.6%) or fibres (44.4%) were observed. MPs had especially blue and black colors, and 55.6% was fluorescent under UV light. Water samples contained especially copolymers and polyethylene terephthalate (PET). However, particles of polyvinyl chloride (PVC), polyacrylamide (PAM) and acrylic were also found (Table 4).

3.3. Possible correlations

No statistical correlation exists between the values of MPs and BPA

Table 3
Anthropogenic microparticles found in Bossea karst system waters during high flow conditions.

Filter	Natural and Regenerated [items/L]	Synthetic [items/L]	N.D. [items/L]	TOT [items/L]
G1 - Cascatella	91.7	0.0	4.2	95.8
G2 - Polla delle Anatre	136.0	0.0	0.0	136.0
G3 - Ernestina	12.0	4.0	8.0	24.0
G4 - Milano	88.0	0.0	0.0	88.0
G5 - Orso	0.0	0.0	0.0	0.0
G6 - Uovo	-	-	-	-
G7 - Baldacchino	123.1	0.0	3.8	126.9
G8 - Batteri	0.0	7.7	0.0	7.7
S1A	48.0	6.0	2.0	56.0
S1B	0.0	0.0	4.5	4.5
RIO1 - SX	104.0	4.0	12.0	120.0
RIO1 - DX	2.0	2.0	4.0	8.0
RIO2	59.3	0.0	13.0	72.2
Mean	55.3	2.0	4.3	61.6
St. Dev.	49.9	2.7	4.4	49.6
%	90.7	3.6	5.8	100.0
Cave				
Mean	64.4	1.7	2.3	68.3
St. Dev.	54.7	2.8	2.9	52.7
%	94.2	2.4	3.3	100.0
Springs				
Mean	24.0	3.0	3.3	30.3
St. Dev.	24.0	3.0	1.3	25.7
%	79.3	9.9	10.8	100.0
Surface watercourses				
Mean	55.1	2.0	9.7	66.7
St. Dev.	41.7	1.6	4.0	45.9
%	82.5	3.0	14.5	100.0

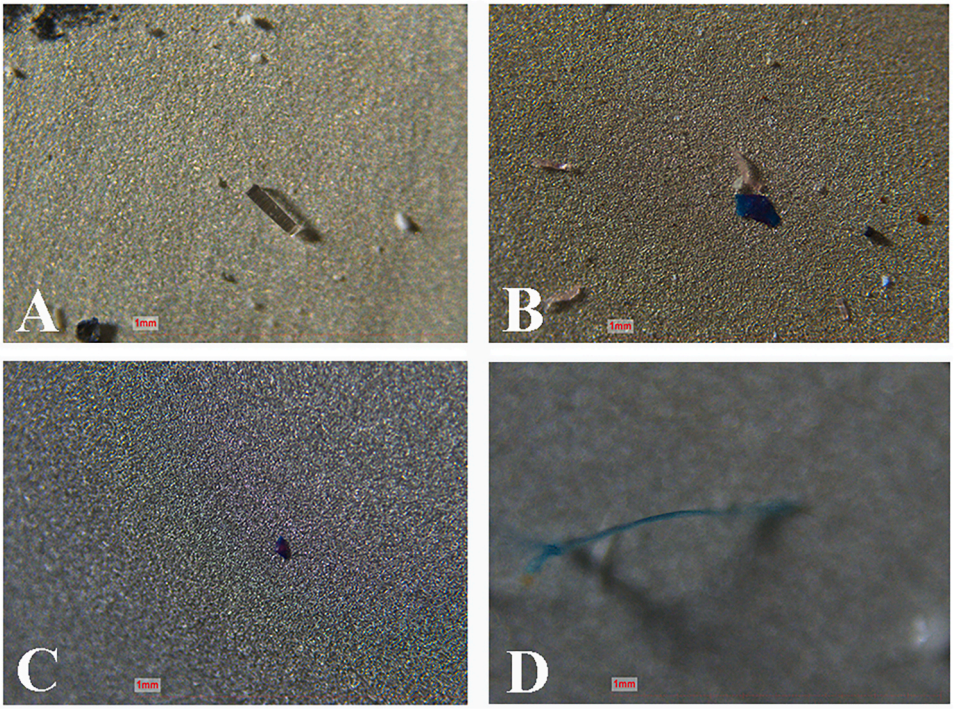


Fig. 3. Images of anthropogenic particles found in Bossea cave waters under microscope. A: transparent polyacrylamide fibre from Rio Roccia Bianca surface watercourse; B: blue copolymer fragment from the overflow; C:blue ethylene vinyl alcohol fragment from Ernestina lake, inside the tourist area of the cave; D: light blue cotton fibre from Polla delle Anatre sampling point, inside the tourist area of the cave.

Table 4
Characteristic of microplastics found in the water sample of the Bossea karst system.

Sampling point	Shape	Size	Colour	Fluorescence	Type
G3 - Ernestina	fragment	0.05	blue	blue	EVOH (Ethylene vinyl alcohol)
G8 - Batteri	fragment	0.05	black	no	copolymer
G8 - Batteri	fragment	0.08	grey	blue	copolymer
S1A	fragment	0.05	blue	no	acrylic
S1A	fragment	0.16	blue	no	copolymer
S1A	fibre	0.16	black	no	PVC (polyvinyl chloride)
RIO1 - SX	fibre	0.74	yellow	yellow	PET (polyethylene terephthalate)
RIO1 - SX	fibre	0.26	transparent	blue	PAM (polyacrylamide)
RIO1 - DX	fibre	0.66	black	no	PET

found in the samples ($rs = -0.275$, $p = 0.413$), as well as with the type of MPs found (Table 4).

4. Discussion

The sampling strategy was designed to provide a first high-resolution snapshot of bisphenols and MP occurrence during a hydrologically relevant event, which is particularly important in karst systems where rapid infiltration and strong event-driven variability are expected. Despite the relatively limited number of samples ($n = 13$ for MPs and $n = 13$ for bisphenols) collected during a single rainfall event, the results cannot be considered fully representative of all hydrological conditions. Nevertheless, they provide valuable baseline information on bisphenols and MP presence under recharge conditions. Additional sampling campaigns covering different hydrological scenarios (e.g., baseflow vs. stormflow conditions and seasonal variability) are necessary to fully characterize contamination dynamics in the studied karst system.

BPA was found in the majority of the examined water samples, highlighting the widespread occurrence of this contaminant in both karst surface and subterranean aquatic karst environments, especially inside the cave. However, the sampling points affected by BPA pollution within the cave were located in both tourist and non-tourist sections, and the highest concentrations were also observed across both areas, suggesting that localized visitor footprint alone cannot account for the entire spatial distribution of the chemical. Contaminants were detected both in underground river waters and in waters coming from fractures in the cave walls and ceilings at multiple and spatially distinct locations within the cave system. The inflows are fed by different and spatially heterogeneous surface recharge areas, reflecting the complex and highly compartmentalized nature of karst drainage systems. This pattern highlights the presence of multiple independent infiltration pathways within the karst network, where water—and associated contaminants—can bypass extensive subsurface filtering and rapidly enter the underground environment. As a result, pollutant distribution within the cave system is strongly influenced by the variability of recharge sources and the structural heterogeneity of the epikarst and fracture network, leading to marked spatial differences even within relatively short distances.

Comparisons with other karst areas are challenging since methods, sampling points and environmental conditions may vary concentrations over time. Moreover, there is a lack of studies regarding karst systems. However, some assumptions can be made. Data found using the words “bisphenol”, “BPA”, “karst”, “cave” and “groundwater” in the search engines of scientific online databases were reported in Table 5 for

Table 5
Comparison of data about maximum BPA concentration in worldwide groundwaters.

Country	Maximum concentration (μg/L)	References
Zambia	0.001	Sorensen et al. (2015)
China	0.027	Du et al. (2016) Dong et al. (2018)
China	0.036	Li et al. (2015) Dong et al. (2018)
Poland	0.149	Ślósarczyk and Jakóbczyk-Karpierz (2026)
New Zeland	0.280	Moreau et al. (2019)
USA	0.430	Bexfield et al. (2019)
South Africa	wet season 0.460	Ligavha-Mbelengwa et al. (2025)
Czech Republic	0.655	Oppeltová et al. (2024)
Austria	0.930	Hohenblum et al. (2004)
Germany	1.136	Osenbrück et al. (2007)
France	1.360	Lapworth et al. (2015)
Spain	1.500	Latorre et al. (2003)
Europe	2.299	Loos et al. (2010)
Tennessee, USA	2.300	DRINKING WATER UNIT (2018)
USA	2.550	Barnes et al. (2008)
South Africa	Dry season 6.600	Ligavha-Mbelengwa et al. (2025)
UK	20.000	Stuart et al. (2011)
Italy	29.000	This study
England	100.000	Lapworth et al. (2015)
Japan	740.000	Michałowicz (2014) Kawagoshi et al. (2003)

groundwaters and Table 6 for karst systems. All data found regarded BPA concentrations; data about other bisphenols were not found.

BPA concentrations found in the Bossea karst system waters are higher than most of groundwater and karst system waters investigated so far (Tables 3 and 4), with the exception of groundwaters analyzed in UK (Lapworth et al., 2015; Stuart et al., 2011), and Japan, where groundwaters were contaminated with leachate derived from refuse dump (Kawagoshi et al., 2003).

BPA pollution in the environment was investigated especially in atmosphere and water (Michałowicz, 2014). In surface waters, BPA was generally detected at low concentrations (e.g. 28.7-98.4 ng/L, Rocha et al. (2013)), however, other investigations have demonstrated substantial contamination, especially near urban centers (Michałowicz, 2014). In urban effluents, main sources are linked especially with atmospheric fallout, sewer overflows, and runoff (Cladière et al., 2013). Significantly higher concentrations of BPA were detected even in industrial wastewater and in groundwater, reaching even levels of 36.7 mg/kg in raw sewage sludges (Lee and Peart, 2000; Michałowicz, 2014).

No statistically significant correlation was observed between MPs and BPA concentrations in this research, as well as with the type of MPs found. This may be linked to the fact that the samples were taken in parallel. Specifically, BPA and MPs analyses were performed on samples collected simultaneously from the same sites and under the same environmental conditions. However, despite the parallel sampling design, no significant correlation between BPA and MPs levels was observed. This lack of correlation may be explained by the differences between the samples.

In addition, BPA detected at the investigated sampling sites may not directly originate from local MPs degradation processes, but rather from upstream contamination sources. Therefore, additional sources of BPA should be considered within this system.

Another potential explanation is that BPA migration within the karst system is not synchronized with that of MPs due to their different physicochemical properties, transport mechanisms, and interactions with the aqueous phases. While dissolved bisphenols behave as pure solutes moving with the conservative flow, MPs are hydrophobic particles subject to gravitational settling, cave conduit deposition, and episodic resuspension during flood pulses. These divergent transport kinetics naturally disrupt any direct statistical correlation, even when the contaminants share a parallel entry into the system. While tourism in show caves may contribute directly to MP contamination (Balestra and Bellopede, 2022, 2023; Christman, 2019; Fraissinet et al., 2025) its contribution to BPA levels cannot be directly inferred without targeted source-tracing or spatially resolved sampling. BPA is a widespread contaminant with multiple potential sources, and it is not specifically or directly associated with tourism-related inputs in the same way as MPs, which can be more directly linked to human presence and littering activities.

In this area, identifying specific pathways of environmental pollution remains complex, because the Bossea system in located in a sparsely populated mountain area. Nonetheless, regional and local anthropogenic activities must be evaluated. In atmosphere, BPA is emitted mainly as a result of industrial activity, with variable concentrations, polluting waters and lands one deposited (Michałowicz, 2014). At about 15 km from Bossea, a paper mill is present, and at least three plastic industries operate at about 20 km, while another one, at less than 20 km from Bossea, was closed in 2022.

BPA is widely used in the paper industry, primarily associated with thermal paper production. In thermal papers, such as receipts, tickets, and labels, BPA acts as a color developer within the heat-sensitive coating. These materials represent the main source of BPA release from the paper sector. During recycling, thermal paper residues often enter mixed paper waste streams, leading to BPA contamination in recycled paper products such as tissues, paper towels, and packaging. Consequently, BPA can be detected in everyday paper-based materials

Table 6
Comparison of data about maximum BPA concentration in karst system waters from different areas of the world.

Country	Type	Maximum concentration (μg/L)	References
Zambia	Wells, karstified dolostone	0.001	Sorensen et al. (2015)
China	Karst groundwater, pore water	0.027	Du et al. (2016) Dong et al. (2018)
China	Groundwater, Loam	0.036	Li et al. (2015) Dong et al. (2018)
Czech Republic	Surface karst waters (borehole, 8 m depth)	0.144	Oppeltová et al. (2024)
Poland	Deep fractured-karst Triassic carbonate aquifer	0.149	Ślósarczyk and Jakóbczyk-Karpierz (2026)
Czech Republic	Thermal karst waters (cave, 60 m depth)	0.242	Oppeltová et al. (2024)
USA	Wells, carbonate aquifers	0.430	Bexfield et al. (2019)
Czech Republic	Groundwater (borehole, 100 m depth)	0.655	Oppeltová et al. (2024)
France	Chalk groundwater	1.360	Lapworth et al. (2015)
Italy	Overflow from a karst cave	10.000	This study
Italy	Surface small karst mountain stream	16.200	This study
Italy	Water coming from a karst cave fracture in the rocks	28.200	This study
Italy	Karst cave underground river	29.000	This study
England	Chalk groundwater	100.000	Lapworth et al. (2015)

not directly manufactured with the compound. Industrial wastewater and sludge generated during paper processing also serve as secondary environmental sources, enabling BPA dispersal into soil and aquatic systems. Additionally, minor contributions may derive from epoxy resins, inks, and coatings used in paper finishing. As the Bossea system is hydrogeologically upstream of the closest active paper mill, any potential industrial contribution from these facilities would likely rely on regional atmospheric transport and subsequent wet deposition onto the catchment area. Moreover, although the paper mill near the Bossea system does not directly manufacture materials that should contain BPA, however, it uses recycled paper for its products, which may contain residual traces of the compound.

BPA is one the most highly produced chemicals worldwide used in the production of polycarbonates and epoxy resins (Hoekstra and Simoneau, 2013; Huang et al., 2012; Michałowicz, 2014). Furthermore, BPA is extensively utilized in the production of polyacrylates, and polyesters, and is incorporated into packaging, beverage bottles and lacquered coatings for metal cans (Arnold et al., 2013; Cooper et al., 2011; Makris et al., 2013; Michałowicz, 2014; Niu et al., 2012).

The plastic industries near the Bossea system produce composite process materials and technical films, which could contain BPA. However, in absence of direct measurements of atmospheric deposition, it is not possible to definitively attribute BPA contamination to some source or to exclude other potential inputs. Influence of other potential contamination sources could be linked to agricultural activities or tourist waste. Nearby snow systems in the ski slopes cannot be ruled out either, as they may have internal coatings with epoxy resins on tanks or pipes, gaskets, sealants or industrial paints with BPA-based epoxy components. However, these quantities should be very low and localized. Once pollution reaches groundwater, the persistence of pollutants in these environments can be significantly prolonged due to the markedly lower hydrodynamic activity and slower transport processes compared to surface waters (Gleeson et al., 2016), including substantially longer residence times within subsurface aquifers. Therefore, further studies are needed to assess BPA pollution in this area, their possible sources, and their potential effects on water chemistry and species, especially groundwater ones.

Moreover, sediments represent an important reservoir and sink for pollutants in karst subterranean environments, such as MPs and MFs (Balestra, 2026; Balestra et al., 2024a, 2024b; Hasenmueller et al., 2023) and their inclusion would provide valuable additional information on pollutant distribution and fate within karst systems. Accordingly, parallel sediment investigations should be considered a priority for future research in karst systems.

BPAP and BPC were not detected in any of the analyzed water samples. The non-detection of these pollutants may be attributed either to their absolute absence in the investigated catchment or to environmental concentrations remaining below the analytical limits of detection. To ensure that these non-detections were not an artifact of methodological limitations, the applicability and high extraction efficiency of the AptaStick platform for both BPAP and BPC were rigorously verified during prior laboratory validation phases via spiking experiments in matrix waters, as detailed in Ochoa-Esteso et al. (2026). In the field, their low environmental occurrence likely reflects limited local inputs, together with potential sorption processes onto solid phases (such as cave sediments) and/or chemical transformation during subsurface transport within the karst system, which can further deplete their presence in the aqueous phase.

Regarding MPs pollution, although it was not possible to sample in the same places as the previous time (Balestra et al., 2023), due to an important heavy rain event, it was possible to observe that interesting differences in MP abundances and characteristics are present (Table 7).

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

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

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

5. Conclusion

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

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

Table 7
Differences between microplastic pollution in waters found in this study (high flow conditions) and previous research (lean period).

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

the possible link with MP transport and contamination.

In karst regions, strong linkages between surface and subterranean systems require the development of more robust and integrated protection strategies. Pollution monitoring in karst systems should be prioritized to support habitat and species conservation, as well as sustainable water resource management, by extending analyses to a wider range of surface and subsurface sites and assessing contamination dynamics over time.

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CRediT authorship contribution statement

Valentina Balestra: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Validation, Visualization, Writing – original draft, Writing – review & editing. **Clara Ochoa-Esteso:** Formal analysis, Writing – review & editing. **María Jesús Lerma-García:** Methodology, Writing – review & editing. **Rossana Bellopede:** Resources, Writing – review & editing. **Enrique Javier Carrasco-Correa:** Conceptualization, Funding acquisition, Methodology, Resources, Validation, Writing – original draft, Writing – review & editing.

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.

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Data availability

Data will be made available on request.

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